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GUIDANCE DOCUMENT ON QUANTITATIVE METHODS FOR EVALUATING THE ACTIVITY OF MICROBICIDES USED ON HARD NON-POROUS SURFACES

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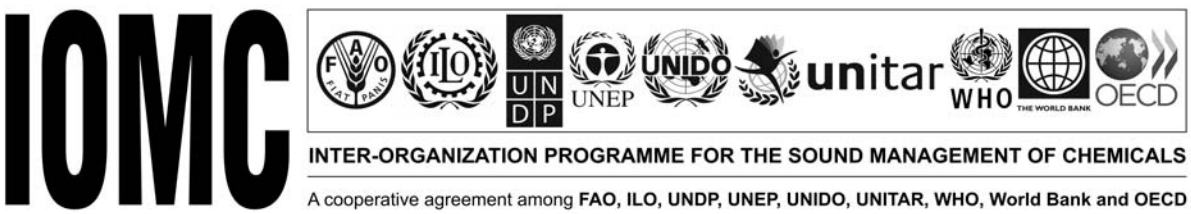

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris 2013

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FOREWORD

This *Guidance Document on Quantitative Methods for Evaluating the Activity of Microbicides used on Hard Non-Porous Surfaces* has been developed under the auspices of the Task Force on Biocides (TFB) and approved by the Working Group of National Co-ordinators of the Test Guidelines Programme (WNT). The document is partitioned into the four sections based on the microbial agent:

A. Quantitative Method for Evaluating Bactericidal Activity of Microbicides used on Hard Non-Porous Surfaces

B. Quantitative Method for Evaluating Mycobactericidal Activity of Microbicides used on Hard Non-Porous Surfaces

C. Quantitative Method for Evaluating Fungicidal Activity of Microbicides used on Hard Non-Porous Surfaces

D. Quantitative Method for Evaluating Virucidal Activity of Microbicides used on Hard Non-Porous Surfaces

In 2007, the United States initiated an international effort to select and evaluate a test method for the purpose of harmonisation. An OECD *ad hoc* Validation Management Group was established involving experts from Canada, France, Germany, Switzerland and the United States (Chair of the VMG). Between 2008 and 2010, a series of international ring trials were conducted to determine method performance and to refine the method. Several commenting rounds of the draft test methods and the preparation of a validation report followed the ring trials. Guidance on how to utilise the test method (*e.g.* number of replications and number of labs) was also drafted. These documents were submitted for the first time to the WNT in April 2011. Note: the *Report of Validation of Efficacy Methods for Antimicrobials used on Hard Surfaces* was approved and published in the series on *Testing and Assessment*, No. 154.

Based on a recommendation by the WNT, a meeting of experts and regulators was held in September 2011 to discuss technical concerns and deficiencies, and to facilitate a path forward. To help resolve the outstanding issues, the United States Environmental Protection Agency (EPA) led a collaborative study in 2012 – the 11-laboratory study only involved the bactericidal test method. The final report on the EPA collaborative study was submitted for the WNT's consideration. A commenting round of the revised bactericidal test method took place in October 2012 – January 2013. A draft *Guidance Document on Quantitative Methods for Evaluating the Activity of Microbicides used on Hard Non-porous Surfaces*, containing a Preface on guidance for the conduct of the tests and four test methods (bactericidal, mycobactericidal, fungicidal and virucidal) was approved by the 25th WNT Meeting in April 2013. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on June 14, 2013.

The OECD member countries are encouraged to use the test methods described into this Guidance Document. The Secretariat will survey the member countries in order to get feedback on the performance of the method and technical recommendations designed to improve the procedures. For this purpose, it is anticipated that a Questionnaire will be made publicly available within one or two years (http://www.oecd.org/env/ehs/).

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

TABLE OF CONTENTS

PREFACE

INTRODUCTION

1. Microbicides are routinely used on hard, non-porous surfaces to interrupt the spread of pathogens in a wide range of sites including healthcare, food production, restaurants, farms, veterinary clinics, homes, schools, and other commercial and institutional settings. Over the years, a number of laboratory test methods have been developed to assess the effectiveness of hard, non-porous surface microbicides. These methods have ranged from testing microorganisms in suspension to drying them on hard carriers and exposing them to the microbicide. Regulatory authorities throughout the world have relied upon data generated using these methods to approve applications/licenses for sale and distribution of these products in commerce. To date, there is no single method for this purpose that can be used and accepted in all OECD member countries. The Guidance Document will be improved in light of the experience gained.

2. In this Guidance Document, four methods are described that permit the evaluation of efficacy of microbicides used on hard non-porous surfaces. They are:

A Quantitative method for evaluating bactericidal activity of microbicides used on hard non-porous surfaces

B Quantitative method for evaluating mycobactericidal activity of microbicides used on hard non-porous surfaces

C Quantitative method for evaluating fungicidal activity of microbicides used on hard non-porous surfaces

D Quantitative method for evaluating virucidal activity of microbicides used on hard non-porous surfaces

3. Information is provided on assessing the microbicidal activity of products on hard non-porous surfaces using a quantitative procedure. To support label claims for specific fields of application, additional information may be needed.

4. General considerations including those common to all four methods are given hereafter. Details of individual methods are given under headings A to D.

GENERAL CONSIDERATIONS

5. The four test methods use a quantitative method for evaluating the bactericidal, mycobactericidal, fungicidal, and virucidal activities of microbicides to be used on hard, non-porous surfaces.

6. The four test methods should be performed by personnel with training in microbiology and aseptic techniques. In order to ensure the validity of test results, training and proficiency testing should be run per a laboratory's internal Standard Operating Procedures; internal SOPs may require a more frequent proficiency testing schedule. Proficiency testing should preferably be conducted on an annual basis. The methods should be conducted in facilities that are well equipped to handle infectious microorganisms at the appropriate biosafety level (1) (2).

7. All labware and those parts of equipment coming into direct contact with test organisms, media and reagents must be sterile. All equipment must be maintained and calibrated, as necessary. Maintaining

good quality control of all media, reagents, and equipment used in these methods is necessary to ensure the validity of the test results.

SCOPE OF APPLICATION OF THE TESTS

8. The four test methods are designed to be used in testing the microbicidal activity of a wide range of formulations used on hard, non-porous surfaces. Each of the methods was validated in a multi-laboratory ring-trial against representative microorganism(s): Bacteria – *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus hirae*; Mycobacterium – *Mycobacterium terrae*; Fungus – *Aspergillus brasiliensis*; Virus – Adenovirus Type 5. These microorganisms are considered to be the basic representatives for the four test methods, however, individual regulatory authorities may require additional or alternative microorganisms to be tested to support label claims.

9. The test methods have been validated for liquid forms of products only. For non-foaming/ foaming aerosols and pump/trigger spray products, the test substance is obtained by dispensing the product into a sterile vessel for collection. Using aseptic techniques, 50 µl of the liquid is removed from the vessel and deposited on the inoculated test carrier. Foaming products that the foam does not break down within two minutes will require further discussion and a separate validation.

a) The methods are not appropriate for pre-saturated towelettes/wipes at this time; such an application type will require further discussion and a separate validation.

b) These methods are designed to test the microbicidal activity of liquid formulations and not the dispensing devices or mechanisms of application.

10. No standardized test protocol can fully reflect all possible variations and challenges a microbicide may encounter in the field. Nevertheless, these test methods include the following levels of stringency to better predict the in-use performance of products on hard, non-porous surfaces: (a) the topography of carriers of brushed stainless steel is to assess the ability of the test substance to access target organisms on textured surfaces, (b) the ratio between the surface area of the disk carrier and the volume of test substance is kept relatively low to better reflect how products are often applied, and (c) the test microbial suspension contains an added soil load to simulate the presence of the residues of body fluids and other substances on pre-cleaned surfaces. However, even the most effective products may fail to work in the field if used inappropriately. Any modifications to the specific instructions listed in the test methods should be validated according to your standard operating procedures and included in the final test report.

STUDY DESIGN

11. These methods are fully quantitative and avoid 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. The methods use disks (1 cm in diameter) of brushed stainless steel as a default carrier to represent hard, non-porous environmental surfaces. The carriers should be brushed on both sides; however, if one side is smooth, the inoculum must be placed on the brushed surface. The use of these small flat carriers allows for their complete immersion and elution in relatively small volumes of eluents. The incorporation of membrane filtration permits the processing of entire eluate volumes and more efficient removal of any residue of the test substance.

12. 10 µL of the test organism in a soil load is placed at the centre of each carrier. 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. If the test substance (or control fluid) does not dome over the inoculum , use the pipet tip to gently spread it without touching the surface, ensuring coverage of the

inoculum. Discard carriers where the test substance spills over the sides of the carrier or if the surface of the carrier is touched with the pipet tip. Do not touch the carrier with the pipet tip. The contact time depends on the label claim (see paragraph 18), and the default temperature is 20-25 °C (see paragraph 17). A neutralizer, validated before or while performing the actual microbicide testing is added at the end of the contact time and the disks are then eluted in the neutralizer. Most (for viruses) and all (for bacteria, fungi, and mycobacterium) of the eluate volume from each disk are 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. Regulatory authorities may require other contact times depending on the label claims.

Discussion of the control carriers

13. Control carriers are used to determine the count of viable organisms on the carriers after the inoculum has been dried. They are prepared and treated in the same manner as the test carriers with the exception that the control carriers receive 50 µL phosphate-buffered saline (PBS) (EBSS for virucidal testing) instead of the test substance. The count obtained from the control carriers is used to calculate the log_{10} reduction following treatment with the test substance. No less than four control carriers are used in each test. The mean viability count on the dried control carriers must be between 0.5 log_{10} and 1.5 log_{10} higher than the defined performance standard. The upper limit of 1.5 logs is set to exclude the influence of too high an inoculum on the results and to enable a fair comparison of the test substances. The basis to which these numbers (0.5 log_{10} – 1.5 log_{10}) are added will vary depending upon the log_{10} reduction required to meet the performance standard as defined. If the upper limit is exceeded but the product demonstrates the desired performance standard, the test is considered valid. However, if the higher limit is exceeded and the product fails to demonstrated the desired performance standard, the test is considered to be invalid and should be repeated.

14. At a minimum, three test carriers for one concentration/dilution of a test substance are recommended for these methods per test run (repetition). Individual regulatory authorities may require a higher number of test carriers.

Soil load

15. All microbial test suspensions for inoculating the carriers are to contain the three-component soil load specified in the methods. The total protein content of the mixture is roughly equal to that in 5% bovine serum and will satisfy label claims as a one-step cleaner/disinfectant. The 5% soil load simulates the presence of light to moderate soil on surfaces. The mixture can be adjusted to 0.5% to support label claims that specify a pre-cleaning step prior to use of the disinfectant. A minimum of 5% soil load is required for one-step cleaner/disinfectant products used in the medical area (hospitals, healthcare facilities, etc.). Regulatory authorities may require other types and levels of soil depending on the label claims. All sources of soil must be standardized and commercially available.

Diluent for test substances

16. The standard hardness of water is to be 375 ppm (as $CaCO_3$), with a range of +5%/-10% (338-394 ppm). The hard water must be used within 24 hours of preparation and titrated on the day of use., to ensure it has been prepared properly and meets the targeted hardness level. Individual regulatory authorities may accept or ask for lower or higher water hardness for certain types of products.

Temperature

17. The default temperature of 20 – 25°C should be maintained during the course of the experiment, including the inoculation of carriers, exposure of carriers to test substance, and neutralisation confirmation testing. However, regulatory authorities may require other temperatures depending on the label claims.

Contact time

18. The selected contact times for the test and control carriers will be determined by the recommended/proposed label claims and use directions, and should reflect the realities of field applications based on the labelled use sites. Different contact times may be required by regulatory authorities depending on the product application.

Concentration of the test substance

19. The test substance should be prepared and tested according to the label directions for use.

Internal reference standard

20. An internal reference standard e.g. two levels of lab grade sodium hypochlorite) should be tested periodically (*e.g.* weekly) or concurrently with the test substance on the same day. The reference standard shall display both high efficacy and low efficacy; this will allow for a determination of method performance and analyst proficiency. The concentration of both levels of sodium hypochlorite should be determined on the day of test. A five minute contact time, three part soil, and 375 ppm hard water will be used. Other active ingredients with known levels of efficacy (high and low efficacy) may also be used for an internal reference standard. Prior to the routine use of a new reference standard, the generation of multi-laboratory collaborative data is necessary.

Recovery

21. For the purpose of determining significant differences of recovery of viable microbes the analyst should conduct in parallel, the assay using filtration and direct plating with treated carriers. For direct plating each 10 mL volume will be aliquoted in ten 1 mL aliquots and direct plated on the same recovery media as used in filtration. The counts derived from direct plating will be compared to those filtered. This comparison is only required to be conducted one time per test substance. If recovery using direct plating is significantly higher than filtration, then direct plating will be used for the assay. Following the generation of sufficient data the need to conduct comparative analysis will be reconsidered.

Testing additional microorganisms

22. The recommended test organisms for use in regulated testing are specified in the individual test methods and were the test organisms used in the ring-trials. These test organisms also have a long history of being used for regulated testing of disinfectants, and are relatively safe and easy to work with. Prior to initiating the tests, applicants should check with their regulating authority to determine whether additional or alternative test organisms and test parameters will be required 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) are acceptable alternatives. If other test organisms are employed, the growth and recovery media, incubation requirements and any other test parameters should be detailed as necessary. Table 1 provides a list – which is not exhaustive – of additional or alternative microorganisms that may be used in the test or required by regulatory authorities. Use the appropriate TG for the test organisms.

Table 1

Organism (ATCC #)	Culture Medium or Cell Line	Recovery Medium or Cell Line

Hepatitis A Virus Strain HM-175 (VR-1402)	FRhK-4 (CRL-1688)	FRhK-4 (CRL-1688)
Murine Parvovirus (VR-767)	A9 cells	A9 cells
Murine Norovirus Strain S99 www.fli.bund.de	RAW 264.7	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+}	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)	MA-104 (CRL-2378) or CV-1 (CCL-70)
SV40 (VR-305)	BSC-1 (CCL-26)	BSC-1 (CCL-26)
Candida albicans (ATCC 10231)	Malt Extract Agar	Malt Extract Agar
Trichophyton mentagrophytes (ATCC 9533)	Sabouraud's Dextrose Agar, incubated for 12 days at 29 ± 2˚C	Sabouraud's Dextrose Agar, plates observed first after 72 hrs and final reading recorded after 10 days at 29 ± 2˚C
Aspergillus brasiliensis (ATCC 16404)	Potato dextrose agar at 24 ± 2˚C	Potato dextrose agar at 24 ± 2˚C
Escherichia coli (ATCC 11229)	Nutrient broth	Nutrient agar

Note: Some of the viruses on this list may need to be dried in an environmental chamber, instead of in the laminar flow hood, in order to achieve the appropriate titre to meet the performance standard. Although only one virus (Adenovirus 5) was tested in the ring-trial, the other viruses listed above are also non-enveloped viruses with the potential to spread via contaminated hard, non-porous surfaces. They together represent major classes of enteric and respiratory viruses. 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. Individual regulatory authorities may require other viruses, from the list above, to be tested (see test method D: Quantitative method for evaluating virucidal activity of microbicides used on hard non-porous surfaces).

Number of test runs and test days

23. It is recommended that applicants have their products tested in multiple laboratories and/or on multiple test days to achieve a higher confidence level for their recommendations of use. Check with your regulatory authority to determine if multiple tests at multiple labs and/or more than one batch of test substance is required. This information should be determined prior to testing.

Proposed Performance standards

24. Table 2 provides the target proposed performance standards (arithmetic mean log reduction) for each of the four test methods (regulatory authorities may require different arithmetic mean log reductions):

Table 2

CLAIM	Disinfectant	Disinfectant	Disinfectant	Disinfectant
	Bacteria	Viruses	Fungi	Mycobacteria
Performance standard (Arithmetic mean log reduction)	4	3	4	4

Note: The average log reduction must meet the standard performance. In cases where a carrier fails to meet the performance criteria but the average survivors of the test carriers meets the performance standard, the test is considered valid.

TEST REPORTS

25. Any submissions requesting label claims must accompany detailed results of all tests of the products, including all controls (such as neutralisation) and raw data from testing as described in the test with evidence for confirmation of neutralisation. Check with individual regulatory authorities for content requirements and formatting.

26. 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) and/or date of manufacture and/or expiration date.

- Chemical name and relative concentrations of active ingredients; such information may come from the manufacturer, product label or material safety datasheet (SDS).

Details on the Test Method

Test organism

- Source

- Scientific name and strain number

- Growth and recovery media

- Preparation of the stock and working culture

Test Conditions

- Temperature

- Contact time
- Soil load (type and amount)
- Hard Water level and titration

Results

- CFU/infective units per carrier
- Log_{10} Reduction
- Neutralisation confirmation
- Copies of the raw data

Controls

- Sterility, viability, purity controls should all be performed according to the laboratories internal Standard Operating Procedures
- Cytotoxicity should be reported, when evident, for virucidal testing.
- Identification of the target microorganism
- Identification of any contaminants

Conclusion

REFERENCES

(1) Biosafety in Microbiology and Biomedical Laboratories (2007) 5th Ed., U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention and National Institutes of Health.

(2) Laboratory Biosafety Guidelines 3rd Edition, 2004: http://www.phac-aspc.gc.ca/ols-bsl/lbg-ldmbl/

GLOSSARY

BSA: Bovine Serum Albumin

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

CFU: Colony Forming Unit.

Control fluid (PBS) is the fluid (which is harmless to the test organism) placed on the control carriers in place of the test substance.

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 vial to recover these from 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 Buffered Saline

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.

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

Test organism: A specific strain of microorganism used in testing. It also may be referred to as a *surrogate, simulant, target* or *marker microbe.*

Test suspension is suspension of the test organism prepared specifically for use in test.

A "QUANTITATIVE METHOD FOR EVALUATING BACTERICIDAL 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 neutraliser 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 method is designed for testing the bactericidal activity of substances to be used on hard, non-porous surfaces (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. 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 twenty-seven laboratories from eight member countries participated and a validation report (5) was produced. Further, a collaborative evaluation of the bactericidal test method was conducted in 2012; the summary of the study is presented as an Annex to the validation report.

3. Details on relevant materials and reagents and the preparation of the test organisms are found in Annexes 2 to 5.

Requirements for test substance

4. The following information on the test substance should be determined (see regulatory authorities for specific requirements):

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 name and relative concentrations of active ingredients; such information may come from product label or 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 3, 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.

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) i) Directions for determination and verification of neutralisation of test substance.

j) Specifications for performance criteria when available.

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

l) Soil load to be used in testing.

m) Test substance diluent (if applicable).

INITIAL CONSIDERATIONS AND LIMITATIONS

6. The method employs disks of brushed stainless steel with magnetic properties.

7. Surrogate test organisms are specified herein; however, test organisms more relevant to other settings, *e.g.*, dairy, baking or brewing industries are permitted in consultation with the target regulatory agency.

8. 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.

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

10. The method has been validated for testing liquid formulations. For testing other product forms refer to paragraph 9 of the Preface under "Scope of Application of the Tests".

PRINCIPLE OF THE TEST

11. 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) Determination and verification of neutralisation.

d) Inoculation, drying and transfer of the carriers.

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

12. 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. The use of small flat carriers allows for their complete immersion and elution in relatively small volumes of eluents. The incorporation of membrane filtration permits the processing of entire eluate volumes and more efficient removal of any residue of the test substance.

13. 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 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. Arithmetic mean $\log_{10}$ reductions in the numbers of viable test organisms following exposure to the test substance are calculated in relation to the mean number of viable of test organisms on the control carriers.

TEST PROCEDURE

14. The effectiveness of the neutralizer is determined prior to or during testing. For verification purposes the activity of the neutralizer will be verified per the methodology given in Annex 1.

Preparation and sterilisation of carriers

15. The carriers are soaked in a suitable detergent solution (*e.g.* Liquinox or equivalent) free from any antimicrobial activity for 2-4 hours to degrease and then rinsed thoroughly in distilled or

deionised water. Carriers should be visually checked for abnormalities (*e.g.* rust, chipping) and discarded if observed. At least four control carriers and three test carriers are used for each test organism and contact time/temperature.

16. 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 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, the inoculum on it runs over the edge or the inoculum is off-centre. The carriers are single use and should be discarded following testing.

Preparation of test organisms

17. The test organisms listed below were used in the ring trial and could be used for regulated testing. However, other specific test organisms 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) are acceptable alternatives. The maintenance of bacterial cultures is described in Annex 2. If other test organisms are employed, adapt growth and recovery media, incubation requirements and any other test parameters as necessary.

Pseudomonas aeruginosa (ATCC 15442)

18. For this test organism, the culture medium is prepared as follows:

- prepare synthetic broth containing 10% glucose or dextrose.
- add 100 µL of stock culture to 10 mL of broth and incubate for 18 h – 24 h at 36 ± 1°C.
- Remove visible pellicle using vacuum suction. Using a serological pipette, withdraw the remaining broth being careful to avoid any sediment on the bottom of the tube.

Staphylococcus aureus (ATCC 6538)

19. For this test organism, the culture medium is prepared as follows:

- prepare a soybean-casein digest broth (equivalent to Trypticase Soya Broth);
- add 100 µL of stock culture to 10 mL of broth and incubate for 18 h-24 h at 36 ± 1°C.

  Note: Golden yellow colonies are selected from semi-solid media (only strains producing mostly yellowish colonies on media are used – see Annex 2).

Enterococcus hirae (ATCC 10541)

20. For this test organism, the culture medium is prepared as follows:

- prepare a soybean-casein digest broth (equivalent to Trypticase Soya Broth);
- add 100 µL of stock culture to 10 mL of broth and incubate for 18 h – 24 h at 36 ± 1°C.

Preparation of Test Suspension

21. Following incubation, use the broth cultures to prepare a test suspension for each organism. The broth cultures should be centrifuged as described below to achieve the desired level of viable organisms on the dried carrier count.

- Test organisms require centrifugation of the broth culture to obtain the required number of viable cells. The product of centrifugation (*g* force) and time for which it is applied (t minutes) determines the organism's sedimentation rate. The centrifugation should be between 5000 and 10000 g_N for 20 ± 5 minutes and resuspend the pellets in PBS. Centrifugation for less than 5000 g_N may result in incomplete sedimentation of the test bacteria. If multiple tubes are centrifuged, pool the resuspended pellets.

- To achieve carrier counts in the range of 4.5 to 5.5 logs, resuspend the pellet in 10 mL PBS. Use this culture to prepare the inoculum. If necessary, the culture may be either concentrated or diluted to achieve the target carrier counts.

  Note: After removing the supernatant, vortex the pellet on a high vortex speed to disaggregate the pellet completely prior to resuspending it in 10 mL. If necessary, add 1 mL of PBS to the pellet to aid in the disaggregation.

22. Initially the approximate CFU count of each freshly prepared microbial test suspension may be estimated spectrophotometrically at a wave length of approximately 650 nm, based on a standard curve specific to the test organism. This may act as a guide to the required dilutions but is confirmed by a plate count assay on the recovery medium to be used in the test. Only a higher number than the defined performance standard allows for statistical evaluation. Microbial counts are confirmed in each test by determining the numbers of viable organisms on each of the control carriers.

23. Prior to inoculation of carriers, the soil load is aseptically added.

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, vortex each component and add 25 µL of BSA, 100 µL of mucin, and 35 µL of yeast extract stocks to 340 µL of the microbial test suspension (see Table 1). The inoculum is vortexed again for 10 seconds.

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

Component	Volume (µL)
Test suspension	340
5% (w/v) BSA solution*	25
0.4% (w/v) Mucin solution*	100
5% (w/v) Yeast extract solution*	35
Total	**500**

*Note: Final concentration of the stock solution.

Inoculation and Drying of Carriers

25. 10 µL of the inoculum are withdrawn with a calibrated positive-displacement pipette (Figure 1), and deposited at the centre of the brushed surface of the 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). Discard inoculated carriers where the inoculum has run off the centre or over the edge of the carrier.

26. The Petri dish is transferred with the inoculated carriers into a desiccator and the lid of the Petri dish is removed (Figure 3). Close the desiccator and check that it is properly sealed. The desiccator is evacuated using a vacuum source to achieve 20 – 25 inches mercury (508 – 635 torr; 677 – 847 mbar; 68000 – 85000 Pascal). Further details on using a desiccator are provided in Annex 5. The inoculated carriers are kept in the evacuated desiccator at 20 – 25°C for 60 ± 10 minutes (Figure 4). If carriers are not dry within the specified time, check the desiccator system.

Exposure of the dried inoculum 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 inoculum after desiccation to the test substance or carrier count control fluid is as follows:

- Using sterile forceps transfer each dried carrier (Figure 5) with the inoculated side up to the flat bottom vial (Figure 6).
- Cap the vial.
- Repeat until all carriers are transferred.
- Carriers can be stored at 20-25°C for up to one hour.
- 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.
- The vial must be in a horizontal position to ensure uniform distribution of test substance onto carrier.
- Deposit 50 µL of the test substance, equilibrated to 20-25°C, over the dried inoculum on each test carrier, ensuring complete coverage, at predetermined staggered intervals (Figure 7); do not touch pipette tip to carrier, and do not cap the vials.
- Test carriers are held at 20-25°C for no more than the selected contact time (see paragraph 18 of the Preface).
- Control carriers are the last to be treated and receive 50 µL PBS, equilibrated to 20-25°C, instead of the test substance; the control carriers are treated in a manner identical to that for the test carriers.

29. The number of test organisms on the dried carriers should be 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 basis to which these numbers (0.5 $\log_{10}$ – 1.5 $\log_{10}$) are added will vary depending upon the log reduction required to demonstrate the passing of the performance standard as defined in paragraph 22 of the Preface, under "Performance standards".

Neutralisation of test substance and elution of test organisms

30. Immediate (within 10 seconds) neutralisation of the test substance is required at the end of the contact time. Use less than 10 seconds as appropriate for shorter contact times. The protocol for the determination and verification of the neutraliser is given in Annex 1. At the end of the contact time, 10 mL* of the neutraliser is added to each vial according to the predetermined schedule. For consistency across laboratories/operators, this is documented as the 10^0 dilution. Note: *For ease of pipetting, 10 mL are used instead of 9.95 mL; this will not significantly affect the result.

31. Briefly (2-3 sec) vortex each vial following the addition of the neutralizer. Following the neutralization of the entire set of carriers, vortex each vial for 30 ± 5 seconds at high speed to recover the inoculum. If possible, each carrier is examined visually and, in case of incomplete elution, further vortexing is performed. Do not remove carrier from the vial.

Dilution and recovery

32. At this stage one mL of the eluate is removed and used for any needed 10-fold (1mL + 9mL) dilutions to get countable numbers of the test organism to determine $\log_{10}$ reductions in viability. The number of dilutions to be made and tested will depend on the initial inoculum and the level of microbicidal activity expected. Dilution and recovery are completed between five and 30 minutes at room temperature after the neutraliser is added to the vial containing the carrier.

33. All samples, control and treated, will be filtered (see Annex 4 for information on membrane filtration system). Direct plating is not allowed. The procedure for dilution and recovery is as follows:

- Prewet each membrane filter with about 10 mL of sterile PBS.
- Separate membrane filters but the same filtration unit may be used for processing the eluate from a given carrier starting with the most dilute sample first. Always filter eluates from control carriers last to reduce risk of contamination of the eluates and filters from the test carriers.
- Prepare dilution vials beforehand by adding diluent and labelling them (Figure 8).
- For the remaining 9.0 mL of the eluate, hold a magnet at the bottom of the vial to keep the carrier in place while pouring the contents of the vial into the membrane filtration system (Figure 9).
- Rinse vial with approximately 20 mL of PBS, vortex for five seconds and keeping magnet in place (while pouring), pour the wash into the same filtration system.
- Repeat this step one more time, swirl, and filter by applying vacuum.
- With the vacuum on, rinse the inside surface of the funnel unit with an additional 40 mL of PBS. Note: If desired, the vacuum may be left on for the duration of the filtration process beginning with the wetting step.
- For dilution tubes, rinse each tube once with approximately 10 mL of PBS and briefly vortex.
- Remove the membrane filter aseptically with sterile forceps and place it carefully over the agar surface of the recovery medium (Soybean-casein digest agar), starting at the edge as illustrated to avoid trapping any air bubbles between the filter and the agar surface (Figure 10).

- Incubate the plates at 36 ± 1°C for 24 – 48 hours and examine for colonies of the test organism.

34. The elution and filtration steps for control carriers are also the same as those described above for the test carriers. However, eluates from control carriers will always require 10-fold dilutions to provide countable plates (15 – 200 CFU/plate).

DATA AND REPORTING

Assessing performance of test substance

35. Performance is assessed by counting surviving bacteria from the each test carrier and comparing the number obtained to the mean of those on the control carriers. All colony counts are recorded. Counts between 15-200 CFU/plate are used in calculations to determine log reductions. However, if counts are less than 15 and no plates demonstrate counts between 15 – 200 CFU/plate, then those counts are used in calculations to determine log reductions. If no survivors, then use <1 CFU in calculations to determine log reductions.

36. Data are summarized in a tabular form showing raw data for each test and control plate. Data are also presented to validate the neutralisation process used in the test.

Calculating Log_{10} reductions

37. A method for determining arithmetic mean log_{10} reduction in the viability count of the test organisms by the test substance in quantitative carrier tests such as this one has been described (6).

Log_{10} Reduction = Average Log_{10} recovered from control carriers – Average Log_{10} recovered from the test carriers.

Test report

38. The test report includes, but not limited to 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 name and relative concentrations of active ingredients.

Details on the test method

Test organism

- Source

- Scientific name and strain number

- Growth and recovery media

- Preparation of the stock and working culture

Test conditions

- Concentration of the test substance
- Temperature
- Contact time
- Test substance diluent (hard water level and titration)
- Soil load

Results

- CFU per carrier
- Log_{10} reduction
- Neutralisation determination and verification
- 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 (2011) Report on validation of efficacy methods for antimicrobials used on hard surfaces – Part I & Part II, [ENV/JM/MONO(2011)34], OECD Environmental Health and Safety Publications, series on Testing and Assessment, No. 154.

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

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

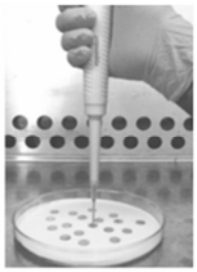


Figure 3 (left): Petri plate lid is removed during drying of carriers

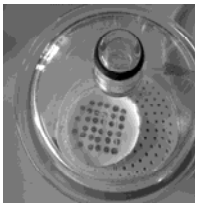


Figure 4 (right): Carriers left in an evacuated desiccator to dry at room temperature for 60±10 minutes.

Figure 5 (left): Carrier with dried inoculum being picked up for placement in flat bottom vial

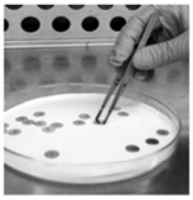


Figure 6 (right): Carrier placed into the flat bottom vial

Figure 7 (left): Dried inoculum on carrier covered with 50 µL of test substance or control fluid

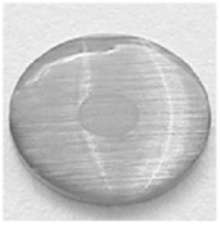


Figure 8 (right): Labelled dilutions vials with diluent; tubes may be used in place vials

Figure 9 (left): Magnet placed on the outside bottom of the vial to hold the default carrier in place while pouring eluate into filter funnel.

Figure 10 (right): Membrane filter being placed on surface of recovery agar to avoid trapping air bubbles underneath the filter.

ANNEX 1

DETERMINATION AND VERIFICATION OF APPROPRIATE NEUTRALISATION

Purpose

Verifying effective neutralisation immediately at the end of the contact time is essential when assessing the microbicidal activity of any test substance. In addition, controls are included to rule out any microbicidal or microbistatic action of the neutraliser itself or by its reaction with the soil load. It is recommended that a suitable neutraliser be identified prior to commencing the actual testing of a given substance. However, additional confirmation of neutralisation may be performed along with or even after such testing.

The neutralisation must be verified separately for each test substance against each type of test organism to be used in the method. In case one neutraliser is incompatible with all the test organisms, more than one neutraliser may be used. If several concentrations of a given test substance are being evaluated, the highest concentration is tested to determine and verify neutralization.

Principle

The neutralizer is added to the carrier vial immediately at the end of the contact time. This dilutes the test substance while also chemically neutralising its microbicidal/microbistatic activity. Except in tests for virucidal activity, the entire eluate is then passed through a membrane filter which is washed with phosphate buffered saline (PBS) to further remove any residues of the test substance; the membrane is placed on a suitable recovery medium.

In the neutraliser determination/verification assay, the test substance is first mixed with a candidate neutraliser. The test organism is then added to the reaction mixture as dried inoculum on a carrier; if desired, additional evaluations may be conducted using the test organism as a liquid. The neutralisation process is deemed acceptable if there is no statistically significant difference between the numbers of viable organisms recovered from the neutralised test substance and the controls including those for any microbistatic or microbicidal effect of the neutraliser itself.

Two separate test suspensions (A and B) are required; A is a diluted suspension of the test organism, and B is the test microbial suspension with the added soil load.

Procedure

(a) Prepare Test Suspension A: Dilute the test microbial suspension with PBS to achieve an average challenge of 20-200 CFU/carrier after the 10 µL inoculum has dried. Prior testing may be required to account for differences in the loss of viability of the different test organisms on drying (see further section: "Requirements for a valid test and possible outcomes"). Test Suspension A should be used within 4 hours of preparation.

(b) Prepare Test Suspension B: First prepare the soil load by combining 25 µL 5% (w/v) BSA, 35 µL 5% (w/v) yeast extract, and 100 µL of 0.4% (w/v) mucin – mix well. Combine

132 µL of Test Suspension A and 68 µL of the soil load (SL). The test microbial suspension with soil load should also achieve an average challenge of 20-200 CFU/carrier after drying.

(c) It is recommended that a calibration curve, using optical density (OD at 650nm), be created to estimate the number of viable organisms in Test Suspension A.

(d) Carrier inoculation: Inoculate at least 10 carriers with Test Suspension A and at least 10 carriers with Test Suspension B by adding 10 µL to each carrier. Follow instructions in the test method for drying the carriers.

(e) Treatment 1a: Titer Control (with SL). Add 10 mL PBS to each of four vials. Add one dried carrier inoculated with Test Suspension B gently to each vial. Vortex each vial for 30 ± 2 seconds. Proceed as in (k) below. If necessary, using another vial, perform this procedure with 10 µL of Test Suspension *B* in place of one of the inoculated carriers.

(f) Treatment 1b: Optional Titer Control (without SL). Add 10 mL PBS to each of four vials. Add one dried carrier inoculated with Test Suspension A gently to each vial Vortex each vial for 30 ± 2 seconds. Proceed as in (k) below. If necessary, using another vial, perform this procedure with 10 µL of Test Suspension *A* in place of one of the inoculated carriers.

(g) Treatment 2: Neutraliser Toxicity Control. Add 10 mL neutraliser to each of three vials. Add one dried carrier inoculated with Test Suspension B gently to each vial. Vortex each vial for 30 ± 2 seconds. Proceed as in (k) below. If necessary, using another vial, perform this procedure with 10 µL of Test Suspension B in place of one of the inoculated carriers.

(h) Treatment 3a: Neutraliser Effectiveness (with SL). Add 50 µL of the test substance to each of three vials. Then add 10 mL neutraliser to each vial and briefly swirl. After 10 seconds, gently add one dried carrier inoculated with Test Suspension B to each vial. Vortex each vial for 30 ± 2 seconds. Proceed as in (k) below. If necessary, using another vial, perform this procedure with 10 µL of Test Suspension B in place of one of the inoculated carriers.

(i) Treatment 3b: Optional Neutraliser Effectiveness (without SL). Add 50 µL of the test substance to each of three vials. Then add 10 mL neutralizer each vial and briefly swirl. After 10 seconds, gently add one dried carrier inoculated with Test Suspension A to each vial. Vortex each vial for 30 ± 2 seconds. Proceed as in (k) below. If necessary, using another vial, perform this procedure with 10 µL of Test Suspension A in place of one of the inoculated carriers.

(j) Treatment 3c: Optional Neutralizer Effectiveness (independent addition of SL). Add 50 µL of the test substance to each of three vials. Then add 10 mL of the neutralizer to each vial followed by the addition of 10 µL of the 3-part soil load to each vial and briefly swirl. After 10 seconds, add one dried carrier inoculated with Test Suspension A to each vial. Vortex each vial for 30 ± 2 seconds. Proceed as in (k) below. If necessary, using another vial, perform this procedure with 10 µL of Test Suspension A in place of one of the inoculated carriers.

(k) Hold the mixtures from (e) through (j) for 10 ± 1 minute at room temperature (22 ± 2°C). Steps (*e.g.*, addition of PBS, neutralizer) should be conducted at timed intervals (*e.g.* 1 min. intervals) to ensure consistent time of contact. At the conclusion of the holding period, vortex each vial for 30 ± 2 seconds and pass each mixture through a separate membrane filter. Use a magnet to prevent carriers from falling onto the filter membrane. Wash each vial with approximately 20 mL PBS and vortex for 5 ± 1 second; filter the washes through the same filter membrane. Repeat once (altogether 40 mL). Finish the filtering process by

rinsing the inside of the funnel unit with about 40 mL and filtering the rinsing liquid through the same filter membrane.

(l) Remove the membrane aseptically with sterile forceps and place it carefully over the surface of the recovery medium. Avoid trapping any air bubbles between the filter and the agar surface. Incubate the plates under the conditions appropriate for the test organism used.

(m) Examine the plates after incubation and record as CFU per carrier and – if necessary – per sample of the Test Suspension A or B. Calculate the averages for each set of test conditions with carriers.

Requirements for a Valid Test and Possible Outcomes

For the assay to be considered valid, ensure that:

(a) The recovered number of CFU in the optional Titer Control with SL (see section 3 (e)) using Test Suspension B yields 20-200 CFU per carrier and, if necessary, when using the microbial suspension as a liquid.

(b) The recovered number of CFU in the Titer Control without SL (see section 3 (f)) using Test Suspension A yields 20-200 CFU per carrier and, if necessary, when using the microbial suspension as a liquid.

For determining and verifying the effectiveness of the neutraliser, ensure that:

(a) The recovered number of CFU in the Neutraliser Toxicity Control [see section 3 (g)] is at least 75% of the Titer Control with SL (see a)). A count lower than 75% indicates that the neutraliser is harmful to the test organism. Note: counts higher than the Titer Control (e.g., 120% of the Titer Control) are also deemed valid.

(b) The recovered number of CFU in the Neutraliser Effectiveness (with SL) [see section 3 (h)] treatment is within 75% of the Titer Control; this verifies effective neutralisation in the presence of SL in the inoculum. The same results are expected for the optional Neutraliser Effectiveness (without SL) [see section 3 (i)] and Neutraliser Effectiveness (independent addition of SL) [see section 3 (j)] assays.

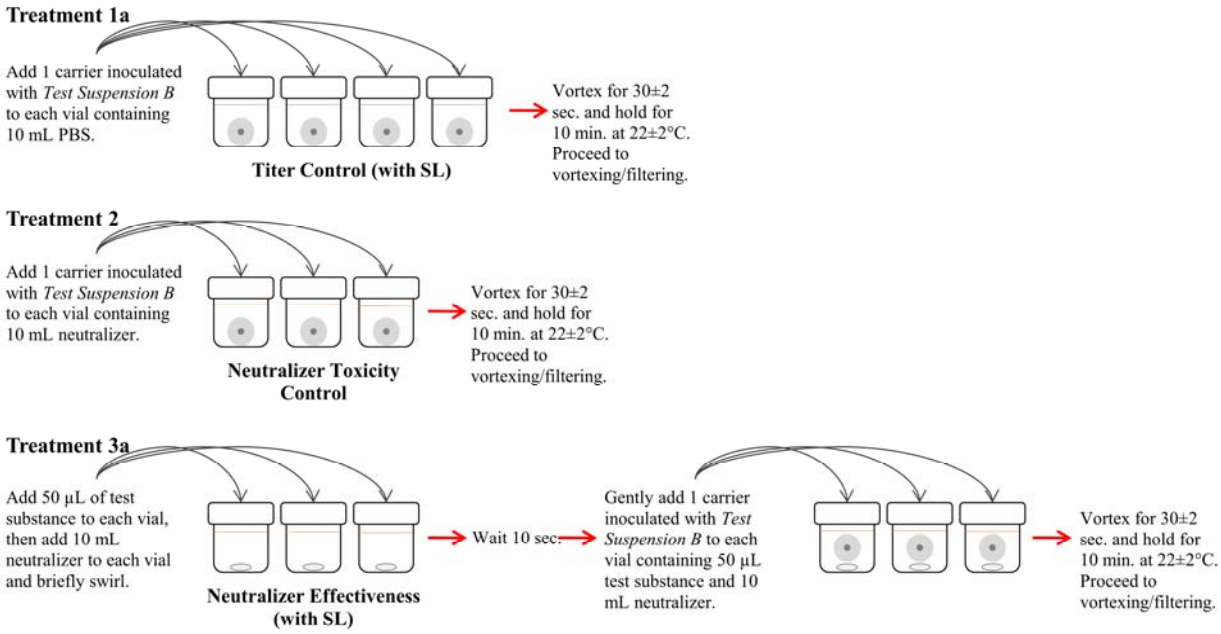
(c) The above criteria must be met. If the criteria are not met, another neutraliser or mixture of neutralisers must be identified and verified.

(d) Always compare the numbers of the Titer Controls determined on carriers with the numbers of the respective Neutraliser Toxicity Controls or Neutraliser Effectiveness assays determined on carriers. If a liquid microbial suspension is used instead of carriers, compare the numbers determined in the respective suspensions with each other accordingly.

If the determination and verification of an appropriate neutraliser is done for the first time – for a given test organism and test substance – all tests have to be performed including the optional tests and the tests with test suspension instead of carrier.

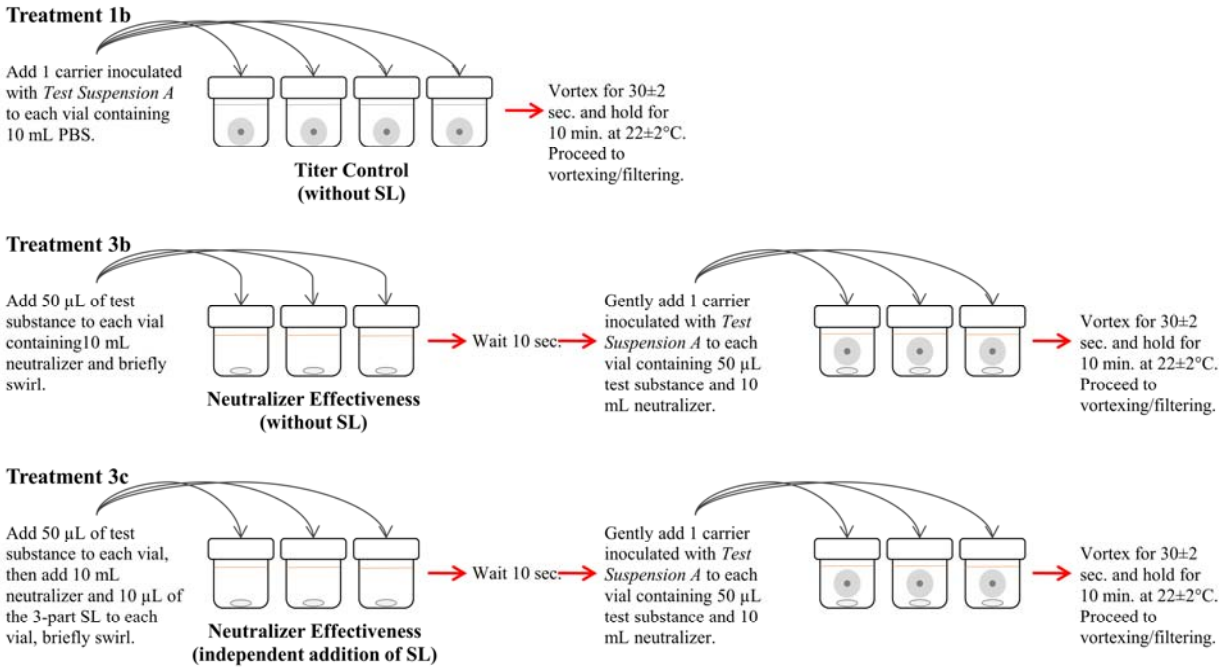
To reduce the workload when determining a suitable neutraliser, it is recommended to start with section 3 (a) and (c). Then the assays with carriers described in section 3 (b), (d), (e), (g) and (h) should be performed. Proceed with the test proper once an appropriate neutraliser has been identified.

OECD Neutralization Assay Flow Chart (required components)



Optional suspension test: Using an additional vial, perform each procedure using 10 µL of the appropriate test suspension in place of the inoculated carrier.

OECD Neutralization Assay Flow Chart (optional components)



Optional suspension test: Using an additional vial, perform each procedure using 10 µL of the appropriate test suspension in place of the inoculated carrier.

ANNEX 2

PROCEDURES FOR MAINTENANCE OF BACTERIAL CULTURES

The source of the test organisms may be the ATCC or another established culture collections such as the National Collection of Type Cultures (NCTC). Proper documentation on the source of the culture(s) and date(s) received by the testing laboratory should be on file.

Requirements

All test organisms for use in the quantitative carrier test should be maintained according to the procedures described here.

The purity and identity of the preserved test organism should be verified during preparation.

Methods

Principle

Upon receipt, the organism should be grown, aliquoted into vials and stored frozen at -70ºC or below. A frozen vial or bead is retrieved when needed and subcultured to make a stock which is subsequently used to prepare the test suspension for testing microbicides.

Material and reagents

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 or beads.

Culture media and reagents

Commercially prepared culture media and any ingredients purchased to make such media in-house are obtained from commercial sources. Chemicals/reagents are of analytical grade or appropriate for microbiological purposes. See Annex 3.

Procedure for preservation of vegetative bacteria

Reconstitution of the freeze-dried test organisms

- Open ampule of freeze dried organism per manufacturer's instructions. Using a tube containing 5-6 mL of TSB, aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth. Mix thoroughly. Incubate broth culture at 36 ± 1ºC for 24 ± 2 hours.

- After incubation, streak a loopful of the suspension on the agar surface of a suitable recovery medium to obtain isolated colonies. Incubate the plates at the required temperature for the time to allow for the growth of the organism; for example, an incubation of 18-24 h at 36 ± 1°C is sufficient for growing *Staphylococcus aureus*.

- Select and sample from representative colonies from the agar plate and assess for purity and identity of the test organism.

- Pick three to five isolated colonies of the test organism and resuspend in 1 mL of an appropriate broth medium. Place 0.1 mL of the suspension on each of several agar plates and perform spread-plating. Incubate the plates at the required temperature for the time to allow for the growth of the organism.

- Store the remaining portion of the suspension in a refrigerator until the culture identification and verification is complete.

Preparation for storage

- Details on cryoprotectant solutions are in Annex 3.

- Following the incubation of the agar plate (see the above section on *Reconstitution of the freeze-dried test organisms*), place 5 mL sterile cryoprotectant solution on the surface of each plate. Resuspend the growth in the solution using a glass spreader but without damaging the agar surface. Aspirate the suspension from the plate with a pipette and place it in a sterile bottle or tube large enough to hold about 30 mL. Repeat the growth harvesting procedure with the remaining plates and continue adding the suspension to the same tube. Mix the contents of the tube thoroughly.

- Immediately after mixing, pipette out 0.5-1.0 mL aliquots of the harvested suspension into separate properly labelled cryovials; these represent the frozen stock cultures.

- Alternatively, beads or commercially available kits (with beads) may be used for cryoprotection.

- Store the cryovials at -70ºC or lower (no more than 18 months). If vials or beads are depleted, a new test organism should be obtained from commercial source.

Preparation of test organisms from frozen stock cultures

- Defrost a cryovial or remove (using a wire or a pair of forceps) a single bead from a cryovial; such defrosting should be rapid to avoid loss in the viability of the preserved cells. Add 100 µL of defrosted material from the cryovial or one bead to 10.0 mL of appropriate broth medium and incubate under conditions suitable for the test organism. This is the working culture.

ANNEX 3

PREPARATION OF SOLUTIONS, REAGENTS AND MEDIA

General Remark: Use only reagent-grade chemicals.

Cryoprotectant solution

a. Cryoprotectant solution:

Beef extract		3.0 g
Tryptone, pancreatic digest of casein		5.0 g
Glycerol ($C_3H_8O_3$) [2]		150.0 g
Water	to	1000.0 g

Dissolve the constituents in boiling water. Sterilise in the autoclave. After sterilisation the pH of the solution should be equivalent to 6.9 ± 0.2 when measured at 20ºC ± 1ºC.

b. Culture Broth with 15% Glycerol ($C_3H_8O_3$)

Polysorbate-80 solution, consisting of:

Polysorbate-80		0.5 g
Water	to	1000.0 g

Sterilise by autoclaving.

Growth and recovery media and supplements

Required materials can be purchased from commercial sources. These materials may vary among suppliers or lots, and usage should be tracked as a part of proper quality assurance procedures. Conduct sterility tests on each new batch of liquid or semi-solid media by incubating at least two randomly selected broth tubes and agar plates for at least five days at 36 ± 1°C. Also check the ability of each new batch of medium to support the growth by inoculating it with 10-50 colonies of the test organism and incubating it at the required temperature.

a. Tryptone Soya Agar (TSA) for bacteria

Tryptone-soy broth powder	30 g
Bacteriological agar	15 g
Water	1000 mL

Add all to water; stir to dissolve on a hot plate. Sterilise by autoclaving.

b. Tryptone Soya Broth (TSB) for bacteria (Same as Soybean-Casein Digest Broth)

Tryptone, pancreatic digest of casein	17.0 g
Soya peptone, papaic digest of Soybean meal	3.0 g
Sodium chloride (NaCl)	5.0 g
Water	800.0 mL
Dipotassium phosphate (K_2HPO_4)	2.5 g
Glucose	2.5 g
Water	to 1000.0 mL

Sterilise by autoclaving. After sterilisation the pH of the medium should be equivalent to 7.2 ± 0.2 when measured at 20 ± 1ºC.

c. AOAC Synthetic broth (SB) or equivalent for *Pseudomonas aeruginosa*

Synthetic broth	17.0 g
Water	1 L
Sterile 10% glucose solution	10.0 mL

Add dehydrated synthetic broth powder to de-ionized water and bring to a boil while stirring to dissolve. Sterilise by autoclaving for 20 minutes. After cooling, aseptically add 10 mL sterile glucose solution to the medium. After sterilisation the pH of the medium should be equivalent to 7.1 ± 0.1 when measured at room temperature.

Alternatively, synthetic broth may be prepared as follows:

Solution A. Dissolve 0.05 g L-cystine, 0.37 g DL-methionine, 0.4 g L-arginine·HCl, 0.3 g DL-histidine, 0.85 g L-lysine·HCl, 0.21 g L-tyrosine, 0.5 g DL-threonine, 1.0 g DL-valine, 0.8 g L-leucine, 0.44 g DL-isoleucine, 0.06 g glycine, 0.61 g DL-serine, 0.43 g DL-alanine, 1.3 g L-glutamic acid·HCl, 0.45 g L-aspartic acid, 0.26 g DL-phenylalanine, 0.05 g DL-tryptophan, and 0.05 g L-proline in 500 mL H_2O containing 18 mL 1 N NaOH.

Solution B. Dissolve 3.0 g NaCl, 0.2 g KCl, 0.1 g $MgSO_4 \cdot 7\ H_2O$, 1.5 g KH_2PO_4, 4.0 g Na_2HPO_4, 0.01 g thiamine·HCl, and 0.01 niacinamide in 500 mL H_2O.

Mix Solutions A and B, final pH should be 7.1 ± 0.1, dispense in 10 mL portions in 20 × 150 mm tubes, and steam sterilize 20 min at 121°C. After cooling, aseptically add 0.1 mL sterile 10% glucose (dextrose) solution per tube.

Sterile 10% glucose solution

Add 10.0 g of dextrose to a 100 mL volumetric flask, fill with de-ionized water to the 100 mL mark and mix until dissolved. Filter sterilise using a 0.2 µm filter. Use to prepare synthetic broth.

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 PBS with Tween-80 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 and then mixed in equal volumes. Non-PBS based neutralizers may be use as deemed necessary.

Phosphate buffered saline (PBS)

Add 1.25 mL of PB stock solution and 8.75 g of NaCl to a volumetric flask, fill with distilled or deionised water to the 1000 mL mark, and mix; adjust pH to 6.5-7.5, if necessary. Sterilise by filtration or autoclaving. Alternative PBS formulations with the same pH may be used.

Phosphate buffered (PB) stock solution

Dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) 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 distilled or 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 6.5 – 7.5):

a. Add 0.5 g yeast extract to 10 mL of PBS (*low* molecular weight component), mix, and pass through a 0.2 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 0.2 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, autoclave and aliquot. Store at either 4 ± 2 °C or -20 ± 2 °C.

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

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 diluted test substance should be used ≤ 3 hours of preparation. The following is based on CEN method EN 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 anhydrous magnesium chloride ($MgCl_2$) (or 42.36 g $MgCl_2 \cdot 6H_2O$) and 46.24 g calcium chloride ($CaCl_2$) in water and dilute to 1000 mL. Sterilise by membrane filtration. 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 24 hours.

Note: The final hardness expressed as calcium carbonate ($CaCO_3$) is 375 mg/L +5%/-10%. Other levels of water hardness may be used as appropriate. Hach kit analysis may be used to determine the concentration of water hardness (as mg/L $CaCO_3$).

Complexometric Determination of the Water Hardness

Method

During the complexometric titration the calcium and magnesium ions are converted (transported) with a standard solution containing complex forming ligands (for example ethylene di-amine tetra acetic acid = EDTA). The end point of the titration is made visible by a change of the solution's colour after adding metal indicators.

Reagents

- The standard solution of the di-sodium salt of ethylene di-amine tetra acetic acid (1 ml solution corresponds to 1° dH ["German hardness"] for a sample quantity of 100 ml of the solution).
- Indicator pellets (Eriochrome Black T).
- Ammoniac buffer solution, pH 10.

Performance of the titration

Dilute one indicator pellet in 100 ml of the water to be analyzed. After the pellet has dissolved the solution is mixed with 5 ml of the ammoniac buffer solution. The thus prepared sample will be titrated with the EDTA- standard solution when the change of colour is green (until the moment when the colour becomes green).

Evaluation of the result

The numbers of millilitres of the measuring solution used indicates directly the water hardness in °dH. The numbers of millilitres of the measuring solution used indicates directly the water hardness in °dH. One °dH corresponds to 17.85 mg/L $CaCO_3$ and 21.06 °dH corresponds to 375 mg/L $CaCO_3$.

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 4

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.

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.

Bunsen burner: with a gas source and flame igniter.

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 typically identical in their topography and finish. If carriers are not brushed on both sides, inoculate the brushed side with the test organism. Refer to Annex 6 for specifications.

Centrifuge: to sediment the test organism(s) for concentration, or washing, or both.

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

Colony Counter (optional): for example, Quebec Colony Counter.

Cryovials

Desiccator: Vacuum source may be a pump or central supply.

Desiccant: Silica gel (silicon dioxide) placed in the bottom chamber of the desiccator to assist in the drying of the carriers. Fresh desiccant is required for adequate drying.

Dispenser: for dispensing sterile 10 mL aliquots of diluent/eluent.

Flasks: volumetric flasks and side arm flask to collect filtrate.

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.

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 ± 2°C to dry clean and wrapped sterile glassware.

Incubators: an incubator to maintain a temperature of 36 ± 1°C.

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. Polyethersulfone membranes with 0.2 or 0.45 µm pore diameter will 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.

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

Petri plates (plastic): 100 mm × 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, 10-1000 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.

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 commercial 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 at least 20 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 approximately 25 mm in neck diameter and hold at least 20 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).

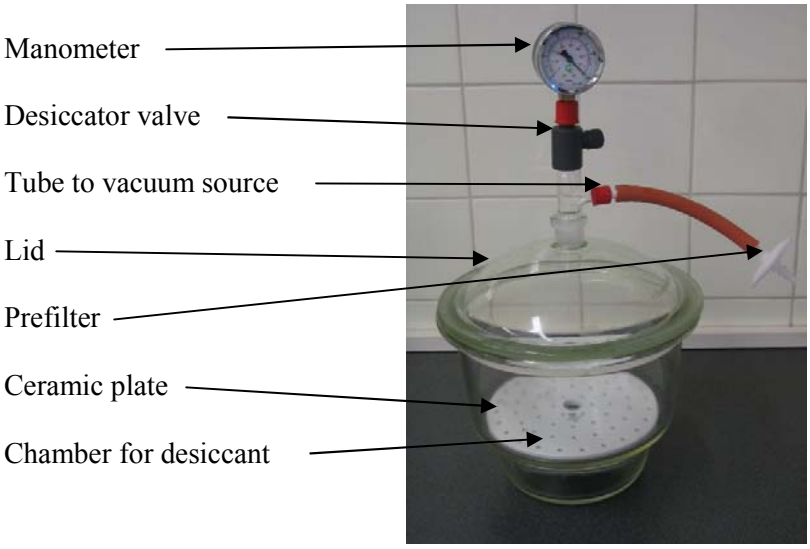
Water bath, capable of reaching and maintaining a temperature of 45 ± 1ºC to keep agar media from solidifying when making culture plates.

ANNEX 5

INSTRUCTIONS FOR USING A DESICCATOR TO VACCUM-DRY INOCULATED CARRIERS

Description

A desiccator is a container which is hermetically sealed with a lid. Inside is a ceramic plate for placing the carriers loaded with the inoculum to be dried. The desiccator is connected by a tube to either a central vacuum supply or a vacuum pump to evacuate the air. A manometer mounted on the lid shows the level of vacuum inside the desiccators.



Procedure

1. Use a thin and even layer of silicon grease on the contact surfaces between the lid and the desiccator for a good seal and also for easy removal of the lid at the end of the carrier drying process.

2. For drying, remove the lid and put the loaded carriers into the desiccator.

3. Replace the lid and close the desiccator valve.

4. Connect the tube to the vacuum source, close the desiccator valve and start the vacuum (the prefilter on the vacuum line is to prevent the escape of any microorganisms from the carriers into the air; each prefilter can be reused after autoclaving, but no more than five times). The evacuation of the air should continue for the entire drying period to ensure complete drying of the

carriers (60 minutes or until visibly dry). If the desiccator lid and all the connections are properly sealed the manometer should register in about five minutes a reading of 80-130 mbar (tables for converting mbars to "torr", "Pascal" or "inches mercury" are readily available on the Internet). If carriers are not dry within the specified time, check the desiccator system (*e.g.* refresh dessicant if necessary).

5. At the end of the drying period, switch off the vacuum source and open the desiccator valve.

ANNEX 6

CARRIER SPECIFICATIONS AND MAINTENANCE

- Ferritic stainless steel: Consist of chromium (17%) and iron and essentially nickel-free.
- AISI Type 430 (European equivalent name X6Cr17 and number 1.4016) belongs to Group 2, which is the most widely used family of ferritic alloys.
- Dimensions: 1 cm (0.39370 inch) in diameter; 0.7 mm (0.27559 inch) thick.
- AISI 430 - ASTM A240; Japanese Industrial Standard (JIS) G4305; EN 10088-2
- No. 4 Finish (EN 10088-2 1J/2J): A ground unidirectional finish obtained with 150 grit abrasive (AISI).
- Passivation: A soak in a mild acid bath for a few minutes to remove any impurities and accumulated debris from the disk surface.
- Tumbling: To remove the punching burrs from the edges of the discs they are tumbled in a barrel together with ceramic chips and a cleanser.

B "QUANTITATIVE METHOD FOR EVALUATING MYCOBACTERICIDAL 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 neutraliser 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 method is designed for testing the mycobactericidal activity of substances to be used on hard, non-porous surfaces (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. 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 twenty-seven laboratories from eight member countries participated and a validation report (5) was produced. Further, a collaborative evaluation of the bactericidal test method was conducted in 2012; the summary of the study is presented as an Annex to the validation report.

3. Details on relevant materials and reagents and the preparation of the test organisms are found in Annexes 2 to 5.

Requirements for test substance

4. The following information on the test substance should be determined (see regulatory authorities for specific requirements):

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 name and relative concentrations of active ingredients; such information may come from product label or 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 3, 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.

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 determination and verification of neutralisation of test substance.

j) Specifications for performance criteria when available.

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

l) Soil load to be used in testing.

m) Test substance diluent (if applicable)

INITIAL CONSIDERATIONS AND LIMITATIONS

6. The method employs disks of brushed stainless steel with magnetic properties.

7. Surrogate test organisms are specified herein; however, test organisms more relevant to other settings, e.g., dairy, baking or brewing industries are permitted in consultation with the target regulatory agency.

8. 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.

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

10. The method has been validated for testing liquid formulations. For testing other product forms, refer to paragraph 9 of the Preface, under "Scope of Application of the Tests".

PRINCIPLE OF THE TEST

11. 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) Determination and verification of neutralisation.

d) Inoculation, drying and transfer of the carriers.

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

12. 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. The use of small flat carriers allows for their complete immersion and elution in relatively small volumes of eluents. The incorporation of membrane filtration permits the processing of entire eluate volumes and more efficient removal of any residue of the test substance.

13. 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 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. Arithmetic mean $\log_{10}$ reductions in the numbers of viable test organisms following exposure to the test substance are calculated in relation to the mean number of viable of test organisms on the control carriers.

TEST PROCEDURE

14. The effectiveness of the neutralizer is determined prior to or during testing. For verification purposes the activity of the neutralizer will be verified per the methodology given in Annex 1.

Preparation and sterilisation of carriers

15. The carriers are soaked in a suitable detergent solution (*e.g.* Liquinox or equivalent) free from any antimicrobial activity for 2-4 hours to degrease and then rinsed thoroughly in distilled or deionised water. Carriers should be visually checked for abnormalities (e.g. rust, chipping) and discarded if observed. At least four control carriers and three test carriers are used for each test organism and contact time/temperature.

16. 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 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 or the inoculum is off-centre. The carriers are single use and should be discarded following testing.

Preparation of test organisms

17. The test organisms listed below were used in the ring trial and could be used for regulated testing. However, other specific test organisms 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) are acceptable alternatives. The maintenance of bacterial cultures is described in Annex 2. If other test organisms are employed, adapt growth and recovery media, incubation requirements and any other test parameters as necessary.

Mycobacterium terrae (ATCC 15755)

18. For this test organism, Middlebrook 7H9 broth containing glycerol and ADC Enrichment (100 mL aliquots in each of four culture flasks) are inoculated with 1 mL of stock culture and incubated (without shaking) at 36 ± 1°C for 20-22 days.

Preparation of Test Suspension

19. Following incubation, use the broth culture to prepare the test suspension. The broth culture should be centrifuged to achieve the desired level of viable organisms on the dried carrier count. Centrifuge the broth culture at between 5000 and 10000 g_N for 20 ± 5 minutes.

20. Supernatant is decanted and the cells washed by resuspending in sterile distilled/deionised water. This washing step is repeated two more times (if multiple tubes are centrifuged, pool the resuspended pellets).

21. The final pellet is resuspended in 10 mL of PBS. Five to seven mL of the resuspended suspension is placed in bijou bottle (or equivalent) with 10 glass beads and sterile PBS and vortexed for 45-60 seconds to break up clumps of the cells. This suspension should be used when prepared and not stored.

22. Initially the approximate CFU count of each freshly prepared microbial test suspension may be estimated spectrophotometrically at a wave length of approximately 650 nm, based on a standard curve specific to the test organism. This may act as a guide to the required dilutions but is confirmed by a plate count assay on the recovery medium to be used in the test. Only a higher number than the defined performance standard allows for statistical evaluation. Microbial counts should be confirmed in each test by determining the numbers of viable organisms on each of the control carriers.

23. Prior to inoculation of carriers, the soil load is aseptically added.

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, vortex each component and add 25 µL of BSA, 100 µL of mucin, and 35 µL of yeast extract stocks should be added to 340 µL of the microbial test suspension (see Table 1). The inoculum is vortexed again for 10 seconds.

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

Component	Volume (µL)
Test suspension	340
5% (w/v) BSA solution*	25
0.4% (w/v) Mucin solution*	100
5% (w/v) Yeast extrac*t	35
Total	**500**

*Note: final concentration of the stock solution.

Inoculation and drying of carriers

25. 10 µL of the inoculum are withdrawn with a calibrated positive-displacement pipette (Figure 1), and deposited at the centre of the brushed surface of the 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). Discard inoculated carriers where the inoculum has run off the centre or over the edge of the carrier.

26. The Petri dish is transferred with the inoculated carriers into a desiccator and the lid of the Petri dish is removed (Figure 3). Close the desiccator and check that it is properly sealed. The desiccator is evacuated using a vacuum source to achieve 20-25 inches mercury (508-635 torr; 677-847 mbar; 68000-85000 Pascal). Further details on using a desiccator are provided in Annex 5. The inoculated carriers are kept in the evacuated desiccator at 20-25°C for 60 ± 10 minutes (Figure 4). If carriers are not dry within the specified time, check the desiccator system.

Exposure of the dried inoculum 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 inoculum after desiccation to the test substance or carrier count control fluid is as follows:

- Using sterile forceps, transfer each dried carrier (Figure 5) with the inoculated side up to the flat bottom vial (Figure 6).
- Cap the vial.
- Repeat until all carriers are transferred.
- Carriers can be stored at 20-25°C for up to one hour.

- 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.

- The vial must be in a horizontal position to ensure uniform distribution of test substance onto carrier.

- Deposit 50 µL of the test substance, equilibrated to 20-25°C, over the dried inoculum on each test carrier, ensuring complete coverage, at predetermined staggered intervals (Figure 7); do not touch pipette tip to carrier, and do not cap the vials.

- Test carriers are held at 20-25°C for no more than the selected contact time (see paragraph 18 of the Preface).

- Control carriers are the last to be treated and receive 50 µL PBS, equilibrated to 20-25°C, instead of the test substance; the control carriers are treated in a manner identical to that for the test carriers.

29. The number of test organisms on the dried carriers should be 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 basis to which these numbers (0.5 $\log_{10}$ – 1.5 $\log_{10}$) are added will vary depending upon the log reduction required to demonstrate the passing of the performance standard as defined in paragraph 22 of the Preface, under "Performance Standards".

Neutralisation of test substance and elution of test organisms

30. Immediate (within 10 seconds) neutralisation of the test substance is required at the end of the contact time. Use less than 10 seconds as appropriate for shorter contact times. The protocol for the determination and verification of the neutraliser is given in Annex 1. At the end of the contact time, 10 mL* of the neutraliser is added to each vial according to the predetermined schedule. For consistency across laboratories/operators, this is documented as the 10^0 dilution. Note: *For ease of pipetting, 10 mL are used instead of 9.95 mL; this will not significantly affect the result.

31. Briefly (2-3 sec) vortex each vial following the addition of the neutralizer. Following the neutralization of the entire set of carriers, vortex each vial for 30 ± 5 seconds at high speed to recover the inoculum. If possible, each vial is capped and vortexed for approximately 30 seconds to recover the inoculum. Each carrier is examined visually and, in case of incomplete elution, further vortexing is performed. Do not remove carrier from vial.

Dilution and recovery

32. At this stage one mL of the eluate is removed and used for any needed 10-fold (1mL + 9mL) dilutions to get countable numbers of the test organism to determine $\log_{10}$ reductions in viability. The number of dilutions to be made and tested will depend on the initial inoculum and the level of microbicidal activity expected. Dilution and recovery are completed between five and 30 minutes at room temperature after the neutraliser is added to the vial containing the carrier.

33. All samples, control and treated, will be filtered (see Annex 4 for information on membrane filtration system). Direct plating is not allowed. The procedure for dilution and recovery is as follows:

- Prewet each membrane filter by passing through it about 10 mL of sterile PBS.
- Separate membrane filters but the same filtration unit may be used for processing the eluate

from a given carrier starting with the most dilute sample first. Always filter eluates from control carriers last to reduce risk of contamination of the eluates and filters from the test carriers.

- Prepare dilution vials before hand by adding diluent and labelling them (Figure 8).

- For the remaining 9.0 mL of the eluate, hold a magnet at the bottom of the vial to keep the carrier in place while pouring the contents of the vial into the membrane filtration system (Figure 9).

- Rinse vial with approximately 20 mL of PBS, vortex for five seconds and keeping magnet in place (while pouring), pour the wash into the same filtration system.

- Repeat this step one more time, swirl, and filter by applying vacuum.

- With vacuum on, rinse the inside surface of the funnel unit with an additional 40 mL of PBS. Note: If desired, the vacuum may be left on for the duration of the filtration process beginning with the wetting step.

- For dilution tubes, rinse each tube once with ~10 mL of PBS and briefly vortex.

- Remove the membrane filter aseptically with sterile forceps and place it carefully over the agar surface of the recovery medium (Middlebrook 7H11 Agar supplemented with OADC should be used as a Post-Exposure Recovery Medium), starting at the edge as illustrated to avoid trapping any air bubbles between the filter and the agar surface (Figure 10).

- Incubate the plates at 36 ± 1°C for 13-15 days and examine for colonies of the test organism.

34. The elution and filtration steps for control carriers are also the same as those described above for the test carriers. However, eluates from control carriers will always require 10-fold dilutions to provide countable plates (15-200 CFU/plate).

DATA AND REPORTING

Assessing performance of test substance

35. Performance is assessed by counting surviving bacteria from the each test carrier and comparing the number obtained to the mean of those on the control carriers. All colony counts are recorded. Counts between 15-200 are used in calculations to determine log reductions. However, if counts are less than 15 and no plates demonstrate counts between 15-200 CFU/plate, then those counts are used in calculations to determine log reductions. If no survivors, then use <1 CFU in calculations to determine log reductions.

36. Data are summarized in a tabular form showing raw data for each test and control plate. Data are also presented to validate the neutralisation process used in the test.

Calculating Log_{10} reductions

37. A method for determining arithmetic mean log_{10} reduction in the viability count of the test organisms by the test substance in quantitative carrier tests such as this one has been described (6).

Log_{10} Reduction = Average Log_{10} recovered from control carriers - Average Log_{10} recovered from the test carriers

Test report

38. The test report includes, but not limited to 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 name and relative concentrations of active ingredients.

Details on the test method

Test organism

- Source
- Scientific name and strain number
- Growth and recovery media
- Preparation of the stock and working culture

Test conditions

- Concentration of the test substance
- Temperature
- Contact time
- Test substance diluent (hard water level and titration)
- Soil load

Results

- CFU per carrier
- Log_{10} reduction
- Neutralisation determination and verification
- 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 (2011) Report on validation of efficacy methods for antimicrobials used on hard surfaces – Part I & Part II, [ENV/JM/MONO(2011)34], OECD Environmental Health and Safety Publications, series on Testing and Assessment, No. 154.

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

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

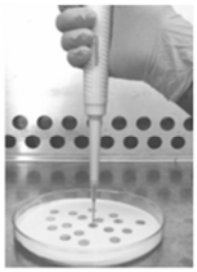


Figure 3 (left): Petri plate lid is removed during drying of carriers

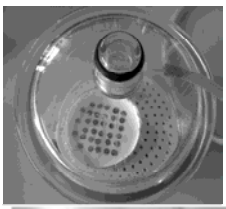


Figure 4 (right): Carriers left in an evacuated desiccator to dry at room temperature for 60±10 minutes.

Figure 5 (left): Carrier with dried inoculum being picked up for placement in flat bottom vial

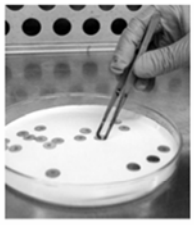


Figure 6 (right): Carrier placed into the flat bottom vial

Figure 7 (left): Dried inoculum on carrier covered with 50 µL of test substance or control fluid

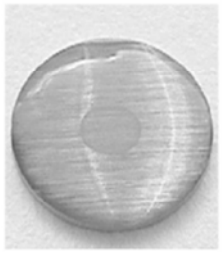


Figure 8 (right): Labelled dilutions vials with diluent; tubes may be used in place vials

Figure 9 (left): Magnet placed on the outside bottom of the vial to hold the default carrier in place while pouring eluate into filter funnel.

Figure 10 (right): Membrane filter being placed on surface of recovery agar to avoid trapping air bubbles underneath the filter.

ANNEX 1

DETERMINATION AND VERIFICATION OF APPROPRIATE NEUTRALIZATION

Purpose

The neutralization of the active ingredients found in antimicrobial products is one of the most important steps in product efficacy testing. A neutralization process and/or chemical agent (e.g., combined use of dilution, chemical neutralization, membrane filtration and rinsing of the membrane filters) are used in each test to inactivate the product's active ingredients, a process essential to achieving the desired contact time and full recovery of viable test microorganism. In addition, the neutralizer itself or in combination with the organic burden must not exhibit bacteriostatic activity against the test microbe as bacteriostatic activity may bias the efficacy data. It is preferable to perform the neutralization assay prior to or concurrently with product testing.

The neutralization should be confirmed and verified separately for each test substance against each test organism to be used in the method. Furthermore, it might be necessary to employ different neutralizers for different test organisms. If several concentrations of a given test substance are being evaluated, the highest concentration is tested to determine and verify neutralization. If the concentration(s) is (are) unknown, the test may be performed with different concentrations of the test substance in parallel.

Principle

The neutralizer is added to the carrier vial immediately at the end of the contact time. This dilutes the test substance while also chemically neutralising its microbicidal/microbistatic activity. Except in tests for virucidal activity, the entire eluate is then passed through a membrane filter which is washed with phosphate buffered saline (PBS) to further remove any residues of the test substance; the membrane is placed on a suitable recovery medium.

In the neutraliser determination/verification assay, the test substance is first mixed with a candidate neutraliser. The test organism is then added to the reaction mixture as dried inoculum on a carrier; if desired, additional evaluations may be conducted using the test organism as a liquid. The neutralisation process is deemed acceptable if there is no statistically significant difference between the numbers of viable organisms recovered from the neutralised test substance and the controls including those for any microbistatic or microbicidal effect of the neutraliser itself.

Two separate test suspensions (A and B) are required; A is a diluted suspension of the test organism, and B is the test microbial suspension with the added soil load.

Procedure

(a) Prepare Test Suspension A: Dilute the test microbial suspension with PBS to achieve an average challenge of 20-200 CFU/carrier after the 10 µL inoculum has dried. Prior testing may be required to account for differences in the loss of viability of the different test organisms on drying (see further section: "Requirements for a valid test and possible outcomes"). Test Suspension A should be used within 4 hours of preparation.

(b) Prepare Test Suspension B: First prepare the soil load by combining 25 µL 5% (w/v) BSA, 35 µL 5% (w/v) yeast extract, and 100 µL of 0.4% (w/v) mucin – mix well. Combine 132 µL of Test Suspension A and 68 µL of the soil load (SL). The test microbial suspension with soil load should also achieve an average challenge of 20-200 CFU/carrier after drying.

(c) If desired, estimate the number of viable organisms in Test Suspensions A and B by measuring the optical density (OD at 650 nm) of the suspensions and creating a calibration curve.

(d) Carrier inoculation: Inoculate at least 10 carriers with Test Suspension A and at least 10 carriers with Test Suspension B by adding 10 µL to each carrier. Follow instructions in the test method for drying the carriers.

(e) Treatment 1: Titer Control (with OSL). Add one dried carrier inoculated with Test Suspension B to each of three vials. Add 10 mL PBS to each vial. Vortex each vial for 20 ± 2 seconds. Proceed as in (k) below. If deemed necessary, using another vial, perform this procedure with 10 µL of Test Suspension B in place of the inoculated carrier.

(f) Treatment 1a: Optional Titer Control (without OSL). Add one dried carrier inoculated with Test Suspension A to each of three vials. Add 10 mL PBS to each vial. Vortex each vial for 20 ± 2 seconds. Proceed as in (k) below. If deemed necessary, using another vial, perform this procedure with 10 µL of Test Suspension A in place of the inoculated carrier.

(g) Treatment 2: Neutralizer Toxicity Control. Add one dried carrier inoculated with Test Suspension B to each of three vials. Add 10 mL neutralizer to each vial. Vortex each vial for 20 ± 2 seconds. Proceed as in (k) below. If deemed necessary, using another vial, perform this procedure with 10 µL of Test Suspension B in place of the inoculated carrier.

(h) Treatment 3: Neutralizer Effectiveness (with OSL). Add 50 µL of the test substance to each of three vials then add 10 mL neutralizer to each vial. After 10 seconds, gently add one dried carrier inoculated with Test Suspension B to each vial. Vortex each vial for 20 ± 2 seconds. Proceed as in (k) below. If deemed necessary, using another vial, perform this procedure with 10 µL of Test Suspension B in place of the inoculated carrier.

(i) Treatment 3a: Optional Neutralizer Effectiveness (without OSL). Add 50 µL of the test substance to each of three vials then add 10 mL neutralizer each vial. After 10 seconds, gently add one dried carrier inoculated with Test Suspension A to each vial. Vortex each vial for 20 ± 2 seconds. Proceed as in (k) below. If deemed necessary, using another vial, perform this procedure with 10 µL of Test Suspension A in place of the inoculated carrier.

(j) Treatment 3b: Optional Neutralizer Effectiveness (independent addition of OSL). Add 50 µL of the test substance to each of three vials, and then add 10 mL of the neutralizer to each vial followed by the addition of 10 µL of the 3-part soil load to each vial. After 10 ± 2 seconds, add one dried carrier inoculated with Test Suspension A to each vial. Vortex each vial for 20 seconds. Proceed as in (k) below. If deemed necessary, using another vial, perform this procedure with 10 µL of Test

Suspension A in place of the inoculated carrier.

(k) Hold the mixtures from (e) through (j) for 10 ± 1 minutes at room temperature (22 ± 2 °C). Steps (*e.g.*, addition of PBS, neutralizer) should be conducted at timed intervals (*e.g.*, 1 min. intervals) to ensure consistent time of contact. At the conclusion of the holding period, vortex each vial for 20 ± 2 seconds and pass each mixture through a separate membrane filter. Use a magnet to prevent carriers from falling onto the filter membrane. Wash each vial and the inside of the filter apparatus with approximately 50 mL PBS; filter the washes through the same filter membrane.

(l) Remove the membrane filter aseptically with sterile forceps and place it carefully over the surface of the recovery medium. Avoid trapping any air bubbles between the filter and the agar surface. Incubate the plates under the conditions appropriate for the test organism used.

(m) Examine the plates after incubation and record as CFU per vial. Calculate the averages for each set of test conditions.

Requirements for a Valid Test and Possible Outcomes

For the assay to be considered valid, ensure that:

a) The recovered number of CFU in the optional Titer Control without OSL using Test Suspension A yields approximately 20-200 CFU/carrier.

b) The recovered number of CFU in the Titer Control with OSL using Test Suspension B yields approximately 20-200 CFU/carrier.

For determining and/or verifying the effectiveness of the neutralizer:

a) The recovered number of CFU in the Neutralizer Toxicity Control is at least 75% of the Titer Control. A count lower than 75%indicates that the neutralizer is harmful to the test organism. Note: counts higher than the Titer Control (*e.g.*, 120% of the titer control) are also deemed valid.

b) The recovered number of CFU in the Neutralizer Effectiveness (with OSL) treatment is within 75% of the Titer Control; this verifies effective neutralization in the presence of the soil load in the inoculum. The same results are expected for the optional Neutralizer Effectiveness (without OSL) and Neutralizer Effectiveness (independent addition of OSL) assays.

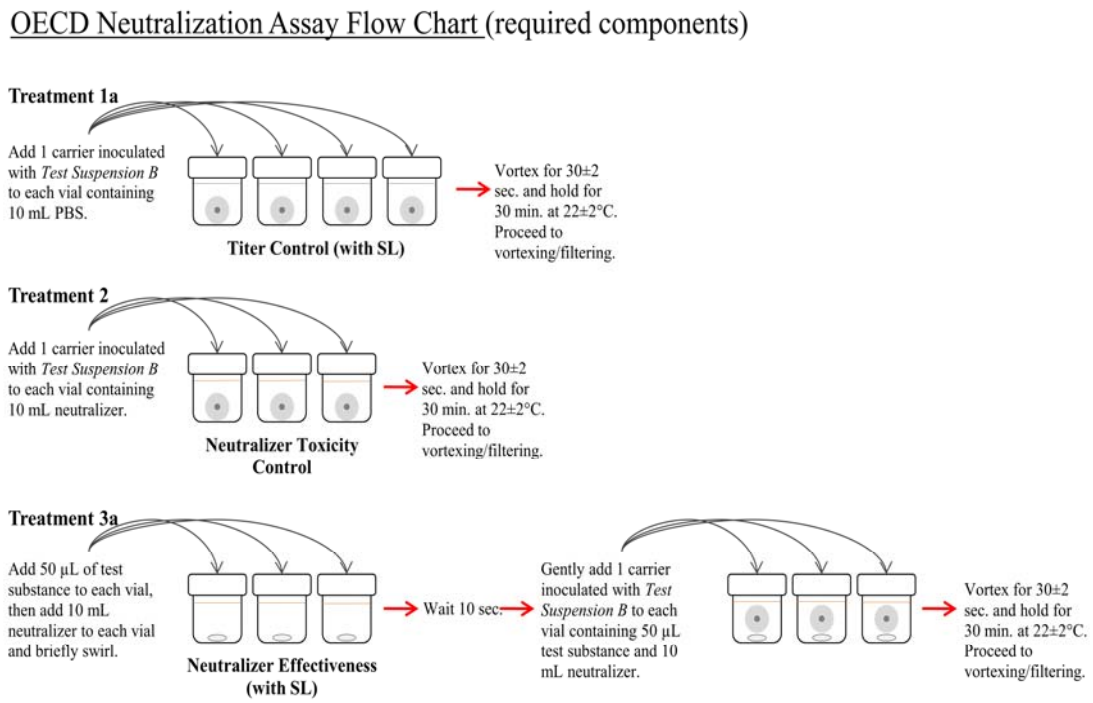
c) The above criteria must be met. If the criteria are not met, another neutralizer or mixture of neutralizers must be identified and verified.

d) Always compare the numbers of the Titer Controls determined on carriers with the numbers of the respective Neutraliser Toxicity Controls or Neutraliser Effectiveness assays determined on carriers. If a liquid microbial suspension is used instead of carriers, compare the numbers determined in the respective suspensions with each other accordingly.

If the determination and verification of an appropriate neutraliser is done for the first time – for a given test organism and test substance – all tests have to be performed including the optional tests and the tests with test suspension instead of carrier.

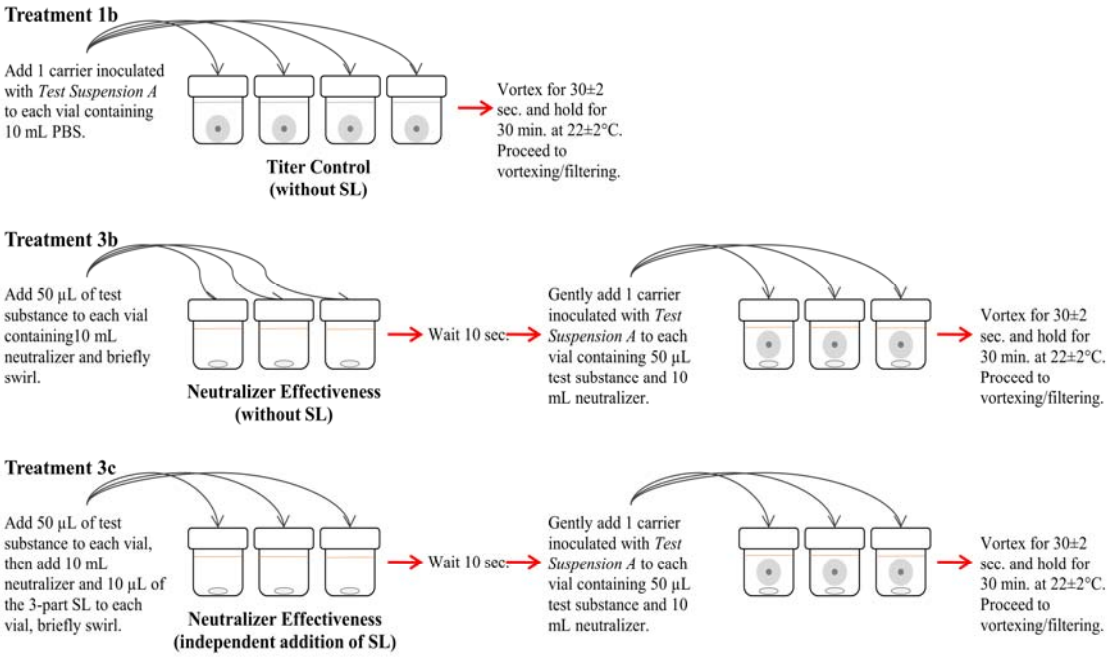
To reduce the workload when determining a suitable neutraliser, it is recommended to start with section 3 (a) and (c). Then the assays with carriers described in section 3 (b), (d), (e), (g) and (h) should be performed. Proceed with the test proper once an appropriate neutraliser has been identified.

OECD Neutralisation Assay Flow Chart



Optional suspension test: Using an additional vial, perform each procedure using 10 µL of the appropriate test suspension in place of the inoculated carrier.

OECD Neutralization Assay Flow Chart (optional components)



Optional suspension test: Using an additional vial, perform each procedure using 10 µL of the appropriate test suspension in place of the inoculated carrier.

ANNEX 2

PROCEDURES FOR MAINTENANCE OF MYCOBACTERIAL CULTURES

The source of the test organisms may be the ATCC or another established culture collections such as the National Collection of Type Cultures (NCTC). Proper documentation on the source of the culture(s) and date(s) received by the testing laboratory should be on file.

Requirements

All test organisms for use in the quantitative carrier test should be maintained according to the procedures described here.

The purity and identity of the preserved test organism should be verified during preparation.

Methods

Principle

Upon receipt, the organism should be grown, aliquoted into vials and stored frozen at -70ºC or below. A frozen vial or beads is retrieved when needed and subcultured to make a stock which is subsequently used to prepare working cultures for testing microbicides.

Material and reagents

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 or beads.

Culture media and reagents

Commercially prepared culture media and any ingredients purchased to make such media in-house are obtained from commercial sources. Chemicals/reagents are of analytical grade or appropriate for microbiological purposes. See Annex 3.

Procedure for preservation of vegetative bacteria

Reconstitution of the freeze-dried test organisms

- Open ampule of freeze dried organism per manufacturer's instructions. Using a tube containing 5-6 mL of the suspension medium MADC, aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture.

- Inoculate one agar plate (M7H9 or M7H11) with 0.1 mL of the test organism suspension and spread to achieve a lawn of growth. Streak a loopful of the suspension on the agar surface of a suitable recovery medium (M7H9 or M7H11) to obtain isolated colonies for purity check. Incubate for 20-22 days at 36 ± 1°C.

- Select and sample from representative colonies from the agar plate and assess for purity and identity of the test organism.

- Store the remaining portion of the suspension in a refrigerator until the culture identification and verification is complete

Preparation for storage

- Details on cryoprotectant solutions are in Annex 3.

- At the end of the incubation period, add 5 mL MADC to the surface of each agar plate. It is recommended to inoculate multiple agar plates to prepare sufficient number of cryovials for several tests. Resuspend the cells in this solution using a glass spreader and aspirate the cell suspension from the surface of the agar. Be careful not to disrupt the agar during harvesting. Transfer suspension into a sterile vessel. Repeat by adding another 5 mL of MADC to the agar plates, resuspend the cells, aspirate suspension and pool with the initial cell suspension. Mix the contents of the tube thoroughly.

- Immediately after mixing, pipette out 1.0-1.5 mL aliquots of the harvested suspension into separate properly labelled cryovials; these represent the frozen stock cultures.

- Alternatively, beads or commercially available kits (with beads) may be used for cryoprotection.

- Store the cryovials at -70ºC or lower (no more than 18 months). If vials or beads are depleted, a new test organism should be obtained from commercial source.

Preparation of test organisms from frozen stock cultures

- Defrost the cryovial or remove (using a wire or a pair of forceps) a single bead from a cryovial; such defrosting should be rapid to avoid loss in the viability of the preserved cells. One bead or 1 mL of thawed culture may be used to directly inoculate four separate flasks of 100 mL MADC broth per the efficacy test methodology. Incubate at 36 ± 1°C for 20-22 days.

ANNEX 3

PREPARATION OF SOLUTIONS, REAGENTS AND MEDIA

General Remark: Use only reagent-grade chemicals.

Cryoprotectant solution

Cryoprotectant solution:

Beef extract	3.0 g
Tryptone, pancreatic digest of casein	5.0 g
Glycerol ($C_3H_8O_3$) [2]	150.0 g
Water	to 1000.0 g

Dissolve the constituents in boiling water. Sterilise in the autoclave. After sterilisation the pH of the solution should be equivalent to 6.9 ± 0.2 when measured at 20ºC ± 1ºC.

Growth and recovery media and supplements

Required materials can be purchased from commercial sources. These materials may vary among suppliers or lots, and usage should be tracked as a part of proper quality assurance procedures. Conduct sterility tests on each new batch of liquid or semi-solid media by incubating at least two randomly selected broth tubes and agar plates for at least five days at 36 ± 1°C. Also check the ability of each new batch of medium to support the growth by inoculating it with 10-50 colonies of the test organism and incubating it at the required temperature.

Middlebrook 7H9 Broth with 10% ADC enrichment and glycerol (MADC)

Middlebrook 7H9 broth powder	4.7g
Glycerol ($C_3H_8O_3$)	150.0g
Water	750.0mL

Sterilise in autoclave. Add under aseptic conditions, 100 mL Middlebrook ADC enrichment and then add sterilised water up to 1000 mL. The pH of the medium should be 6.6 ± 0.2.

Middlebrook 7H11 broth

Middlebrook 7H11 broth powder	19.0 g
Glycerol ($C_3H_8O_3$)	5.0 mL
Water	895.0 mL

Dissolve 19 g of dehydrated medium in 900 mL H_2O containing 5 mL glycerol: Heat to boiling to dissolve completely. Autoclave sterilise. Cool sterile media to 45-50 °C, add 100 mL of Middlebrook ADC Enrichment under aseptic conditions and mix thoroughly.

Oleic Acid Albumin Dextrose Catalase Complex (OADC)

Solution I*: Albumin Dextrose Catalase Complex (ADCC)*

Bovine serum albumin fraction V (Fisher Scientific: BP1600-100)	50.0 g
NaCl (Sigma-Aldrich: EC 231-598-3)	8.1 g
Glucose (Sigma®: EC 200-075-1)	20.0 g
Catalase (Bovine) (Sigma: C-10 EC1.11.1.6)	0.04 g
Deionised water (dd H_2O)	900.0 mL

Add all to distilled or deionised H_2O and stir to dissolve on a stir plate

Solution II: *Sodium oleate solution*

Oleic Acid (Fisher Scientific: CAS 112-80-1)	0.6 mL
6 M NaOH (Sigma: EC215-185-5; S 5881)	0.6 mL
Deionised water (dd H_2O)	30.0 mL

Add all to a 200 mL Erlenmeyer flask and incubate in a water bath at 50°C until solution is clear

Add solution II to solution I. Adjust pH to ~7.0 with 1N NaOH. Add dd H_2O up to one L and filter to sterilise. Store at 4 ºC.

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 PBS with Tween-80 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 and then mixed in equal volumes. Non-PBS based neutralizers may be used as deemed necessary.

Phosphate buffered saline (PBS)

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

Phosphate buffered (PB) stock solution

Dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) 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 distilled or 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 6.5-7.5):

a. Add 0.5 g yeast extract to 10 mL of PBS (*low* molecular weight component), mix, and pass through a 0.2 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 0.2 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, autoclave and aliquot. Store at either 4 ± 2 °C or -20 ± 2 °C.

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

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 diluted test substance should be used ≤3 hours of preparation. The following is based on CEN method EN 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 anhydrous magnesium chloride ($MgCl_2$) (or 42.36 g $MgCl_2 \cdot 6H_2O$) and 46.24 g calcium chloride ($CaCl_2$) in water and dilute to 1000 mL. Sterilise by membrane filtration. 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 24 hours.

Note: The final hardness expressed as calcium carbonate ($CaCO_3$) is 375 mg/L +5%/-10%. Other levels of water hardness may be used as appropriate. Hach kit analysis may be used to determine the concentration of water hardness (as mg/L $CaCO_3$).

Complexometric Determination of the Water Hardness

Method

During the complexometric titration the calcium and magnesium ions are converted *(transported)* with a standard solution containing complex forming ligands (for example ethylene di-amine tetra acetic

acid = EDTA). The end point of the titration is made visible by a change of the solution's colour after adding metal indicators.

Reagents

- The standard solution of the di-sodium salt of ethylene di-amine tetra acetic acid (1 ml solution corresponds to 1° dH ["German hardness"] for a sample quantity of 100 ml of the solution).

- Indicator pellets (Eriochrome Black T).

- Ammoniac buffer solution, pH 10.

Performance of the titration

Dilute one indicator pellet in 100 ml of the water to be analyzed. After the pellet has dissolved the solution is mixed with 5 ml of the ammoniac buffer solution. The thus prepared sample will be titrated with the EDTA- standard solution when the change of colour is green (until the moment when the colour becomes green).

Evaluation of the result

The numbers of millilitres of the measuring solution used indicates directly the water hardness in °dH. One °dH corresponds to 17.85 mg/L $CaCO_3$ and 21.06 °dH corresponds to 375 mg/L $CaCO_3$.

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 4

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.

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

Bijou bottles with ten glass beads (3-5 mm in diameter) in each, or equivalent

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

Bunsen burner: with a gas source and flame igniter.

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 typically identical in their topography and finish. If carriers are not brushed on both sides, inoculate the brushed side with the test organism. Refer to Annex 6 for specifications.

Centrifuge: to sediment the test organism(s) for concentration, or washing, or both.

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

Colony Counter (optional): for example, Quebec Colony Counter.

Cryovials

Desiccator: Vacuum source may be a pump or central supply.

Desiccant: Silica gel (silicon dioxide) placed in the bottom chamber of the desiccator to assist in the drying of the carriers. Fresh desiccant is required for adequate drying.

Dispenser: for dispensing sterile 10 mL aliquots of diluent/eluent.

Flasks: volumetric flasks and side arm flask to collect filtrate.

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.

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 5 mm in diameter.

Gloves: sterile, disposable, for handling test items.

Hot Air Oven: an oven at 60 ± 2 °C to dry clean and wrapped sterile glassware.

Incubators: an incubator to maintain a temperature of 36 ± 1 °C.

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. Polyethersulfone membranes with 0.2 or 0.45 µm pore diameter will 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.

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, 10-1000 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.

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 commercial 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 at least 20 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 approximately 25 mm in neck diameter and hold at least 20 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).

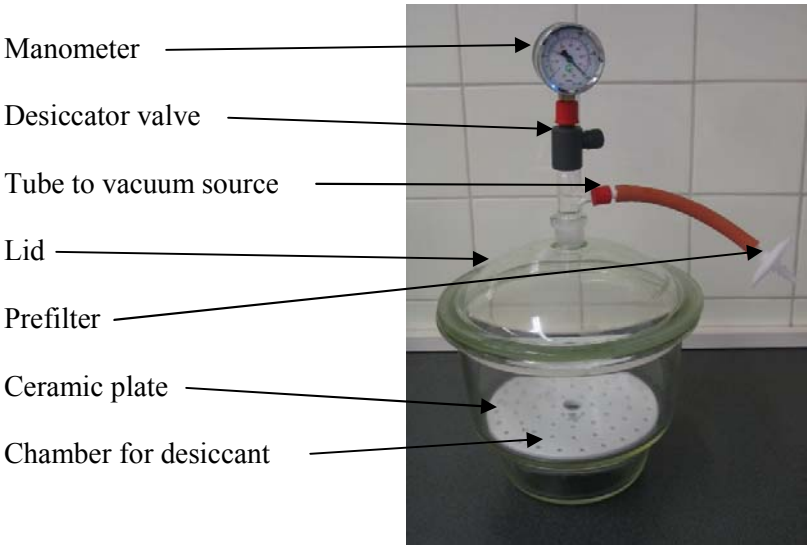
Water bath, capable of reaching and maintaining a temperature of 45 ± 1ºC to keep agar media from solidifying when making culture plates.

ANNEX 5

INSTRUCTIONS FOR USING A DESICCATOR TO VACCUM-DRY INOCULATED CARRIERS

Description

A desiccator is a container which is hermetically sealed with a lid. Inside is a ceramic plate for placing the carriers loaded with the inoculum to be dried. The desiccator is connected by a tube to either a central vacuum supply or a vacuum pump to evacuate the air. A manometer mounted on the lid shows the level of vacuum inside the desiccators. A desiccator is a container which is hermetically sealed with a lid. Inside is a ceramic plate for placing the carriers loaded with the inoculum to be dried. The desiccator is connected by a tube to either a central vacuum supply or a vacuum pump to evacuate the air. A manometer mounted on the lid shows the level of vacuum inside the desiccators.



Procedure

6. Use a thin and even layer of silicon grease on the contact surfaces between the lid and the desiccator for a good seal and also for easy removal of the lid at the end of the carrier drying process.

7. For drying, remove the lid and put the loaded carriers into the desiccator.

8. Replace the lid and close the desiccator valve.

9. Connect the tube to the vacuum source, close the desiccator valve and start the vacuum (the prefilter on the vacuum line is to prevent the escape of any microorganisms from the carriers into

the air; each prefilter can be reused after autoclaving, but no more than five times). The vacuation of the air should continue for the entire drying period (60 minutes or until visibly dry) to ensure complete drying of the carriers. If the desiccator lid and all the connections are properly sealed the manometer should register in about five minutes a reading of 80-130 mbar (tables for converting mbars to "torr", "Pascal" or "inches mercury" are readily available on the Internet). If carriers are not dry within the specified time, check the desiccator system (*e.g.* refresh dessicant if necessary).

10. At the end of the drying period, switch off the vacuum source and open the desiccator valve.

ANNEX 6

CARRIER SPECIFICATIONS AND MAINTENANCE

- Ferritic stainless steel: Consist of chromium (17%) and iron and essentially nickel-free.
- AISI Type 430 (European equivalent name X6Cr17 and number 1.4016) belongs to Group 2, which is the most widely used family of ferritic alloys.
- Dimensions: 1 cm (0.39370 inch) in diameter; 0.7 mm (0.27559 inch) thick.
- AISI 430 - ASTM A240; Japanese Industrial Standard (JIS) G4305; EN 10088-2
- No. 4 Finish (EN 10088-2 1J/2J): A ground unidirectional finish obtained with 150 grit abrasive (AISI).
- Passivation: A soak in a mild acid bath for a few minutes to remove any impurities and accumulated debris from the disk surface.
- Tumbling: To remove the punching burrs from the edges of the discs they are tumbled in a barrel together with ceramic chips and a cleanser.

C "QUANTITATIVE METHOD FOR EVALUATING FUNGICIDAL 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 neutraliser 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 method is designed for testing the fungicidal activity of substances to be used on hard, non-porous surfaces (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. 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 twenty-seven laboratories from eight member countries participated and a validation report (5) was produced. Further, a collaborative evaluation of the bactericidal test method was conducted in 2012; the summary of the study is presented as an Annex to the validation report.

3. Details on relevant materials and reagents and the preparation of the test organisms are found in Annexes 2 to 5.

Requirements for test substance

4. The following information on the test substance should be determined (see regulatory authorities for specific requirements):

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 name and relative concentrations of active ingredients; such information may come from product label or 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 3, 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.

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 determination and verification of neutralisation of test substance.

j) Specifications for performance criteria when available.

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

l) Soil load to be used in testing.

m) Test substance diluent (if applicable)

INITIAL CONSIDERATIONS AND LIMITATIONS

6. The method employs disks of brushed stainless steel with magnetic properties.

7. A surrogate test organism is specified herein; however, test organisms more relevant to other settings, *e.g.* dairy, baking or brewing industries are permitted in consultation with the target regulatory agency.

8. 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.

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

10. The method has been validated for testing liquid formulations. For testing other products forms, refer to paragraph 9 of the Preface, under "Scope of Application of the Tests".

PRINCIPLE OF THE TEST

11. 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 (fungicide) 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) Determination and verification of neutralisation.

d) Inoculation, drying and transfer of the carriers.

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

12. 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. The use of small flat carriers allows for their complete immersion and elution in relatively small volumes of eluents. The incorporation of membrane filtration permits the processing of entire eluate volumes and more efficient removal of any residue of the test substance.

13. 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 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. Arithmetic mean $\log_{10}$ reductions in the numbers of viable test organisms following exposure to the test substance are calculated in relation to the mean number of viable of test organisms on the control carriers.

TEST PROCEDURE

14. The effectiveness of the neutralizer is determined prior to testing. For verification purposes the activity of the neutralizer will be verified per the methodology given in Annex 1.

Preparation and sterilisation of carriers

15. The carriers are soaked in a suitable detergent solution (*e.g.* Liquinox or equivalent) free from any antimicrobial activity for 2-4 hours to degrease and then rinsed thoroughly in distilled or deionised water. Carriers should be visually checked for abnormalities (*e.g.* rust, chipping) and discarded if observed. At least four control carriers and three test carriers are used for each test organism and contact time/temperature.

16. 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 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 or the inoculum is off-centre. The carriers are single use and should be discarded following testing.

Preparation of test organisms

17. The test organisms listed below were used in the ring trial and could be used for regulated testing. However, other specific test organisms 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) are acceptable alternatives. The maintenance of fungal cultures is described in Annex 2. If other test organisms are employed, adapt growth and recovery media, incubation requirements and any other test parameters as necessary.

Aspergillus niger (ATCC 64958)

18. For this test organism, the culture medium is prepared as follows:

- Growth and recovery media – plates of Sabouraud's Dextrose Agar.

- The stock culture of *A. niger* is maintained on a Sabouraud's Dextrose Agar plate at 4 ± 2°C. At one-month intervals, fresh agar plates are inoculated and incubated for ten days at 30 ± 2°C. Conidial suspensions are prepared from these stocks.

19. To prepare a conidial suspension, a loop of the test fungus is inoculated at the centre of each of four or more Sabouraud's Dextrose plates and then incubated at 30 ± 2°C for ten days. The mycelial matt is removed from the plates with a sterile spatula and transferred to a 250 mL conical flask containing 25-50 mL PBS and 5-7 glass beads. The flask is shaken vigorously enough to break off the conidia from the hyphae.

20. The resulting suspension is filtered through sterile absorbent cotton and conidia are collected in the filtrate. The suspension is standardized as needed for testing by diluting it with PBS. The conidial concentration in the suspension may be estimated by using any suitable means (e.g. haemocytometer, microscopic evaluation, plate count, spectrophotometrically). Conidial suspensions stored at 4 ± 2°C can be used for up to four weeks to inoculate carriers.

Preparation of Test Suspension

21. Following storage, use the conidial suspension to prepare a test suspension of the test organism. The conidial suspension should be centrifuged as described below to achieve the desired level of viable organisms on the dried carrier count. Test organism requires centrifugation of the broth culture to

obtain the required number of viable conidia. The product of centrifugation (*g* force) and time for which it is applied (t minutes) determines the organism's sedimentation rate. The centrifugation should be between 5000 and 10000 g_N for 20 ± 5 minutes and resuspend the pellets in PBS. Centrifugation for less than 5000 g_N may result in incomplete sedimentation of the test fungi. If multiple tubes are centrifuged, pool the resuspended pellets.

22. Initially the approximate CFU count of each freshly prepared microbial test suspension may be estimated spectrophotometrically at a wave length of approximately 650 nm, based on a standard curve specific to the test organism. This may act as a guide to the required dilutions but is confirmed by a plate count assay on the recovery medium to be used in the test. Only a higher number than the defined performance standard allows for statistical evaluation. Microbial counts are confirmed in each test by determining the numbers of viable organisms on each of the control carriers.

23. Prior to the inoculation of carriers, the soil load is aseptically added.

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, vortex each component and add 25 µL of BSA, 100 µL of mucin, and 35 µL of yeast extract stocks should be added to 340 µL of the microbial test suspension (see Table 1). The inoculum is vortexed again for 10 seconds.

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

Component	Volume (µL)
Test suspension	340
5% (w/v) BSA solution*	25
0.4% (w/v) Mucin solution*	100
5% (w/v) Yeast extract*	35
Total	**500**

*Note: Final concentration of the stock solution

Inoculation and drying of carriers

25. 10 µL of the inoculum are withdrawn with a calibrated positive-displacement pipette (Figure 1), and deposited at the centre of the brushed surface of the 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). Discard inoculated carriers where the inoculum has run off the centre or over the edge of the carrier.

26. The Petri dish is transferred with the inoculated carriers into a desiccator and the lid of the Petri dish is removed (Figure 3). Close the desiccator and check that it is properly sealed. The desiccator is evacuated using a vacuum source to achieve 20-25 inches mercury (508-635 torr; 677-847 mbar; 68000-85000 Pascal). Further details on using a desiccator are provided in Annex 5. The inoculated carriers are kept in the evacuated desiccator at 20-25°C for 60±10 minutes (Figure 4). If carriers are not dry within the specified time, check the desiccator system.

Exposure of the dried inoculum 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 inoculum after desiccation to the test substance or carrier count control fluid is as follows:

- Using sterile forceps, transfer each dried carrier (Figure 5) with the inoculated side up to the flat bottom vial (Figure 6).
- Cap the vial.
- Repeat until all carriers are transferred.
- Carriers can be stored at 20-25°C for up to one hour.
- 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.
- The vial must be in a horizontal position to ensure uniform distribution of test substance onto carrier.
- Deposit 50 µL of the test substance, equilibrated to 20-25°C, over the dried inoculum on each test carrier, ensuring complete coverage, at predetermined staggered intervals (Figure 7); do not touch pipette tip to carrier and do not cap the vials.
- Test carriers are held at 20-25°C for no more than the selected contact time (see paragraph 18 of the Preface).
- Control carriers are the last to be treated and receive 50 µL PBS, equilibrated to 20-25°C, instead of the test substance; the control carriers treated in a manner identical to that for the test carriers.

29. The number of the test organisms on the dried carriers should be 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 basis to which these numbers (0.5 $\log_{10}$ – 1.5 $\log_{10}$) are added will vary depending upon the log reduction required to demonstrate the passing of the performance standard as defined in paragraph 22 of the Preface under "Performance standards".

Neutralisation of test substance and elution of test organisms

30. Immediate (within 10 seconds) neutralisation of the test substance is required at the end of the contact time. Use less than 10 seconds as appropriate for shorter contact times. The protocol for the determination and verification of the neutraliser is given in Annex 1. At the end of the contact time, 10 mL* of the neutraliser is added to each vial according to the predetermined schedule. For consistency across laboratories/operators, this is documented as the 10^0 dilution. Note: *For ease of pipetting, 10 mL are used instead of 9.95 mL; this will not significantly affect the result.

31. Briefly (2-3 sec) vortex each vial following the addition of the neutralizer. Following the neutralization of the entire set of carriers, vortex each vial for 30 ± 5 seconds at high speed to recover

the inoculum. If possible, each carrier is examined visually and, in case of incomplete elution, further vortexing is performed. Do not remove carrier from the vial.

Dilution and recovery

32. At this stage one mL of the eluate is removed and used for any needed 10-fold (1mL + 9mL) dilutions to get countable numbers of the test organism to determine $\log_{10}$ reductions in viability. The number of dilutions to be made and tested will depend on the initial inoculum and the level of microbicidal activity expected. Dilution and recovery are completed between five and 30 minutes at room temperature after the neutraliser is added to the vial containing the carrier.

33. All samples, control and treated, will be filtered (see Annex 4 for information on membrane filtration system). Direct plating is not allowed. The procedure for dilution and recovery is as follows:

- Prewet each membrane filter by passing through it about 10 mL of sterile PBS.
- Separate membrane filters but the same filtration unit may be used for processing the eluate from a given carrier starting with the most dilute sample first. Always filter eluates from control carriers last to reduce risk of contamination of the eluates and filters from the test carriers.
- Prepare dilution vials before hand by adding diluent and labelling them (Figure 8).
- For the remaining 9.0 mL of the eluate, hold a magnet at the bottom of the vial to keep the carrier in place while pouring the contents of the vial into the membrane filtration system (Figure 9).
- Rinse vial with approximately 20 mL of PBS, vortex for five seconds and keeping magnet in place (while pouring), pour the wash into the same filtration system.
- Repeat this step one more time, swirl, and filter by applying vacuum.
- With the vacuum on, rinse the inside surface of the funnel unit with an additional 40 mL of PBS. Note: If desired, the vacuum may be left on for the duration of the filtration process beginning with the wetting step.
- For dilution tubes, rinse each tube once with ~10 mL of PBS and briefly vortex.
- Remove the membrane filter aseptically with sterile forceps and place it carefully over the agar surface of the recovery medium (Sabouraud's Dextrose Agar), starting at the edge as illustrated to avoid trapping any air bubbles between the filter and the agar surface (Figure 10).
- Incubate the plates at 30 ± 2°C for 7-9 days and examine for colonies of the test organism; to rule out the presence of any late growing stressed or injured organisms.

34. The elution and filtration steps for control carriers are also the same as those described above for the test carriers. However, eluates from control carriers will always require 10-fold dilutions to provide countable plates (10-50 CFU/plate).

DATA AND REPORTING

Assessing performance of test substance

35. Performance is assessed by counting surviving bacteria from the each test carrier and comparing the number obtained to the mean of those on the control carriers. All colony counts are recorded. Counts between 10-50 CFU/plate are used in calculations to determine log reductions. However, if counts are less than 10 and no plates demonstrate counts between 10-50 CFU/plate, then those counts are used in calculations to determine log reductions. If no survivors, then use < 1 CFU in calculations to determine log reductions.

36. Data are summarized in a tabular form showing raw data for each test and control plate. Data are also presented to validate the neutralisation process used in the test.

Calculating Log_{10} reductions

37. A method for determining arithmetic mean log_{10} reduction in the viability count of the test organisms by the test substance in quantitative carrier tests such as this one has been described (6).

Log_{10} Reduction = Average Log_{10} recovered from control carriers - Average Log_{10} recovered from the test carriers

Test report

38. The test report includes, but not limited to 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 name and relative concentrations of active ingredients.

Details on the test method

Test organism

- Source
- Scientific name and strain number
- Growth and recovery media
- Preparation of the stock and working culture

Test conditions

- Concentration of the test substance
- Temperature
- Contact time
- Test substance diluent (hard water level and titration)
- Soil load

Results

- CFU per carrier
- Log_{10} reduction
- Neutralisation determination and verification
- 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 (2011) Report on validation of efficacy methods for antimicrobials used on hard surfaces – Part I & Part II, [ENV/JM/MONO(2011)34], OECD Environmental Health and Safety Publications, series on Testing and Assessment, No. 154.

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

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

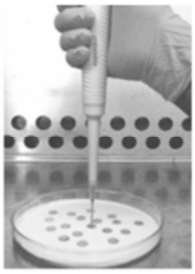


Figure 3 (left): Petri plate lid is removed during drying of carriers

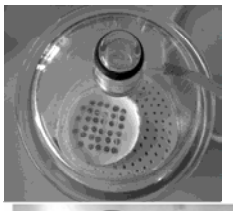


Figure 4 (right): Carriers left in an evacuated desiccator to dry at room temperature for 60±10 minutes.

Figure 5 (left): Carrier with dried inoculum being picked up for placement in flat bottom vial

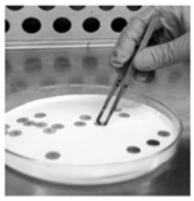


Figure 6 (right): Carrier placed into the flat bottom vial

Figure 7 (left): Dried inoculum on carrier covered with 50 µL of test substance or control fluid

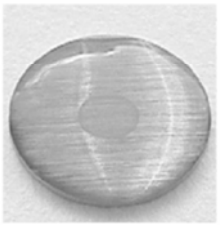


Figure 8 (right): Labelled dilutions vials with diluent; tubes may be used in place vials

Figure 9 (left): Magnet placed on the outside bottom of the vial to hold the default carrier in place while pouring eluate into filter funnel.

Figure 10 (right): Membrane filter being placed on surface of recovery agar to avoid trapping air bubbles underneath the filter.

ANNEX 1

DETERMINATION AND VERIFICATION OF APPROPRIATE NEUTRALIZATION

Purpose

The neutralization of the active ingredients found in antimicrobial products is one of the most important steps in product efficacy testing. A neutralization process and/or chemical agent (*e.g.* combined use of dilution, chemical neutralization, membrane filtration and rinsing of the membrane filters) are used in each test to inactivate the product's active ingredients, a process essential to achieving the desired contact time and full recovery of viable test microorganism. In addition, the neutralizer itself or in combination with the organic burden must not exhibit bacteriostatic activity against the test microbe as bacteriostatic activity may bias the efficacy data. It is preferable to perform the neutralization assay prior to or concurrently with product testing.

The neutralization should be confirmed and verified separately for each test substance against each test organism to be used in the method. Furthermore, it might be necessary to employ different neutralizers for different test organisms. If several concentrations of a given test substance are being evaluated, the highest concentration is tested to determine and verify neutralization. If the concentration(s) is (are) unknown, the test may be performed with different concentrations of the test substance in parallel.

Principle

The neutralizer is added to the carrier vial immediately at the end of the contact time. This dilutes the test substance while also chemically neutralising its microbicidal/microbistatic activity. Except in tests for virucidal activity, the entire eluate is then passed through a membrane filter which is washed with phosphate buffered saline (PBS) to further remove any residues of the test substance; the membrane is placed on a suitable recovery medium.

In the neutraliser determination/verification assay, the test substance is first mixed with a candidate neutraliser. The test organism is then added to the reaction mixture as dried inoculum on a carrier; if desired, additional evaluations may be conducted using the test organism as a liquid. The neutralisation process is deemed acceptable if there is no statistically significant difference between the numbers of viable organisms recovered from the neutralised test substance and the controls including those for any microbistatic or microbicidal effect of the neutraliser itself.

Two separate test suspensions (A and B) are required; A is a diluted suspension of the test organism, and B is the test microbial suspension with the added soil load.

Procedure

(a) Prepare Test Suspension A: Dilute the test microbial suspension with PBS to achieve an average challenge of 20-200 CFU/carrier after the 10 µL inoculum has dried. Prior testing may be required to account for differences in the loss of viability of the different test organisms on drying (see further section: "Requirements for a valid test and possible outcomes"). Test Suspension A should be used within 4 hours of preparation.

(b) Prepare Test Suspension B: First prepare the soil load by combining 25 µL 5% (w/v) BSA, 35 µL 5% (w/v) yeast extract, and 100 µL of 0.4% (w/v) mucin – mix well. Combine 132 µL of Test Suspension A and 68 µL of the soil load (SL). The test microbial suspension with soil load should also achieve an average challenge of 20-200 CFU/carrier after drying.

(c) If desired, estimate the number of viable organisms in Test Suspensions A and B by measuring the optical density (OD at 650nm) of the suspensions and creating a calibration curve.

(d) Carrier inoculation: Inoculate at least 10 carriers with Test Suspension A and at least 10 carriers with Test Suspension B by adding 10 µL to each carrier. Follow instructions in the test method for drying the carriers.

(e) Treatment 1: Titer Control (with OSL). Add one dried carrier inoculated with Test Suspension B to each of three vials. Add 10 mL PBS to each vial. Vortex each vial for 20 ± 2 seconds. Proceed as in (k) below. If deemed necessary, using another vial, perform this procedure with 10 µL of Test Suspension B in place of the inoculated carrier.

(f) Treatment 1a: Optional Titer Control (without OSL). Add one dried carrier inoculated with Test Suspension A to each of three vials. Add 10 mL PBS to each vial. Vortex each vial for 20 ± 2 seconds. Proceed as in (k) below. If deemed necessary, using another vial, perform this procedure with 10 µL of Test Suspension A in place of the inoculated carrier.

(g) Treatment 2: Neutralizer Toxicity Control. Add one dried carrier inoculated with Test Suspension B to each of three vials. Add 10 mL neutralizer to each vial. Vortex each vial for 20 ± 2 seconds. Proceed as in (k) below. If deemed necessary, using another vial, perform this procedure with 10 µL of Test Suspension B in place of the inoculated carrier.

(h) Treatment 3: Neutralizer Effectiveness (with OSL). Add 50 µL of the test substance to each of three vials then add 10 mL neutralizer to each vial. After 10 seconds, gently add one dried carrier inoculated with Test Suspension B to each vial. Vortex each vial for 20 ± 2 seconds. Proceed as in (k) below. If deemed necessary, using another vial, perform this procedure with 10 µL of Test Suspension B in place of the inoculated carrier.

(i) Treatment 3a: Optional Neutralizer Effectiveness (without OSL). Add 50 µL of the test substance to each of three vials then add 10 mL neutralizer each vial. After 10 seconds, gently add one dried carrier inoculated with Test Suspension A to each vial. Vortex each vial for 20 ± 2 seconds. Proceed as in (k) below. If deemed necessary, using another vial, perform this procedure with 10 µL of Test Suspension A in place of the inoculated carrier.

(j) Treatment 3b: Optional Neutralizer Effectiveness (independent addition of OSL). Add 50 µL of the test substance to each of three vials, and then add 10 mL of the neutralizer to each vial followed by the addition of 10 µL of the 3-part soil load to each vial. After 10 ± 2 seconds, add one dried carrier inoculated with Test Suspension A to each vial. Vortex each vial for 20 seconds. Proceed as in (k) below. If deemed necessary, using another vial, perform this procedure with 10 µL of Test Suspension A in place of the inoculated carrier.

(k) Hold the mixtures from (e) through (j) for 10 ± 1 minute at room temperature (22 ± 2°C). Steps (*e.g.*, addition of PBS, neutralizer) should be conducted at timed intervals (*e.g.*, 1 min. intervals) to ensure consistent time of contact. At the conclusion of the holding period, vortex each vial for 20 ± 2 seconds and pass each mixture through a separate membrane filter. Use a magnet to prevent carriers from falling onto the filter membrane. Wash each vial and the inside of the filter apparatus with approximately 50 mL PBS; filter the washes through the same filter membrane.

(l) Remove the membrane filter aseptically with sterile forceps and place it carefully over the surface of the recovery medium. Avoid trapping any air bubbles between the filter and the agar surface. Incubate the plates under the conditions appropriate for the test organism used.

(m) Examine the plates after incubation and record as CFU per vial. Calculate the averages for each set of test conditions.

Requirements for a Valid Test and Possible Outcomes

For the assay to be considered valid, ensure that:

a) The recovered number of CFU in the optional Titer Control without OSL using Test Suspension A yields approximately 20-200 CFU/carrier.

b) The recovered number of CFU in the Titer Control with OSL using Test Suspension B yields approximately 20-200 CFU/carrier.

For determining and/or verifying the effectiveness of the neutralizer:

a) The recovered number of CFU in the Neutralizer Toxicity Control is at least 75% of the Titer Control. A count lower than 75% indicates that the neutralizer is harmful to the test organism. Note: counts higher than the Titer Control (e.g., 120% of the titer control) are also deemed valid.

b) The recovered number of CFU in the Neutralizer Effectiveness (with OSL) treatment is within 75% of the Titer Control; this verifies effective neutralization in the presence of the soil load in the inoculum. The same results are expected for the optional Neutralizer Effectiveness (without OSL) and Neutralizer Effectiveness (independent addition of OSL) assays.

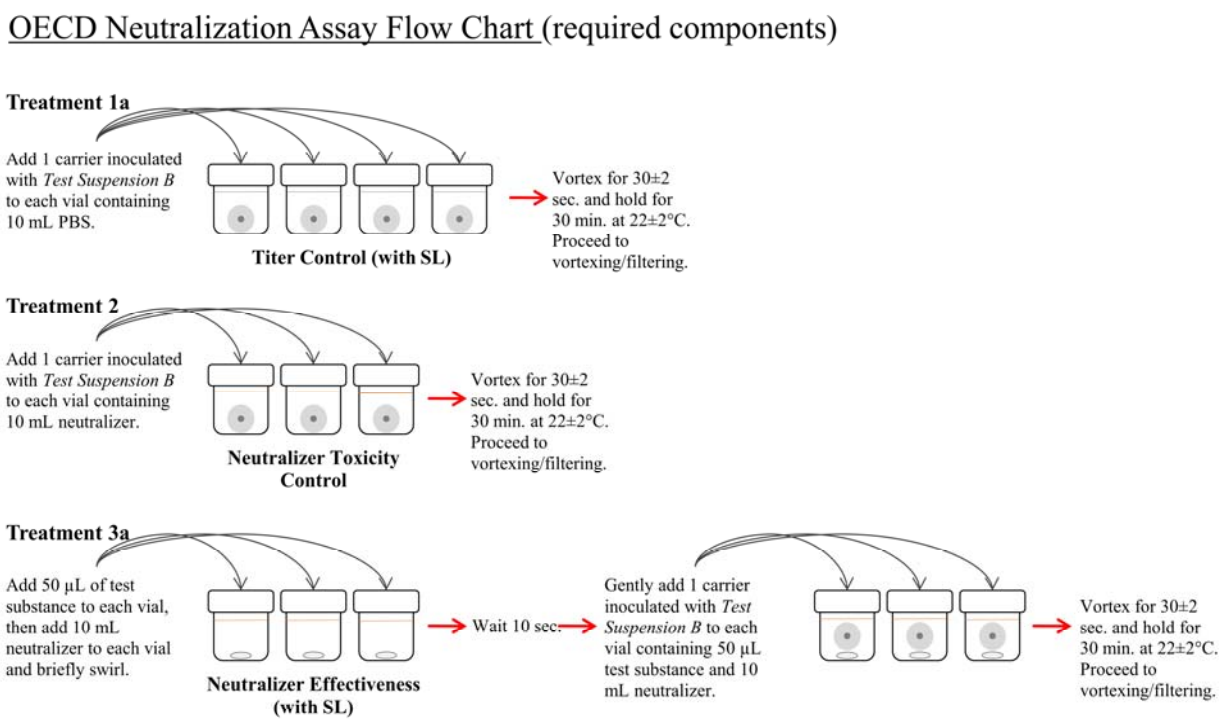
c) The above criteria must be met. If the criteria are not met, another neutralizer or mixture of neutralizers must be identified and verified.

d) Always compare the numbers of the Titer Controls determined on carriers with the numbers of the respective Neutraliser Toxicity Controls or Neutraliser Effectiveness assays determined on carriers. If a liquid microbial suspension is used instead of carriers, compare the numbers determined in the respective suspensions with each other accordingly.

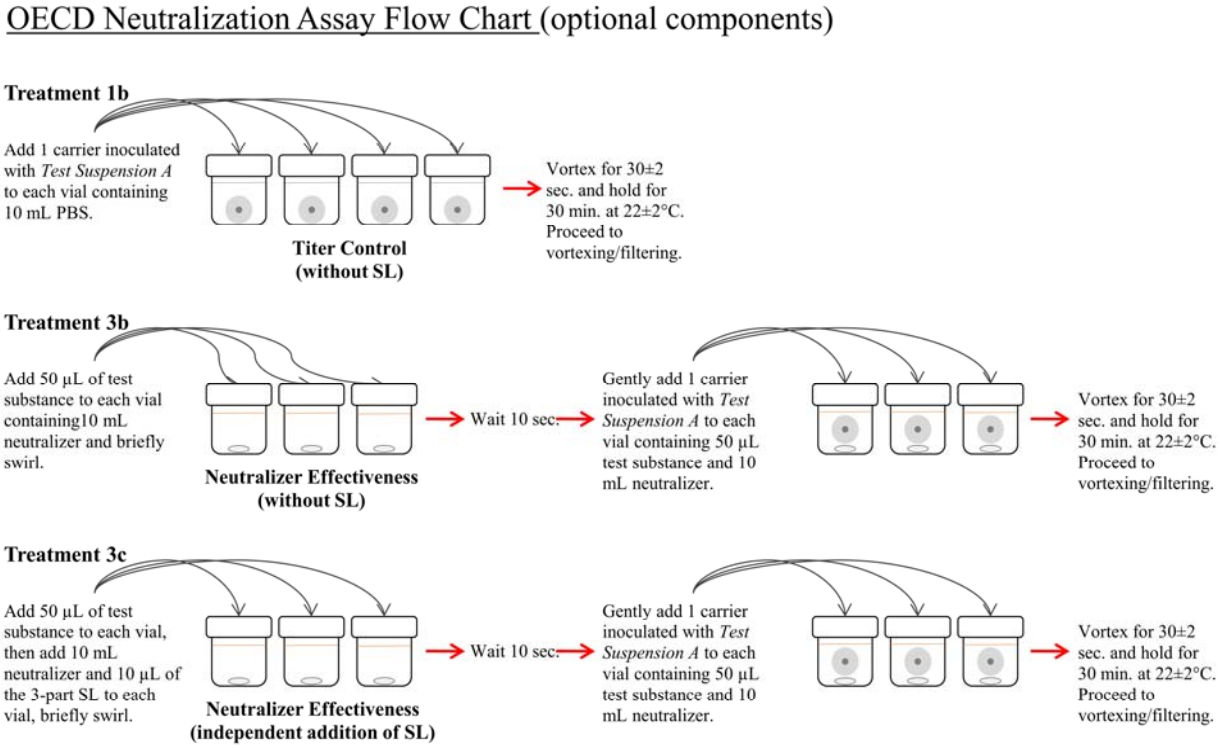
If the determination and verification of an appropriate neutraliser is done for the first time – for a given test organism and test substance – all tests have to be performed including the optional tests and the tests with test suspension instead of carrier.

To reduce the workload when determining a suitable neutraliser, it is recommended to start with section 3 (a) and (c). Then the assays with carriers described in section 3 (b), (d), (e), (g) and (h) should be performed. Proceed with the test proper once an appropriate neutraliser has been identified.

OECD Neutralisation Assay Flow Chart



Optional suspension test: Using an additional vial, perform each procedure using 10 µL of the appropriate test suspension in place of the inoculated carrier.



Optional suspension test: Using an additional vial, perform each procedure using 10 µL of the appropriate test suspension in place of the inoculated carrier.

ANNEX 2

PROCEDURES FOR MAINTENANCE OF FILAMENTOUS FUNGI

The source of the test organisms may be the ATCC or another established culture collections such as the National Collection of Type Cultures (NCTC). Proper documentation on the source of the culture(s) and date(s) received by the testing laboratory should be on file.

Requirements

All test organisms for use in the quantitative carrier test should be maintained according to the procedures described here.

The purity and identity of the preserved test organism should be verified during preparation.

Methods

Principle

Upon receipt, the organism should be grown, aliquoted into vials and stored frozen at -70ºC or below. A frozen vial or bead is retrieved when needed and subcultured to make a stock which is subsequently used to prepare working cultures for testing microbicides.

Material and reagents

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 or beads.

Culture media and reagents

Commercially prepared culture media and any ingredients purchased to make such media in-house are obtained from commercial sources. Chemicals/reagents are of analytical grade or appropriate for microbiological purposes. See Annex 3.

Procedure for preservation of filamentous fungi

Reconstitution of the freeze-dried test organisms

- Open ampule of freeze dried organism per manufacturer's instructions. Using a tube containing 5-6 mL of the suspension medium MEB, aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture.

- Inoculate one agar plate (Sabouraud's Dextrose) with a loopful of the test suspension and streak to achieve single colonies. Incubate at 30 ± 2°C for ten days. Select and sample from representative colonies from agar plate and assess purity and identity of test organism.

- Store the remaining portion of the suspension in a refrigerator for store until the culture identification and verification is complete.

Preparation for storage

a) Add polysorbate-80 solution to the surface of one or both of the agar plates (see the above section on *Reconstitution of the freeze dried test organism*) – 10 mL per plate – and detach conidia into the solution with the help of a glass spatula or glass beads. Transfer the suspension into the flask and shake gently for one minute together with approximately 5 g of beads.

b) Filter the suspension through a fritted filter directly or indirectly (via containers) into centrifuge tubes. Centrifuge the filtered suspension at 2000 g_N for 20 minutes. Discard the supernatant. Re-suspend the conidia in 10 mL or more – depending on the volume needed per year – in cryoprotectant solution and mix for 30 s. (Details of the cryoprotectant solution are in Annex 3).

c) Immediately after mixing, pipette 0.5-1.0 mL quantities of the diluted suspension (a) into separate properly labelled cryovials; these represent the frozen stock cultures.

An alternative way of storage is by coating of beads: pipette 1 mL of suspension into a cryovial containing two beads. Shake the vial to distribute the suspension onto the beads. Allow to stand for 30 minutes at 20°C. Remove the excess cryoprotectant solution with a pipette.

d) Place and store cryovials at a temperature of -70°C or lower (no more than 18 months). If vials or beads are depleted, a new test organism should be obtained from commercial source.

Preparation of test organisms from frozen stock cultures

a) Defrost the cryovial or remove (using a wire or forceps) a single bead of a cryovial. Inoculate agar plate(s) or slope(s) with this suspension/coated bead and incubate 30 ± 2°C for ten days.

b) To prepare a conidial suspension, a loop of the test fungus is inoculated at the centre of each of four or more Sabouraud's Dextrose plates and then incubated at 30 ± 2°C for ten days. The mycelial mass is removed from the plates with a sterile spatula and transferred to a 250 mL conical flask containing 25-50 mL PBS and 5-7 glass beads. The flask is shaken vigorously enough to break off the conidia from the hyphae. The resulting suspension is filtered through sterile absorbent cotton and conidia are collected in the filtrate. The suspension is standardized as needed for testing by diluting it with PBS so that it contains about 1 x 10^7 conidia per mL. Conidial suspensions stored at 4 ± 2°C can be used for up to four weeks to inoculate carriers.

ANNEX 3

PREPARATION OF SOLUTIONS, REAGENTS AND MEDIA

General Remark: Use only reagent-grade chemicals.

Cryoprotectant solution

Cryoprotectant solution:

Beef extract	3.0 g
Tryptone, pancreatic digest of casein	5.0 g
Glycerol ($C_3H_8O_3$) [2]	150.0 g
Water	to 1000.0 g

Dissolve the constituents in boiling water. Sterilise in the autoclave. After sterilisation the pH of the solution should be equivalent to 6.9 ± 0.2 when measured at 20ºC ± 1ºC.

Growth and recovery media and supplements

Required materials can be purchased from commercial sources. These materials may vary among suppliers or lots, and usage should be tracked as a part of proper quality assurance procedures. Conduct sterility tests on each new batch of liquid or semi-solid media by incubating at least two randomly selected broth tubes and agar plates for at least five days at 36 ± 1°C. Also check the ability of each new batch of medium to support the growth by inoculating it with 10-50 colonies of the test organism and incubating it at the required temperature.

c. Malt Extract Broth

(MEB) Malt extract	20 g
Water	to 1000 mL

d. Sabouraud Dextrose Agar

BBL™ Sabouraud Dextrose Agar	65 g
Deionised Water (dd H_20)	to 1000 mL

Dissolve powder in water; boil for one minute on a hot stir plate then autoclave to sterilise.

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 PBS with Tween-80 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 and then mixed in equal volumes. Non-PBS based neutralizers may be used as deemed necessary.

Phosphate buffered saline (PBS)

Add 1.25 mL of PB stock solution and 8.75 g of NaCl to a volumetric flask, fill with distilled or deionised water to the 1000 mL mark, and mix; adjust pH to 6.5-7.5, if necessary. Sterilise by filtration or autoclaving . Alternative PBS formulations with the same pH may be used.

Phosphate buffered (PB) stock solution

Dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) 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 distilled or 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 6.5-7.5):

a. Add 0.5 g yeast extract to 10 mL of PBS (*low* molecular weight component), mix, and pass through a 0.2 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 0.2 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, autoclave and aliquot. Store at either 4 ± 2°C or -20 ± 2°C.

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

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 diluted test substance should be used ≤ 3 hours of preparation. The following is based on CEN method EN 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 anhydrous magnesium chloride ($MgCl_2$) (or 42.36 g $MgCl_2 \cdot 6H_2O$) and 46.24 g calcium chloride ($CaCl_2$) in water and dilute to 1000 mL. Sterilise by membrane filtration. 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 24 hours.

Note: The final hardness expressed as calcium carbonate ($CaCO_3$) is 375 mg/L +5%/-10%. Other levels of water hardness may be used as appropriate. Hach kit analysis may be used to determine the concentration of water hardness (as mg/L $CaCO_3$).

Complexometric Determination of the Water Hardness

Method

During the complexometric titration the calcium and magnesium ions are converted (transported) with a standard solution containing complex forming ligands (for example ethylene di-amine tetra acetic acid = EDTA). The end point of the titration is made visible by a change of the solution's colour after adding metal indicators.

Reagents

- The standard solution of the di-sodium salt of ethylene di-amine tetra acetic acid (1 ml solution corresponds to 1 °dH ["German hardness"] for a sample quantity of 100 ml of the solution).
- Indicator pellets (Eriochrome Black T).
- Ammoniac buffer solution, pH 10.

Performance of the titration

Dilute one indicator pellet in 100 ml of the water to be analyzed. After the pellet has dissolved the solution is mixed with 5 ml of the ammoniac buffer solution. The thus prepared sample will be titrated with the EDTA- standard solution when the change of colour is green (until the moment when the colour becomes green).

Evaluation of the result

The numbers of millilitres of the measuring solution used indicates directly the water hardness in °dH. One °dH corresponds to 17.85 mg/L $CaCO_3$ and 21.06 °dH corresponds to 375 mg/L $CaCO_3$.

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 4

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.

Absorbent cotton

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.

Bunsen burner: with a gas source and flame igniter

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 typically identical in their topography and finish. If carriers are not brushed on both sides, inoculate the brushed side with the test organism. Refer to Annex VII for specifications.

Centrifuge: to sediment the test organism(s) for concentration, or washing, or both.

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

Colony Counter (optional): for example, Quebec Colony Counter.

Cryovials

Desiccator: Vacuum source may be a pump or central supply.

Desiccant: Silica gel (silicon dioxide) placed in the bottom chamber of the desiccator to assist in the drying of the carriers. Fresh desiccant is required for adequate drying.

Dispenser: for dispensing sterile 10 mL aliquots of diluent/eluent.

Flasks: volumetric flasks and side arm flask to collect filtrate

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.

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 ± 2°C to dry clean and wrapped sterile glassware.

Incubators: an incubator to maintain a temperature of 36 ± 1°C.

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. Polyethersulfone membranes with 0.2 or 0.45 µm pore diameter will 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.

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, 10-1000 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.

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

Saline: 0.85% Normal Saline

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

Silicone grease for desiccator

Spatula (stainless steel)

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 commercial 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 at least 20 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 approximately 25 mm in neck diameter and hold at least 20 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).

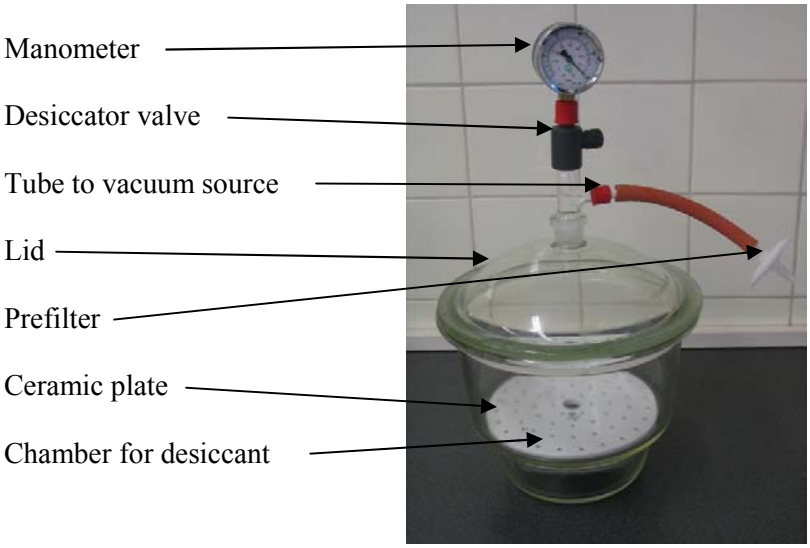
Water bath, capable of reaching and maintaining a temperature of 45 ± 1ºC to keep agar media from solidifying when making culture plates.

ANNEX 5

INSTRUCTIONS FOR USING A DESSICATOR TO VACCUM-DRY INOCULATED CARRIERS

Description

A desiccator is a container which is hermetically sealed with a lid. Inside is a ceramic plate for placing the carriers loaded with the inoculum to be dried. The desiccator is connected by a tube to either a central vacuum supply or a vacuum pump to evacuate the air. A manometer mounted on the lid shows the level of vacuum inside the desiccators.



Procedure

1) Use a thin and even layer of silicon grease on the contact surfaces between the lid and the desiccator for a good seal and also for easy removal of the lid at the end of the carrier drying process.

2) For drying, remove the lid and put the loaded carriers into the desiccator.

3) Replace the lid and close the desiccator valve.

4) Connect the tube to the vacuum source, close the desiccator valve and start the vacuum (the prefilter on the vacuum line is to prevent the escape of any microorganisms from the carriers into the air; each prefilter can be reused after autoclaving, but no more than five times). The evacuation of the air should continue for the entire drying period (60 minutes or until visibly dry) to ensure

complete drying of the carriers. If the desiccator lid and all the connections are properly sealed the manometer should register in about five minutes a reading of 80-130 mbar (tables for converting mbars to "torr", "Pascal" or "inches mercury" are readily available on the Internet). If carriers are not dry within the specified time, check the desiccator system (*e.g.* refresh dessicant if necessary.)

5) At the end of the drying period, switch off the vacuum source and open the desiccator valve.

ANNEX 6

CARRIER SPECIFICATIONS AND MAINTENANCE

- Ferritic stainless steel: Consist of chromium (17%) and iron and essentially nickel-free.
- AISI Type 430 (European equivalent name X6Cr17 and number 1.4016) belongs to Group 2, which is the most widely used family of ferritic alloys.
- Dimensions: 1 cm (0.39370 inch) in diameter; 0.7 mm (0.27559 inch) thick.
- AISI 430 - ASTM A240; Japanese Industrial Standard (JIS) G4305; EN 10088-2
- No. 4 Finish (EN 10088-2 1J/2J): A ground unidirectional finish obtained with 150 grit abrasive (AISI).
- Passivation: A soak in a mild acid bath for a few minutes to remove any impurities and accumulated debris from the disk surface.
- Tumbling: To remove the punching burrs from the edges of the discs they are tumbled in a barrel together with ceramic chips and a cleanser.

D "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 neutraliser 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 method is designed for testing the virucidal activity of substances to be used on hard, non-porous surfaces (1) (2). Assessments of virucidal 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. 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 twenty-seven laboratories from eight member countries participated and a validation report (5) was produced. Further, a collaborative evaluation of the bactericidal test method was conducted in 2012; the summary of the study is presented as an Annex to the validation report.

3. Details on relevant materials and reagents and the preparation of the test organisms are found in Annexes 2 to 5.

Requirements for test substance

4. The following information on the test substance should be determined (see regulatory authorities for specific requirements):

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 name and relative concentrations of active ingredients; such information may come from product label or 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 3, 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): host cell lines, 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 $\log_{10}$. When cytotoxicity is evident, a $\geq$ 3 log calculated reduction in the viral titre should be demonstrated beyond the cytotoxic level.

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

d) Specification(s) for default test carriers.

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 determination and verification of neutralisation of test substance.

j) Specifications for performance criteria when available.

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

l) Soil load to be used in testing.

m) Test substance diluent (if applicable).

INITIAL CONSIDERATIONS AND LIMITATIONS

6. The method employs disks of brushed stainless steel with magnetic properties.

7. A virus test organism is specified herein; however, test organisms more relevant to other settings, *e.g.*, dairy, baking or brewing industries are permitted in consultation with the target regulatory agency.

8. The soil load recommended is representative of body secretions and excretions and is also compatible with a wide variety of viruses and their host cells that may be used in testing.

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

10. The method has been validated for testing liquid formulations. For testing other product forms refer to paragraph 9 of the Preface under "Scope of Application of the Tests".

PRINCIPLE OF THE TEST

11. 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. 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 host cell line

c) Preparation of the test organism and inoculum.

d) Determination and verification of neutralisation.

e) Inoculation, drying and transfer of the carriers.

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

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

h) Dilution and recovery of the test organism.

i) Counting the surviving test organisms on test and control carriers and assessing the performance of test substance (*e.g.* PFU, CPE, Agglutination, Haemagglutination endpoints).

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

12. 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. The use of small flat carriers allows for their complete immersion and elution in relatively small volumes of eluents.

13. 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. Arithmetic mean $\log_{10}$ reductions in the numbers of viable test organisms following exposure to the test substance are calculated in relation to the mean of viable test organisms on the control carriers.

TEST PROCEDURE

14. The effectiveness of the neutralizer is determined prior to testing. For verification purposes the activity of the neutralizer will be verified per the methodology given in Annex 1. For viral testing, the neutralisation and cytotoxicity controls should be performed with each assay.

Preparation and sterilisation of carriers

15. The carriers are soaked in a suitable detergent solution (*e.g.* Liquinox or equivalent) free from any antimicrobial activity for 2-4 hours to degrease and then rinsed thoroughly in distilled or deionised water. Carriers should be visually checked for abnormalities (*e.g.* rust, chipping) and discarded if observed. At least four control carriers and three test carriers are used for each test organism and contact time/ temperature.

16. 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 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 or the inoculum is off-centre. The carriers are single use and should be discarded following testing.

Preparation of test organisms

17. Human Adenovirus Type 5 (VR-1516) was used in the ring trial. There are other non-enveloped viruses with a potential to spread via contaminated hard, non-porous surfaces that 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. However, the use of other specific virus types and their respective host cells 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) are acceptable alternatives. Annex 2 describes the maintenance of virus and host cell stocks, although other maintenance procedures may be acceptable. If other test organisms are employed, adapt host cells, incubation requirements and any other test parameters as necessary.

18. 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 2). Culture conditions (*e.g.* cell culture medium, cell line) for virus recovery are generally similar to those for virus propagation.

19. 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.

20. Virus: The selection of the test virus for this method is based on its (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 in cell cultures, or other appropriate endpoints (d) potential public health significance, 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. 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 the individual virus strains as this may lead to variations in results from one laboratory to another.

Method for preparing virus pools

21. Note: Further details are given in Annex 2. Internal Standard Operating Procedures should be followed. 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 virus suspension.

22. Incubate the flask for 60-90 minutes to allow for virus adsorption, if necessary. Add maintenance medium, *e.g.* Eagle Minimal Essential Medium (EMEM) without serum or with 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.

23. Freeze and thaw the flask at least three times as described in Annex III 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, the virus suspension may require concentration by ultra-centrifugation or by other means.

Preparation of inocula

24. 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.

25. The viral suspension is vortexed for 10-30 seconds or until resuspended, but no more than 60 seconds, to evenly distribute the virus particles. To obtain 500 µL of the inoculums with a 5% load, vortex each component and add 25 µL of BSA, 100 µL of mucin, and 35 µL of yeast extract stocks should be 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 components to prepare the inoculum

Component	Volume (µL)
Test virus suspension	340
5% (w/v) BSA solution*	25
0.4% (w/v) Mucin solution*	100
5% (w/v) Yeast extract solution*	35
Total	**500**

*Note: Final concentration of the stock solution.

Inoculation and drying of carriers

26. 10 µL of the inoculum are withdrawn with a calibrated positive-displacement pipette (Figure 1), and deposited at the centre of the brushed surface of the 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). Discard inoculated carriers where the inoculum has run off the centre or over the edge of the carrier.

27. The Petri dish is placed in a biological safety cabinet with the lid ajar and the laminar flow on at 20-25°C for 60 ± 10 minutes. The temperature and humidity should be documented.

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

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

29. The procedure for exposure of the dried inoculum to the test substance or control fluid 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.

- The vial must be in a horizontal position to ensure uniform distribution of test substance onto carrier.

- 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 no more than the selected contact time (see paragraph 18 of the Preface).

- Treat the control carriers 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 time.

- 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.

30. The titre of the test organisms on the dried carriers should be 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 paragraph 22 of the Preface, under "Performance standards".

Neutralisation of test substance and elution of test organisms

31. Immediately (within 10 seconds) neutralise the test substance at the end of the contact time; Use less than 10 seconds as appropriate for shorter contact times. The protocol for the determination and verification of the neutraliser is given in Annex II. At the end of the contact time, add 950 µL of the 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. If no test virus is detected in this un-diluted or any subsequent dilutions, the results be expressed, < 10 $TCID_{50}$ or PFU or below limit of detection.

32. Cap the vials and vortex each vial for approximately 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. Do not remove carrier from the vial.

33. Complete the dilutions of the eluates and their inoculation into cell cultures within 30 minutes after elution of the carriers. Evaluate any interference that the test substance and neutraliser may cause with viral infectivity – see Annex 1.

Dilution and recovery

34. Make 10-fold dilutions of the eluates using plastic cryovials (or equivalent) 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 using at least 4 wells. This is done using a plaque assay system, the most probable number (MPN) method based on tissue culture infective dose 50% ($TCID_{50}$) titration or other appropriate endpoint.

35. 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, $TCID_{50/ml}$ or other appropriate endpoint).

36. Incubate test and control plates at 36 ± 1°C in 5-7% CO_2 (if applicable) for 7-10 days.

DATA AND REPORTING

Assessing performance of test substance

37. Performance is assessed by measuring the level of virus infectivity for 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.

Virus Control Requirements

38. ***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. The use-dilutions of test substances and the soil load must not influence the viability of the host cells.

Calculating Log_{10} reductions

39. A method for determining arithmetic mean 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.

Log_{10} Reduction = Average Log_{10} recovered from control carriers – Average Log_{10} recovered from the test carriers.

Test report

40. The test report should include, but not limited to 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 name 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

- Concentration of the test substance
- Temperature
- Contact time
- Test substance diluent (hard water level and titration)
- Soil load

Results

- PFU or $TCID_{50}$ per carrier or other
- Log_{10} reduction
- Neutralization determination and verification
- 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 (2011) Report on validation of efficacy methods for antimicrobials used on hard surfaces – Part I & Part II, [ENV/JM/MONO(2011)34], OECD Environmental Health and Safety Publications, series on Testing and Assessment, No. 154.

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

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

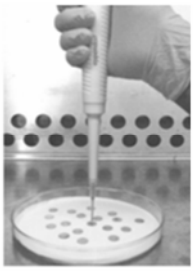


Figure 3 (left): Carrier with dried inoculum being picked up for placement in flat bottom vial

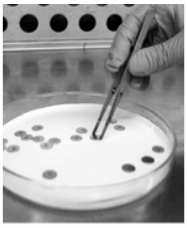


Figure 4 (right): Carrier placed into the flat bottom vial

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

ANNEX 1

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

CONFIRMATION OF NEUTRALISATION OF VIRUCIDAL ACTIVITY

Purpose

This is to confirm that the virucidal activity of the test substance in carrier tests has been brought to undetectable levels at the end of the contact time through the combined use of dilution and chemical neutralisation. The neutralisation protocol should be confirmed separately for each test substance against each type of test virus and its host cells to be used in the method. In case several concentrations of a given test substance are being evaluated, at least the highest concentration is tested to confirm neutralisation.

Application

In the carrier test, an eluent/diluent/neutraliser is added to carrier vials immediately at the end of the contact time. This results in the dilution of the test substance while also chemically neutralising its virucidal activity. While the following is based on virus infectivity assays using plaque-forming units (PFU), the procedure can be adapted to other methods of determining virus infectivity. Use the diluted virus suspension within 4 hours; longer storage at 4 ± 2°C may be possible but must be documented by the testing laboratory.

Procedure

a) Dilute the virus suspension in EBSS to yield countable (30-50) plaques in each 0.1 mL (Test Suspension B) of the inoculum to be placed in each well of a 12-well cluster plate to be used for virus assay. Take 132 µL of suspension B and add to it 68 µL of the soil load to prepare 200 µL of another suspension (Test Suspension C). Separately place 10 µL of Suspension B on each of at least three carriers and Suspension C on each of at least nine carriers and dry them following the instructions in the Test Guide. Since the degree of loss in viability during the drying of the carriers may vary depending on the test organism, run a preliminary test with different numbers of the test organism with and without the soil load to determine the input PFU level desired/carrier after the drying of the inocula.

b) Control for required level of input infective units: Place in each of four flat-bottom plastic vials 1.0 mL of EBSS. Add 10 µL of Suspension C to one vial to assess the input level of infective units. Place one dried carrier in each (Suspension C) of the remaining three vials

to determine the efficiency of virus elution from the carriers. Vortex each vial for 20 seconds. Proceed as in (e) below.

(c) Test for any incompatibility of the neutraliser with the virus and its host cells in the presence of the soil load: Add 1.0 mL of neutraliser under test to four flat-bottom plastic vials. Add 10 µL of Suspension C to one vial to assess safety of the neutraliser for the virus. Place one dried carrier (Suspension C) into each one of the remaining three vials to also determine the efficiency of virus elution from the carriers in the presence of the neutraliser. Vortex each vial for 20 seconds. Proceed as in 'e' below.

(d) Test for neutralisation activity with soil load in the inoculum: Add 1.0 mL of neutraliser under test to four flat-bottom plastic vials, and then add 50 µL of the test substance at the highest concentration used to each vial. Place one carrier with dried Suspension B to each of three vials. Place one carrier (with dried Suspension C) into each of three other vials. This is to determine that the reaction of the neutraliser with the soil load and/or test substance is harmless to the virus and its host cells. Vortex each vial for 20 seconds. Proceed as in 'e' below.

(e) Hold the mixtures from (b), (c) and (d), for 5-30 minutes at room temperature (20-25°C; vortex them again for 20 seconds and assay them for infectious virus.

(f) Count the number of plaques in the test and control wells to confirm neutralisation.

Possible Outcomes

For the proper confirmation of the neutralisation process, ensure that:

- An infective unit count in the mixture from the test for any virucidal activity of neutraliser control (c) is at least 85-115% as compared to the infective unit count in the input control (b). A count lower than 85% would indicate that the neutraliser itself is harmful to the viability of the test virus.

- An infective unit count in the mixture from the neutralisation test without the soil load (d) is at least 85% as compared to the infective unit count in the input control (b). This would indicate that the neutraliser was able to quench the virucidal activity of the test substance in the absence of the soil load.

- An infective unit count in the mixture from the neutralization test with the soil load in the inoculum (d) is at least 85-115% as compared to the infective unit count in the input control (b). This would indicate that the neutraliser was able to quench the virucidal activity of the test substance in the presence of the soil load in the inoculum.

- An infective unit count in the mixture from neutralisation test with soil load (f) is at least 85% as compared to the infective unit count in the input control (b). This would indicate that the neutralizer was able to quench the virucidal activity of the test substance in the presence of the soil load. A lower count of infective units may mean that the neutraliser was unable to quench the virucidal activity or that an interaction between the soil load and the neutraliser may be deleterious to the viability of the test virus.

If all the above criteria are met, the neutralization process is confirmed. If the criteria are not met, find another neutraliser or a mixture of neutralisers to be used.

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.

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.

CHECK FOR INTERFERENCE WITH VIRUS INFECTIVITY

(including the influence of soil load on the host cells)

Purpose

The purpose is to determine if sub-cytotoxic levels of the test substance reduce or enhance 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 virucidal 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: use its highest concentration if more than one level is being tested.

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

   b) Mix 50 µL of the test substance (see point 2) 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 tested.

Table 3

Component	Volume (µL)
Test virus suspension	340
5% (w/v) BSA solution*	25
0.4% (w/v) Mucin solution*	100
5% (w/v) Yeast extract solution*	35
Total	**500**

*Note: Final concentration of the stock solution.

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; if other vessels are used in the assay, adjust the input volumes accordingly.

5. In the remaining six wells, place 100 µL of the neutraliser into three wells and 100 µL of EBSS into three wells to serve 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 and add virus (diluted to give 30-50 plaques in each well) to 9 wells which had separately received the product (six wells) and the neutraliser (three wells). Add 0.1 mL of virus with countable number of plaques to the two remaining wells. 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: use its highest concentration if more than one level is being tested.

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

3. Mix 50 µL of the test substance (see point 2) with 10 µL of the soil load (see Table 3 above) and prepare a further 1:20 and a 1:200 dilution of the test substance in the neutraliser to be tested.

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 enough wells of a 96-well cell culture plate necessary for titration (see below).

5. Put 100 µL EBSS into the number of wells of the same cell culture plate size as used in the test to be used as controls.

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.

Possible outcomes

- Interference with virus infectivity: Any interference by residual amounts of the test formulation 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.

- 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. Another batch of soil load must be prepared using substances of sufficient quality.

- 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 test substance or the alteration of virus receptors on the host cells.

- 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.

ANNEX 2

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. Unless indicated otherwise, the volumes given below are for flasks with a capacity of 75 cm^2. Adjustments will be required if cell culture vessels of other sizes are being used. Laboratories should follow internal Standard Operating Procedures.

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 and cell lines were received, subcultured and frozen as initial stock. In addition, the complete passage history is documented and traceable to the initially frozen vials. See Annex 4.

Culture media and reagents

Commercially prepared culture media and any ingredients purchased to make such media in-house are obtained from commercial sources. Chemicals/reagents are of analytical grade or appropriate for virology purposes. See Annex 3.

Method

- Remove bottle(s) of medium and PBS from refrigerator, tubes of trypsin + EDTA (if needed) from -20 ± 2°C freezer and warm up in water bath at 36 ± 1°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 of passage.

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

- Dispense about 10 mL of calcium/magnesium-free 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 fetal bovine serum and DMSO (if necessary) 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 approximately 30 minutes or so; then move them to -20 ± 2°C freezer and leave them there for 24 hours.

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

- Document the name of the cell line, passage level, number of vials frozen and the date.

REVIVING CELLS FROM LIQUID NITROGEN STORAGE

Purpose

This procedure is for reviving cells frozen in liquid nitrogen. Laboratories should follow internal Standard Operating Procedures.

Procedure

Method

- Warm up the medium to 36 ± 1°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 16-24 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 time before the cells would form a confluent monolayer. The monolayer can be split at that stage.

SUB-CULTURING OF HOST CELLS FOR WORK WITH VIRUSES

Purpose

This procedure describes how to split confluent cell monolayers for subculture. Laboratories should follow internal Standard Operating Procedures.

Procedure

Method

1. Remove bottle(s) of medium, and calcium/magnesium-free PBS from refrigerator or cold room, tubes of trypsin+EDTA from -20 ± 2°C freezer and warm up in water bath or incubator at 36 ± 1°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 calcium/magnesium-free 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 in 5-7% CO_2 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 volume 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

$TCID_{50}$ assay

Virus propagation:

Remove growth medium from confluent monolayer of A549 in a 175 cm^2 culture flask, 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 -36±1°C in in 5-7% CO_2 (gently shake every 10 to 15 min).

At the end of incubation time remove 114noculums and add 25 mL cell culture medium (*e.g.* EMEM) with 2% FBS. Incubate cells for 2 to 4 days at 36±1°C and 5-7% CO_2 (70-95%of all cells will show a distinct CPE at this time).

Virus harvest:

For virus recovery up to 3 rapid freezing (-20 ± 2°C) / thawing (36 ± 1°C) steps are performed to release virus from cells. Transfer the suspension into a 50 mL conical tube, centrifuge for 10 min at 1,000 x*g* (4 ± 2°C) and collect the supernatant as virus stock. Aliquot (1 mL volumes) and store at ≤ -70°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 culture flask 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 or other as appropriate.

Infectivity titre is determined by means of end-point dilution titration in a microtitre-procedure. For this, samples are diluted 1:10 with EMEM with 2-5% FBS (or appropriate) and 100 µL of each dilution are placed in eight wells of a sterile polystyrene flat bottomed cell culture plate (Nunc GmbH & Co. KG). 100 µL (adjusted to provide approx. 10-15 x 10^3cells/well) 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 36 ± 1°C and 5-7 % CO_2 in order to follow the development of cytopathic effects for a period of 7-14 days after inoculation. The wells may be refed during incubation.

Note: 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.

ANNEX 3

PREPARATION OF SOLUTIONS, REAGENTS AND MEDIA

General Remark: Use only reagent-grade chemicals.

Earl's Balanced Salts (EBSS) powder: Made according to manufacturer's instructions

Dulbecco Phosphate Buffered Saline: This is generally more suitable for working with cell cultures and viruses. Made according to manufacturer's instructions

Calcium/magnesium-free PBS: For washing cells before trypsinisation. Made according to manufacturer's instructions

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 PBS with Tween-80 prior to use. The final concentration of polysorbate-80 in the eluent is typically 0.1% v/v. Other concentrations of polysorbate 80 or other neutralisers may be used only after confirmation of the activity. When the neutraliser is heat-sensitive and is aseptically added, the neutraliser and polysorbate-80 should be prepared sterile at double strength in PBS (pH 6.5-7.5) and then mixed in equal volumes. Non-PBS based neutralizers may be used as deemed necessary.

Phosphate buffered saline (PBS)

Add 1.25 mL of PB stock solution and 8.75 g of NaCl to a volumetric flask, fill with distilled or deionised water to the 1000 mL mark, and mix; adjust pH to 6.5-7.5, if necessary. Sterilise by filtration or autoclaving. Alternative PBS formulations with the same pH may be used.

Phosphate buffered (PB) stock solution

Dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 mL of water. Adjust pH to 6.5-7.5 with 0.1 N NaOH or 0.1 N HCl and bring to 1000 mL with distilled or 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 0.2 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 0.2 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, 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.

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 diluted test substance should be used ≤ 3 hours of preparation. The following is based on CEN method EN 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 anhydrous magnesium chloride ($MgCl_2$) (or 42.36 g $MgCl_2 \cdot 6H_2O$) and 46.24 g calcium chloride ($CaCl_2$) in water and dilute to 1000 mL. Sterilise by membrane filtration. 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 24 hours.

Note: The final hardness expressed as calcium carbonate ($CaCO_3$) is 375 mg/L +5%/-10%. Other levels of water hardness may be used as appropriate. Hach kit analysis may be used to determine the concentration of water hardness (as mg/L $CaCO_3$).

Complexometric Determination of the Water Hardness

Method

During the complexometric titration the calcium and magnesium ions are converted *(transported)* with a standard solution containing complexforming ligands (for example ethylene di-amine tetra acetic acid = EDTA). The end point of the titration is made visible by a change of the solution's colour after adding metal indicators.

Reagents

- The standard solution of the di-sodium salt of ethylene di-amine tetra acetic acid (1 ml solution corresponds to 1 °dH ["German hardness"] for a sample quantity of 100 mL of the solution).

- Indicator pellets (Eriochrome Black T)

- Ammoniac buffer solution, pH 10

Performance of the titration

Dilute one indicator pellet in 100 mL of the water to be analyzed. After the pellet has dissolved the solution is mixed with 5 mL of the ammoniac buffer solution. The thus prepared sample will be titrated with the EDTA- standard solution when the change of colour is green (until the moment when the colour becomes green).

Evaluation of the result

The numbers of millilitres of the measuring solution used indicates directly the water hardness in °dH. One °dH corresponds to 17.85 mg/L $CaCO_3$ and 21.06 °dH corresponds to 375 mg/L $CaCO_3$

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 4

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.

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 typically identical in their topography and finish. If carriers are not brushed on both sides, inoculate the brushed side with the test virus. Refer to Annex 5 for specifications.

Cell culture flasks and plasticware

Cell culture Media and supplements: 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 (or range specified in internal Standard Operating Procedure) 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 ± 2°C to dry clean and wrapped sterile glassware.

Incubator: to maintain a temperature of 36 ± 1°C or other appropriate conditions depending on the virus and host cell; 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 5-7% 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. Polyethersulfone membranes with 0.2 or 0.45 µm pore diameter will 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, 10-1000 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 (or range specified in internal Standard Operating Procedure); 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.

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 commercial 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.

Vials or Tubes for Dilution: wide-mouthed and suitable to hold at least 20 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 approximately 25 mm in neck diameter and hold at least 20 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.

ANNEX 5

CARRIER SPECIFICATIONS AND MAINTENANCE

- Ferritic stainless steel: Consist of chromium (17%) and iron and essentially nickel-free.
- AISI Type 430 (European equivalent name X6Cr17 and number 1.4016) belongs to Group 2, which is the most widely used family of ferritic alloys.
- Dimensions: 1 cm (0.39370 inch) in diameter; 0.7 mm (0.27559 inch) thick.
- AISI 430 - ASTM A240; Japanese Industrial Standard (JIS) G4305; EN 10088-2
- No. 4 Finish (EN 10088-2 1J/2J): A ground unidirectional finish obtained with 150 grit abrasive (AISI).
- Passivation: A soak in a mild acid bath for a few minutes to remove any impurities and accumulated debris from the disk surface.
- Tumbling: To remove the punching burrs from the edges of the discs they are tumbled in a barrel together with ceramic chips and a cleanser.