

## DRAFT OECD GUIDELINE FOR THE TESTING OF CHEMICALS

### Quantitative Method for Evaluating Bactericidal Efficacy of Biocides Used on Hard Surfaces

#### SUMMARY

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

#### INTRODUCTION

##### *Background and Scope*

2. This Test Guideline is designed for testing the bactericidal activity of substances to be used on hard surfaces Springthorpe and Sattar 2005 (1), (2). Assessments of microbicidal activity using carrier tests give a better indication of the potential of a given microbicide to perform under field conditions. International harmonisation of test methodology has been developed from meetings (OECD 2002) (3), reports and ongoing national and international initiatives that mandate such testing be quantitative in nature, and has agreed upon performance criteria. Performance criteria may vary depending on the intended use and label claim of the product. Data from such testing can also provide a basis for classification and labelling of a tested formulation. Statistical techniques are employed to ensure data validity. This test has evolved as a modification of a previous standard of ASTM International (2006) (4) following significant international collaboration among OECD member states. A ring trial to validate five new antimicrobial efficacy methods including this one was carried-out from 2007 to 2009 in which thirty-five laboratories from eight member countries participated and a validation report (5) was produced.

3. Definitions used in this Test Guideline are given in Annex I.

##### *Prerequisites for test substance*

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

- 1) The physical state of test substance, its trade name or identification number (ID), lot number(s), source and receipt date at the testing laboratory.
- 2) Chemical nature and relative concentrations of microbicidal ingredients if available; such information may come from product label or manufacturer's safety data sheet

(SDS).

- 3) Conditions and duration (shelf-life) for storage of test substance as specified by the manufacturer; depending on label claim and jurisdiction.
- 4) 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 II, 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 should be clearly documented).

### ***Prerequisites for testing***

5. The following information should also be known before the start of testing:
  1. Specification(s) on test organism(s): source, strain number, growth medium and passage history in test laboratory.
  2. Viability titre of test organism on dried carriers to range from 5.5 to 6.5 log<sub>10</sub> to assure a >5 log<sub>10</sub> reduction of the organism by the test substance.
  3. Directions to prepare suspensions of test organism(s).
  4. Specification(s) for default test carriers (and other optional carriers, if to be used).
  5. Directions to prepare carriers for inoculation.
  6. Directions to inoculate carriers with test organism(s).
  7. Specification for numbers of test and control carriers to be used.
  8. Directions to apply the test substance to assess microbicidal activity.
  9. Directions for neutralisation of test substance and validation of the procedure.
  10. Specification for performance criterion(a) when available.
  11. Temperature(s) and contact time(s) to be used in testing.

### **INITIAL CONSIDERATIONS AND LIMITATIONS**

6. The method employs disks of magnetized stainless steel. Surrogate pathogenic test organisms are specified herein; however, non-pathogenic test organisms more relevant to other settings, e.g., dairy, baking or brewing industries may be permitted.
7. The soil load recommended is representative of body secretions and excretions and is also compatible with a wide variety of organisms that may be used in testing. Other soil loads may be more relevant alternatives for particular industrial applications.
8. Certain jurisdictions require additional and/or alternate tests for formulations to be used on medical devices.
9. The method is suitable for testing liquid formulations and the liquid phase of aerosol, pump and trigger spray products. It is also suitable for testing the expressed liquid of towelette

products.

## **PRINCIPLES OF THE TEST**

10. The viability of test organisms is evaluated when disks receive test organisms in a soil load and are then exposed to the test substance (bactericide). Disks of brushed stainless steel are used to represent hard, non-porous environmental surfaces. This method consists of the following seven consecutive steps:

- 1) Preparation of the carriers.
- 2) Preparation of the test organism and inoculum.
- 3) Inoculation, drying and transfer of the carriers.
- 4) Exposure of the dried inoculum to the test substance or control fluid.
- 5) Neutralisation of the test substance and elution of the test organism.
- 6) Dilution and recovery of the test organism.
- 7) Counting the surviving test organisms and assessing the performance of test substance.

11. This method is fully quantitative and performed in a closed system to avoid any loss of viable test organisms. The level of microbial challenge can also be adjusted in accordance with the desired product performance criterion(a). The use of small flat carriers of test materials 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. The test organism with a soil load is placed at the centre of each carrier. The inoculum is then dried and covered with a relatively small volume of the test substance. Contaminated control carriers receive an equivalent volume of phosphate buffered saline. At the end of the contact time, the test substance is neutralised, the carriers are eluted and the eluates are assayed for viable test organisms. Log<sub>10</sub> reductions in the numbers of viable test organisms following exposure to the test substance are calculated in relation to the mean of viable of test organisms on the control carriers.

## **TEST PROCEDURE**

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

### ***Preparation and Sterilisation of Carriers***

14. The carriers should be soaked in a suitable detergent solution free from any antimicrobial activity and host-cell toxicity for two-four hours to degrease and then rinse them thoroughly in distilled water. Four control carriers and three treatment carriers should be tested for each test organism per treatment..

15. Up to 20 clean carriers should be placed on a sheet of filter paper on the inside bottom surface of a glass Petri dish (150 mm in diameter) or a similar holder. Cover the Petri dish with its lid, wrap the entire dish and sterilise. Extended soaking of the carriers in water or detergent and prolonged rinsing should be avoided to reduce risk of corrosion or rusting. Some

extra carriers should always be prepared for testing in case a carrier is accidentally dropped or the inoculum on it runs over the edge.

***Preparation of test organisms***

16. The test organisms listed below should be used for regulated testing. However, any other specific requirements should be checked before planning the testing. The strain numbers given are for the American Type Culture Collection (ATCC). Equivalent strains from other established culture collections such as the National Collection of Type Cultures (NCTC) might be acceptable alternatives. The maintenance of bacterial cultures is described in Annex IV.

*Pseudomonas aeruginosa* (ATCC 15442)

17. For this test organism, the culture medium should be prepared as follows:

- prepare a 1/1000 dilution of soybean-casein digest broth
- add 100 µL of stock culture to 10 mL of broth and incubate for 72 h – 78 h at 36±1°C to obtain 1.5 10<sup>8</sup> CFU/mL to 5.0 10<sup>8</sup> CFU/mL. The culture can be concentrated, if required, by centrifugation.
- Prior to inoculation of carriers, the soil load should be aseptically added.
- Soybean-casein digest agar should be used as Post-Exposure Recovery Medium -.

18. Under field conditions, bacteria are often under low-nutrient conditions where they survive and grow more slowly and may be more resistant to environmental stressors. This is particularly true for pseudomonads and is reflected by the culture conditions suggested.

*Staphylococcus aureus* (ATCC 6538)

19. Golden yellow colonies should be selected from semi-solid media. The strain to be used should produce mostly yellowish colonies on semi-solid media used in testing.

For this test organism, the culture medium should be prepared as follows:

- prepare a soybean-casein digest broth (same as trypticase soy broth)
  - add 100 µL of stock culture to 10 mL of broth and incubate for 18 h – 24 h at 36±1°C to obtain 1.5 10<sup>8</sup> CFU/mL to 5.0 10<sup>8</sup> CFU/mL.
  - Prior to inoculation of carriers, the soil load should be aseptically added.
- Soybean-casein digest agar should be used as a Post-Exposure Recovery Medium -.

*Enterococcus hirae* (ATCC 10541)

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

- prepare a soybean-casein digest broth
- add 100 µL of stock culture to 10 mL of broth and incubate for 18 h – 24 h at 36±1°C to obtain 1.5 10<sup>8</sup> CFU/mL to 5.0 10<sup>8</sup> CFU/mL.

- Prior to inoculation of carriers, the soil load should be aseptically added.
- Soybean-casein digest agar should be used as Post-Exposure Recovery Medium -.

### ***Preparation of Test Organism Stock Suspension***

21. To prepare stocks, test organisms are harvested by centrifugation of the broth cultures. The product of centrifugation ( $g$  force) and time for which it is applied ( $t$  minutes) controls the organism's sedimentation. The centrifugation should be between 5000 and 10000  $g_N$  for 20 minutes and resuspend the pellets in PBS.
22. Centrifugation for less than 5000  $g_N$  may result in incomplete sedimentation of the test bacteria.
23. Initially the approximate titre of each freshly prepared and homogenized microbial test suspension may be estimated spectrophotometrically at a defined wave length, based on a standard curve specific to the test organism. This can act as a guide to the required dilutions but should be confirmed by a quantitative viability assay on the recovery medium to be used in the test. The concentration of the viable test organism in the dried inoculum should be high enough to meet the required performance criterion. In general, this number should be higher than the defined performance standard to allow for statistical evaluation. Microbial titres should be confirmed in each test by determining the numbers of viable organisms on each of the control carriers.

### ***Inoculation and Drying of Carriers***

24. Test organism suspension should be prepared as described above. The suspension should be vortexed for 10-30 seconds or until resuspended, but no more than 60 seconds, to evenly distribute the cells. To obtain 500  $\mu\text{L}$  of the inoculum, 25  $\mu\text{L}$  of BSA, 100  $\mu\text{L}$  of mucin, and 35  $\mu\text{L}$  of yeast extract stocks should be added to 340  $\mu\text{L}$  of the microbial test suspension (see Table 1). The suspension should be vortexed again for 10 seconds.

Table 1. Volumes of microbial suspension and soil load to prepare the inoculum.

<b>Component</b>	<b>Volume (<math>\mu\text{L}</math>)</b>
Microbial test suspension	340
BSA	25
Mucin	100
Yeast extract	35
<b>TOTAL</b>	<b>500</b>

25. 10  $\mu\text{L}$  of the test organism suspension should be withdrawn with a positive-displacement pipette (Figure 1), and deposited at the centre of a carrier (Figure 2), but the inoculum **should not** be spread with the pipette tip. This operation should be repeated as necessary. For consistency, the same pipette tip should be used throughout the inoculation of a batch of carriers (number of carriers/test). The Petri dish should be transferred with the inoculated carriers into a desiccator.
26. The Petri dish should be placed in a desiccator and the lid of the Petri dish **should be removed** (Figure 3). It should be checked that the desiccator is properly sealed. The

desiccator should be 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 V. The inoculated carriers should be kept in the evacuated desiccator at room temperature for one hour  $\pm$  10 minutes to dry (Figure 4).

### ***Exposure of the dried inoculum to the test substance or control fluid***

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

28. The procedure for exposure of the dried inoculums to the test substance or control fluid is as follows:

- 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 with *Pseudomonas* can be stored at ambient conditions for up to 30 minutes and other vegetative bacteria for up to 1 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.
- Deposit 50  $\mu$ L of the test substance carefully over the dried inoculum on each test carrier, **ensuring complete coverage** (Figure 7), at predetermined staggered intervals.
- **Do not touch pipette tip to carrier.**

Control carriers are treated last and receive 50  $\mu$ L PBS instead. The carriers should be held at desired temperature for required contact period.

*Control carriers* — At least four control carriers should be used in each test.

29. The viability titre of test organism on dried carriers should range from 5.5 to 6.5 log<sub>10</sub> to assure a >5 log<sub>10</sub> reduction of the organism by the test substance.

### ***Neutralisation of test substance and elution of test organisms***

30. Immediate (within 10 $\pm$ 2 seconds) neutralisation is required at the end of the contact time; the protocol for the validation of the neutraliser is given in Annex III. At the end of the contact period, 10 mL\* of a validated neutraliser (containing Polysorbate-80 as specified above) should be added to each vial according to the predetermined schedule.

For consistency across laboratories/operators, this should be documented as the 10<sup>-1</sup> dilution.

31. Each vial should be vortexed for 30 seconds to recover the inoculum. Each carrier should be examined visually and, in case of incomplete elution, further vortexing should be performed.

Dilution and recovery should be completed as soon as possible after neutralisation. Mixtures can be held for 5-30 minutes at room temperature.

\*For ease of pipetting, 10 mL should be used instead of 9.95 mL.

### ***Dilution and recovery***

32. At this stage 1 mL of the eluate should be removed and used for any needed 10-fold (1 + 9) dilutions. The number of dilutions to be made and tested will depend on the initial inoculum and the level of microbicidal activity expected.

If the HGMF system of membrane filtration is used, fewer dilutions will be needed.

33. The procedure for dilution and recovery is as follows:

- Prewet each membrane by passing through it about 10 mL of sterile PBS.
- Use separate membrane filters but the same filtration unit 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 neutralised test sample, 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 about 20 mL of PBS, vortex for five seconds and keeping magnet in place (while pouring), pour the wash into the same filtration system and filter by applying vacuum.
- Repeat this step two more times.
- Rinse the inside surface of the funnel unit with an additional 40 mL of PBS and filter by applying vacuum.
- Remove the membrane filter aseptically with sterile forceps and place it carefully over the agar surface of a suitable 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 the desired temperature for the required length of time.

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 and processing of material from dilutions that will provide countable CFU. To reduce the number of membrane filtrations, dilutions of eluates from control carriers may be spread- or pour-plated only if equivalent numbers of recoverable CFU compared to membrane-filtration can be demonstrated. Spread- or pour-plating may also be used for 10-fold dilutions of the eluates from test carriers except that the 10 mL of the undiluted eluate should be membrane-filtered.

## **DATA AND REPORTING**

### ***Assessing performance of test substance***

35. Bacteria that survive exposure to the test substance are grown on recovery media. 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. Counts should be recorded as CFU per carrier. Bacteria that may be injured can take much longer to form colonies compared to undamaged cells. Thus, where no surviving cells are seen at the end of

the normal incubation period, plates should be re-incubated and re-examined up to five days before discarding.

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

### ***Calculating Log<sub>10</sub> Reductions***

37. A method for determining  $\log_{10}$  in the viability titre of the test organism by the test substance in quantitative carrier tests such as this one has been described (DeVries and Hamilton 1999) (6).

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

### **Test report**

38. The test report should include the following information:

#### *Test and control Substances*

- 1) The physical state of test substance, its trade name or identification number (ID), lot number(s).
- 2) Chemical nature and relative concentrations of microbicidal ingredients if available.

#### *Discussion of the Test Method*

#### *Test organism*

- 1) Source
- 2) Strain number

#### *Test Conditions*

- 1) Temperature
- 2) Contact time

#### *Results*

- 1) CFU per carrier
- 2) Log<sub>10</sub> Reduction

#### *Discussion of the Results*

#### *Conclusion*

Fig. 1 (Left). Ten  $\mu\text{L}$  of the test organism inoculum being removed with a positive-displacement pipette.

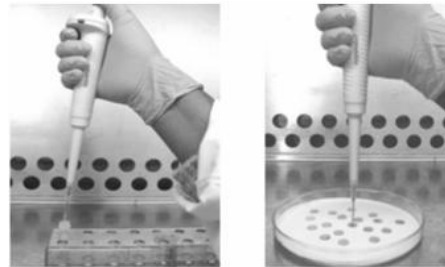


Fig. 2 (Right). The inoculum being placed at the centre of disk carrier.

Fig. 3 (Left). Petri plate lid is removed during drying of carriers.



Fig. 4 (Right). Carriers left in an evacuated desiccator to dry for one hour at room temperature.

Fig. 5 (Left). Carrier with dried inoculum being picked up for placement in flat bottom vial.

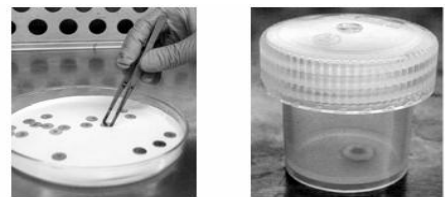


Fig. 6 (Right). Carrier placed into the flat bottom vial.

Fig. 7 (Left). Dried inoculum on carrier covered with 50  $\mu\text{L}$  of test substance or control fluid.

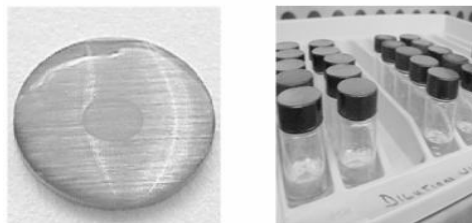


Fig. 8 (Right). Labelled dilutions vials with diluent; tubes may be used in place vials.

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

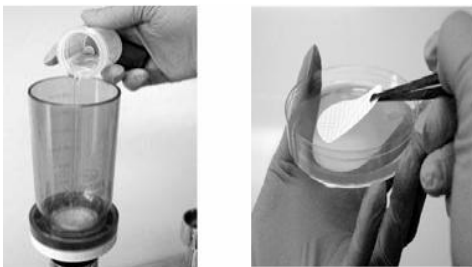


Fig. 10 (Right). Membrane filter being placed on surface of recovery agar to avoid trapping air bubbles underneath the filter.

**REFERENCES**

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## ANNEX I: Definitions and acronyms

- 1) *Carrier* an inanimate surface or object to be inoculated with the test organism.
- 2) CFU: a colony forming unit
- 3) *Eluate* is recovered eluent that contains the test organism.
- 4) *Eluent* is any liquid that is harmless to the test organism(s) and that is added to a carrier to recover these on it.
- 5) *Inoculum*: Test organism in soil load.
- 6) *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.
- 7) *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.
- 8) *Test substance* is a single entity or a formulation that incorporates microbicidal ingredients.
- 9) *Test organism* is one selected for testing; usually for its resistance characteristics. It also may be referred to as a *surrogate*, *simulant*, *target* or *marker microbe*. Ideally, it should be easy and safe to handle, and readily identifiable.

## ANNEX II: Preparation of hard water

**The following is based on CEN method prEN-13727 – Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of bactericidal activity in the medical area - Test method and requirements.**

(See ANNEX VI Preparation of solutions and reagents, section 3)

The paragraph and section numbers in this Annex refer to those in the CEN method

### 1.1 Hard water for dilution of test substance (when required)

The procedure is as follows for preparing one L of hard water:

- 1.1.1. Preparation of Solution A: dissolve 19.84 g magnesium chloride ( $\text{MgCl}_2$ ) and 46.24 g calcium chloride ( $\text{CaCl}_2$ ) in water and dilute to 1 000 mL. Sterilise by membrane filtration or in the autoclave. Store the solution in the refrigerator for no longer than one month;
- 1.1.2. Preparation of Solution B: dissolve 35.02 g sodium bicarbonate ( $\text{NaHCO}_3$ ) in water and dilute to 1 000 mL. Sterilise by membrane filtration. Store the solution in the refrigerator for no longer than one week;
- 1.1.3. Place 600-700 mL of water in a 1 000 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 1 000 mL. The pH of the hard water should be  $7.0 \pm 0.2$  when measured at  $20 \pm 1^\circ\text{C}$ . 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).

1.2. Prepare the hard water under aseptic conditions and use it within 12 h.

**NOTE:** When preparing the product test solutions, addition of the product to the hard water may give a different final water hardness.

## **ANNEX III: Neutraliser Validation Protocol to Determine the Effectiveness of Neutralisation of Microbicide followed by Membrane Filtration and Rinsing in the Disk Carrier Tests for Organisms other than Viruses**

### **1 PURPOSE**

This is to confirm that the microbicidal 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, chemical neutralisation, membrane filtration and rinsing of the membrane filters. The neutralisation protocol should be validated separately for each test substance against each type of organism to be used in the method. In case several levels of a given test substance are being evaluated, it is recommended that neutralisation validation be carried out with the highest concentration to be used in such testing.

### **2 APPLICATION**

In the carrier test, an eluent/neutraliser is added to carrier vial immediately ( $10 \pm 2$  seconds) at the end of the contact time. This results in the dilution of the test substance along with the chemical neutralization of its microbicidal activity. The diluted material is then passed through a membrane filter and the filter is washed with several changes of phosphate buffered saline (PBS).

### **3 PROCEDURE**

#### **Materials:**

- Culture of test organism
- Test substance
- Plates of recovery medium
- Box or tray for dilution vials
- Sterile membrane filters (47 mm in diam. with a pore size of 0.22 or 0.45  $\mu\text{m}$ )
- Side-arm flask to collect filtrate
- Vacuum source and rubber tube for connection
- Flat-ended forceps to pick up membrane filters
- Marker
- Plastic bags for biohazardous waste
- Gloves (optional)
- Water with a standard hardness (see Annex II) to dilute the test substance, if required
- Sterile PBS
- Sterile pipettes and pipettors with sterile tips
- Bunsen burner with a gas source and flame igniter
- Vortex machine
- Sterile automatic dispenser (optional)
- Timer

#### **Method**

- a Titrate the test suspension prior to neutraliser validation to determine the level of CFU. Suspension of vegetative bacteria can be stored at  $4 \pm 2^\circ\text{C}$  for about 48 hours, except for those of *P. aeruginosa*, which should be used on the day they

are prepared.

- b Using that pre-titrated suspension, prepare a 5 mL volume of it in PBS to yield 5 000-1 0000 colony-forming units (CFU)/mL
- c **Control for required level of input CFU:** Add 0.1 mL of the diluted test suspension (b) to 10 mL of PBS; proceed as in (g).
- d **Test for any anti-microbial activity of neutraliser:** Add 0.1 mL of the diluted test suspension (b) to 10 mL of neutraliser; vortex for 10 seconds; proceed as in (g).
- e **Neutralisation without soil load:** Add 50 µL of test substance (at the highest concentration used in the test) to 10 mL of the neutraliser used in the test; vortex for 10 seconds and *immediately* add to it 0.1 mL of diluted test suspension (b); proceed as in (g).
- f **Neutralisation test with soil load:** Add 50 µL of the test substance (at the highest concentration used in the test) and 5.0 µL of the soil load used in the test to 10 mL of the neutraliser used in the test; vortex for 10 seconds and *immediately* add to it 0.1 mL of diluted test suspension (b); proceed as in (g).
- g Hold the mixtures from (c), (d), (e) and (f) for 5-30 minutes at room temperature; vortex them for 10 s and pass them separately through membrane filters. Wash each filter with about 100 mL of PBS.
- h Place each filter on the agar surface of the recovery medium used in the test. Incubate the plates under conditions appropriate for the test organism, and used in the test.
- i Count CFU on the filters and record results.

#### 4 POSSIBLE OUTCOMES

For the proper validation of the neutralisation process:

- The number of CFU in the input CFU control (c) should be in the range of 50-100.
- A CFU count in the mixture from the test for any microbicidal activity of neutralizer control (d) should be at least 85% as compared to the CFU count in the input control (c). A count lower than 85% would indicate that the neutraliser itself is harmful to the viability of the test organism.
- A CFU count in the mixture from the Neutralisation test without the soil load (e) should be at least 85% as compared to the CFU input control (c). This would indicate that the neutraliser was able to quench the microbicidal activity of the test substance in the absence of the soil load.
- A CFU count in the mixture from Neutralisation test with soil load (f) should be at least 85% as compared to the CFU input control (c). This would indicate that the neutraliser was able to quench the microbicidal activity of the test substance in the presence of the soil load. A lower CFU count may mean that the neutraliser was unable to quench the microbicidal activity or that an interaction between the soil load and the neutraliser may be deleterious to the viability of the test organism.

If all the above criteria are met, the neutralisation process is validated. If the criteria are not met, then another neutraliser or a mixture of neutralisers should be found and validated.

## ANNEX IV: Procedures for Maintenance of Bacterial Cultures

### Preparation of Frozen Stock 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.

#### 1. 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 stock culture preparation and periodically during the maintenance period.

#### 2. Methods

##### Principle

Upon receipt, the organism should be grown, aliquoted into vials and stored frozen at  $-70^{\circ}\text{C}$  or below. A frozen vial is retrieved when needed and subcultured to make a stock which is subsequently used to prepare working cultures for testing microbicides. Depending on the nature of the organism and/or its intended use, it may also be possible to prepare working cultures directly prepared from the frozen stock.

#### 3 Material and reagents

##### 3.1 Test Organisms

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

##### 3.2 Culture media and reagents

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

##### 3.3 Water

Reagent-Grade Water:

Any method of preparation of reagent-grade water is acceptable provided that the requisite quality can be met. 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/>). Sterilise the water by autoclaving (20-40 minutes at  $121^{\circ}\text{C}$ ).

## 4. Equipment, apparatus and labware

### General

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.

**Water bath**, capable of reaching and maintaining a temperature of  $45\pm 1^\circ\text{C}$  to keep agar media from solidifying when making culture plates.

**Incubator**, capable of maintaining a temperature of  $36\pm 1^\circ\text{C}$ .

**pH meter**, having an accuracy of calibration of no more than  $\pm 0.1$  pH units.

**NOTE:** A puncture electrode or a flat membrane electrode should be used for measuring the pH of the agar media.

**Electromechanical agitator**, e.g., Vortex™ mixer.

### Sterile forceps or a wire to retrieve beads from cryovials

**Refrigerator**, capable of maintaining a temperature of  $4\pm 2^\circ\text{C}$ .

**Graduated pipettes**, of nominal capacities of 10 mL and 1 mL and 0.1 mL. Calibrated automatic pipettes may be used.

**Petri dishes (plates)**, 90 mm or 100 mm in diameter.

**Glass or ceramic beads**, (3 mm to 4 mm in diameter)

### Volumetric flasks

**Freezer**, capable of maintaining a temperature of  $-70^\circ\text{C}$  or lower.

### Cryovials

## 5 Procedure for preservation of spore-forming and non-spore-forming bacteria (excluding bacterial spores)

### 5.1 Reconstitution of the freeze-dried test organisms

- Resuspend the freeze-dried test organism using 1.0 mL of a suitable broth medium. Place 0.1 mL of the rehydrated suspension in each of two tubes containing 5 mL of a liquid medium suitable for the test organism. Mix thoroughly.

- Streak a loopful of the suspension on the agar surface of a suitable recovery medium to obtain isolated colonies. Pick one or two isolated colonies showing characteristics of the test organism and resuspend the material 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; for example, an incubation of 18-24 h at  $36\pm 1^\circ\text{C}$  is sufficient for growing *Staphylococcus aureus* for *E. coli*. Store the remaining portion of the suspension in a refrigerator until the culture identification and verification is complete.
- Select and sample from representative single colonies from the agar plate and assess purity and identity of the test organism.

### **5.2 Preparation for storage**

Details on cryoprotectant solutions are given below under “Culture Media and Reagents.

At the end of the incubation period, 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 properly labelled cryovials; these represent the STOCK CULTURES

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

Store the cryovials at  $-70^\circ\text{C}$  or lower for a maximum 14 months.

Discard all cryovials if the purity or identity of the organism is in question.

### **5.3 Preparation of test cultures from frozen stock cultures**

- Defrost a cryovial or remove (using a sterile 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. Generate a test culture by adding 100  $\mu\text{L}$  of defrosted material to 10.0 mL of appropriate growth medium and incubate under conditions suitable for the test organism.
- Alternatively, inoculate agar plates (TSA) or TSA slopes with 100  $\mu\text{L}$  of the thawed cryovial suspension or a coated bead and incubate 18-24 h at  $36\pm 1^\circ\text{C}$ ; this is a working culture. The working culture can be stored for up to one week in the refrigerator. Prepare a test organism by inoculating appropriate broth media with a loopful of growth from the agar plate/slope (working culture) and incubate under conditions appropriate for the test organism.
- Inoculate an agar plate with a loopful of the working culture and streak for isolation of colonies. Incubate plate and examine for purity.

## Culture Media And Reagents

### Water

The water should be either deionised distilled water or water with equivalent quality for making reagent solutions and culture media.

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

Tryptone soy broth, consisting of:

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 <sub>2</sub> HPO <sub>4</sub> )	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  $7.2 \pm 0.2$  when measured at  $20^\circ\text{C} \pm 1^\circ\text{C}$ .

### Cryoprotectant solution

(1) Cryoprotectant solution, consisting of:

Beef extract	3.0 g
Tryptone, pancreatic digest of casein	5.0 g
Glycerol (C <sub>3</sub> H <sub>8</sub> O <sub>3</sub> ) [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  $6.9 \pm 0.2$  when measured at  $20^\circ\text{C} \pm 1^\circ\text{C}$ .

2) Culture Broth with 15% Glycerol (C<sub>3</sub>H<sub>8</sub>O<sub>3</sub>)

Polysorbate-80 solution, consisting of:

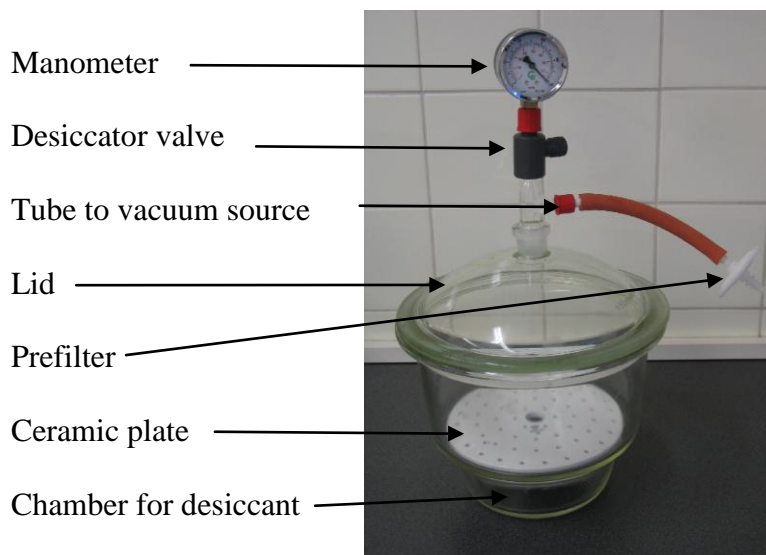
Polysorbate-80	0.5 g
Water	to 1 000.0 g

Sterilise by autoclaving.

## ANNEX V. Instructions for using a Desiccator to Vacuum-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 desiccator.



### 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 (no less than 60 minutes) 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).
- 5) At the end of the drying period, switch off the vacuum source and open the desiccator valve.

## ANNEX VI: Materials and supplies

### *General Equipment and Labware*

- 1) *Analytical balance* – to weigh chemicals and to calibrate inoculum delivery volumes by pipettes. Analytical balances should be calibrated at least annually.
- 2) *Centrifuge* - to sediment the test organism(s) for concentration, or washing, or both.
- 3) *Sterile Polypropylene Centrifuge Tubes with Caps* - 50 mL capacity.
- 4) *Colony Counter* (optional) - for example, Quebec Colony Counter.
- 5) *Desiccator*- Vacuum source may be a pump or central supply.
- 6) *Desiccant* – Silica gel (silicon dioxide) placed in the bottom chamber of the desiccator to assist in the drying of the carriers.
- 7) *Dispenser* — for dispensing sterile 10 mL aliquots of diluent/eluent.
- 8) *Environmental Chamber or Incubator* — to hold the carriers at the desired test temperature.
- 9) *Filtration System for Media and Reagents* — a membrane or cartridge filtration system (0.22 µm pore diameter) for sterilising heat-sensitive solutions.
- 10) *Forceps*, straight or curved and sterile, 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.***
- 11) *Freezers* — a freezer at  $-20\pm 2^{\circ}\text{C}$  for the storage of media and additives. A second freezer at  $-70^{\circ}\text{C}$  or lower to store the stocks of test organisms.
- 12) *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.
- 13) *Gloves* – sterile, disposable, for handling test items.
- 14) *Hot Air Oven* — an oven at  $60^{\circ}\text{C}$  to dry clean and wrapped sterile glassware.
- 15) *Incubators* — an incubator to maintain a temperature of  $36\pm 1^{\circ}\text{C}$ .
- 16) *Biological Safety Cabinet* — suitable for the containment of the test organisms used. Such cabinets require periodic recertification.
- 17) *Magnet* – strong enough to hold the carrier in place in the vial while the liquid is being poured out of it for membrane filtration.
- 18) *Magnetic Stir Plate and Stir Bars* — large enough for a 5-L beaker or Erlenmeyer flask for preparing culture media or other solutions.
- 19) *Markers* - permanent labware marking pens.
- 20) *Membrane Filtration System for Recovery of the Test organisms* - sterile 47- mm

diameter membrane filters and sterile glass, plastic or metal holders for such filters. Membranes with either 0.22  $\mu\text{m}$  or 0.45  $\mu\text{m}$  pore diameter may be used as appropriate for the test organism. Reusable or disposable filtration systems may be used.

**NOTE:** The method described here uses conventional membrane filters. The system with hydrophobic grid membrane filters (HGMF) may also be used for this purpose (Sharpe and Peterkin 1988) (7).

- 21) *Carriers* – Disks (1 cm in diameter) made from 0.7 mm thick sheets of brushed and magnetised stainless steel (AISI #430). Both sides of the carriers are identical in their topography and finish.
- 22) *Test Organisms* – Obtain ATCC organisms directly from ATCC or other credible sources
- 23) *Silicone grease* for desiccator
- 24) *Miscellaneous Laboratory Ware* – pipette tips, plastic vials for storing stocks of microbes, dilution tubes.
- 25) *Petri plates (Pyrex glass) 150 mm in diameter*- for holding and autoclave sterilisation of metal carriers.
- 26) *Sterile Disposable Plastic Petri Dishes* - 100 mm x 15 mm for microbial growth and recovery media.
- 27) *pH Meter* – to measure pH of buffers, eluents and test substance.
- 28) *Pipettors and pipette tips* – to dilute the test substance or test dilutions.
- 29) *Air Displacement Pipettes* — Eppendorf or equivalent, 50–1000  $\mu\text{L}$  with disposable tips – to measure test substance, eluents and diluents as appropriate.
- 30) *Electronic or Non-Electronic Positive Displacement Pipette and tips* — a 10-100  $\mu\text{L}$  pipette and appropriate pipette tips fitted with “plungers” that can dispense accurately 10- $\mu\text{L}$  volumes for inoculation of carriers without the aerosol generation.
- 31) *Refrigerator* —  $4\pm 2^\circ\text{C}$ ; for storage of media, culture plates and reagents.
- 32) *Serological Pipettes* – sterile reusable or single-use pipettes of 1.0, 5.0 and 10.0 mL capacity.
- 33) *Spectrophotometer* - for measuring turbidity of microbial suspensions.
- 34) *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.
- 35) *Timer* — any laboratory timer that can be read in minutes and seconds.
- 36) *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.
- 37) *Vials or Tubes for Dilution* - wide-mouthed and suitable to hold 30 mL easily.

- 38) *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.
- 39) *Vortex Mixer* - to vortex the eluate and rinsing fluid in the carrier vial to ensure efficient recovery of the test organism(s).

### ***Preparation of solutions and reagents***

*Purity of Reagents*—Use only reagent-grade chemicals.

- 1) *Neutraliser and Polysorbate-80 in Eluent*— Chemical neutralisers vary with test substance and should be manufacturer-specified whenever possible. Tween-80 is used in the eluent to help dissociate any microbial clumps that may have formed during testing. The neutraliser is sterilised with or aseptically added to Tween-80 in the PBS eluent prior to use. The final concentration of Tween-80 in the eluent will be 0.1% v/v. When the neutraliser is heat-sensitive and is aseptically added, the neutraliser and Tween-80 should be prepared sterile at double strength in PBS (pH 7.2-7.4) and then mixed in equal volumes.
- 2) *Test Substance* — dilute it first if required for testing and bring it to the test temperature prior to use.
- 3) *Test Substance Diluent* –water with a hardness of 375 (360-390) parts per million (ppm) is to be used. The water for this purpose should be prepared according to the specifications in CEN method prEN-13727. – see Annex II. and its hardness verified.
- 4) *Phosphate Buffer (PB) Stock Solution* — dissolve 34.0 g of potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) in 500 mL of water. Adjust pH to 7.2 ± 0.2 with 0.1 N NaOH or 0.1 N HCl and bring to 1000 mL with deionised water. Alternative phosphate buffers with the same pH may be used.
- 5) *Phosphate Buffered Saline (PBS)* — add 1.25 mL of PB stock solution and 8.75 g of NaCl to a volumetric flask, fill with deionised water to the 1000 mL mark, and mix; adjust pH to 7.2 ± 0.2, if necessary. Sterilise by filtration or autoclaving @121°C for 15-20 min. Alternative PBS formulations with the same pH may be used.
- 6) *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 into it 10-50 colonies of the test organism and incubating it at the required temperature.
- 7) *Water* – either deionised distilled water or water with equivalent quality for making reagent solutions and culture media.
- 8) *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)
- b. Prepare solution and pass through a 0.45  $\mu\text{m}$  pore diameter membrane filter, aliquot and store at either  $4\pm 2^\circ\text{C}$  or  $-20\pm 2^\circ\text{C}$ .
- c. Add 0.5 g bovine serum albumin (BSA) to 10 mL of PBS (high molecular weight component)
- d. Prepare solution and pass through a 0.45  $\mu\text{m}$  pore diameter membrane filter, aliquot and store at either  $4\pm 2^\circ\text{C}$  or  $-20\pm 2^\circ\text{C}$ .
- e. Add 0.04 g mucin (bovine or porcine) to 10 mL of PBS (mucoïd substance). Prepare solution, autoclave (15-20 minutes at  $121^\circ\text{C}$ ), aliquot and store at either  $4\pm 2^\circ\text{C}$  or  $-20\pm 2^\circ\text{C}$ .

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