OECD GUIDELINE FOR THE TESTING OF CHEMICALS

QUANTITATIVE METHOD FOR EVALUATING BACTERICIDAL ACTIVITY OF MICROBICIDES USED ON HARD NON-POREUS SURFACES

INTRODUCTION

Summary

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

Background and Scope

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

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

4. The following information on the test substance should be known before the start of testing:
   a) The physical state of test substance, its trade name or identification number (ID), lot number(s), source and receipt date at the testing laboratory.
   b) Chemical nature and relative concentrations of active ingredients; such information may come from product label or manufacturer’s safety data sheet (SDS).
   c) Conditions and duration (shelf-life) for storage of test substance as specified by the manufacturer; depending on label claim and jurisdiction.
   d) Directions to dilute the test substance to the level(s) at which it is to be tested; unless otherwise indicated by the manufacturer, hard water, as specified in Annex IV, is to be used as the diluent for test substances requiring dilution in water prior to testing (pH and any other adjustments required to prepare the test substance for testing is to be clearly documented).

Prerequisites for testing

5. The following information should also be known before the start of testing:
   a) Specification(s) on test organism(s): source, strain number, growth medium and passage history in test laboratory.
   b) The defined performance standard to adapt the number of test organism on dried carriers to be at least $0.5 \log_{10}$ higher than the defined performance standard, but not higher than $1.5 \log_{10}$.
   c) Directions to prepare suspensions of test organism(s).
   d) Specification(s) for default test carriers (and other optional carriers, if to be used).
   e) Directions to prepare carriers for inoculation.
   f) Directions to inoculate carriers with test organism(s).
   g) Specification for numbers of test and control carriers to be used.
   h) Directions to apply the test substance to assess microbicidal activity.
   i) Directions for neutralisation of test substance and validation of the procedure.
   j) Specification for performance criterion (a) when available.
   k) Temperature(s) and contact time(s) to be used in testing.

INITIAL CONSIDERATIONS AND LIMITATIONS

6. The method employs disks of magnetized stainless steel. Surrogate test organisms are specified herein; however, test organisms more relevant to other settings, e.g., dairy, baking or brewing industries are permitted.
7. The soil load recommended is representative of body secretions and excretions and is also compatible with a wide variety of test organisms that may be used in testing. Other soil loads more relevant for particular applications may be permitted.

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

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

**PRINCIPLE OF THE TEST**

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

   a) Preparation of the carriers.
   b) Preparation of the test organism and inoculum.
   c) Validation of the neutralisation.
   d) Inoculation, drying and transfer of the carriers.
   e) Exposure of the dried inoculum to the test substance and carrier count control fluid (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.

11. This method is fully quantitative and avoids any loss of viable test organisms during the procedure. The level of microbial challenge can also be adjusted in accordance with the desired product performance criterion (a). The use of small flat carriers allows for their complete immersion and elution in relatively small volumes of eluents. 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 defined volume of the test substance equivalent to 641 mL per m². 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. Log₁₀ reductions in the numbers of viable test organisms following exposure to the test substance are calculated in relation to the mean of viable of test organisms on the control carriers.

**TEST PROCEDURE**

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

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

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

Preparation of test organisms

16. The 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) might be acceptable alternatives. The maintenance of bacterial cultures is described in Annex III. If other test organisms are employed, adapt growth and recovery media, incubation requirements and any other test parameters as necessary.

Pseudomonas aeruginosa (ATCC 15442)

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

- prepare a 1/1000 dilution of soybean-casein digest broth; and
- 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 x 10^8 CFU/mL to 5.0 x 10^8 CFU/mL.

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.

Staphylococcus aureus (ATCC 6538)

18. Golden yellow colonies are selected from semi-solid media (only strains producing mostly yellowish colonies on media are used for testing – see Annex III). For this test organism, the culture medium is prepared as follows:

- prepare a soybean-casein digest broth (same as trypticase soy broth); and
- 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 x 10^8 CFU/mL to 5.0 x 10^8 CFU/mL.

Enterococcus hirae (ATCC 10541)

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

- prepare a soybean-casein digest broth; and
- 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 x 10⁷ CFU/mL to 5.0 x 10⁸ CFU/mL.

20. Test organisms, especially *Pseudomonas aeruginosa* may 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 for 20 ± 5 minutes and resuspend the pellets in PBS. Centrifugation for less than 5000 g may result in incomplete sedimentation of the test bacteria.

21. Initially the approximate CFU count 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 may act as a guide to the required dilutions but is confirmed by a quantitative viability 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.

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

23. Soybean-casein digest agar is used as a post-exposure recovery medium.

**Inoculation and drying of carriers**

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

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
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<tbody>
<tr>
<td>Test suspension</td>
<td>340</td>
</tr>
<tr>
<td>BSA</td>
<td>25</td>
</tr>
<tr>
<td>Mucin</td>
<td>100</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>35</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>500</strong></td>
</tr>
</tbody>
</table>

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

26. The Petri dish is 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 VI. The inoculated carriers are kept in the evacuated desiccator at 20-25°C for 60 ± 10 minutes to dry (Figure 4).
Exposure of the dried inoculums to the test substance or carrier count control fluid

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

28. The procedure for exposure of the dried inoculums to the test substance or carrier count control fluid 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 with *Pseudomonas* can be stored at 20-25°C for up to 15 minutes and other vegetative bacteria 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;
- 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 selected contact period;
- control carriers are the last to be treated and receive 50 µL PBS, equilibrated to 20-25°C, instead of the test substance; the carriers are held at 20-25°C for selected contact period; and
- at least four control carriers are used in each test.

29. A number of the test organisms on the dried carriers between 0.5 log<sub>10</sub> and 1.5 log<sub>10</sub> 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<sub>10</sub> – 1.5 log<sub>10</sub>) are added will vary depending upon the log reduction required to demonstrate the passing of the performance standard as defined in the Guidance Document.

Neutralisation of test substance and elution of test organisms

30. Immediate (within 10 ± 2 seconds) neutralisation of the test substance is required at the end of the contact time; the protocol for the validation of the neutraliser is given in Annex II. At the end of the contact period, 10 mL* of a validated neutraliser is added to each vial according to the predetermined schedule. For consistency across laboratories/operators, this is documented as the 10<sup>0</sup> dilution. Note: *For ease of pipetting, 10 mL are used instead of 9.95 mL; this will not significantly affect the result.

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

32. At this stage one mL of the eluate is removed and used for any needed 10-fold (1mL + 9mL) dilutions. 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 neutralisation.

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 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 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 the 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.

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 (15-300 CFU). To reduce the number of membrane filtrations, dilutions of eluates from test and control carriers may be spread or pour plated, except that the 9 mL of the undiluted eluate is always membrane-filtered.

**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 carrier counts are recorded; however only counts between 15-300 are used in calculations to determine log reductions. Examine the plates after 48 ± 4 hours and record 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 incubation period, plates are re-incubated and re-examined after an additional three days before discarding.
36. Data are summarized in a tabular form showing raw data for each test and control carrier. Data are also presented to validate the neutralisation process used in the test.

**Calculating Log_{10} reductions**

37. A method for determining 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).

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

**Test report**

38. The test report includes the following information:

**Test and control substances**

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

**Details on the test method**

**Test organism**

- Source
- Strain number
- Growth and recovery media

**Test conditions**

- Temperature
- Contact time

**Results**

- CFU per carrier
- Log_{10} reduction
- Neutralisation validation
- Copies of the raw data

**Conclusion**
REFERENCES


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

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 I

DEFINITIONS AND ABBREVIATIONS

BSA: Bovine Serum Albumin

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

CFU: Colony Forming Unit.

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

Eluate is recovered eluent that contains the test organism.

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

Inoculum: Test organism in soil load.

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

PBS: Phosphate Buffer Saline

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

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

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

NEUTRALISATION VALIDATION

Purpose

This is to confirm that the microbicidal and/or microbistatic 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 test organism 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 in the neutralisation validation.

Application

In the carrier test, an eluent/diluent/neutraliser is added to carrier vial immediately (10 ± 2 seconds) at the end of the contact time. This results in the dilution of the test substance while also chemically neutralising its microbicidal and/or microbistatic activity. The diluted material is then passed through a membrane filter and the filter is washed with phosphate buffered saline (PBS) to further remove any residues of the test substance.

Procedure

a Estimate the number of viable organisms of the test suspension by counting CFU or measuring the optical density (calibration curve) prior to neutralisation validation to determine the level of CFU. Suspension of vegetative bacteria can be stored at 4 ± 2°C for about 48 hours, except for those of *P. aeruginosa*, which should be used on the day they are prepared.

b Using that suspension, prepare a 5 mL volume of it in PBS to yield 200-1000 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 20-25°C; 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.

**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 15-125.

- 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 III

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.

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 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. See Annex V.

Culture media and reagents

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

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. 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 one or two 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 for store up to six weeks until the culture identification and verification is complete.

Preparation for storage

- Details on cryoprotectant solutions are is Annex IV.

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

- Store the cryovials at -70°C or lower for a maximum 14 months.

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.

- The working culture can be stored for up to one week in the refrigerator.
• Alternatively, inoculate agar plates (TSA) or TSA slopes with 100 μL of the thawed cryovial suspension or a coated bead and incubate 18-24 h at 36 ± 1°C;

• A new working culture could be prepared by inoculating appropriate broth media with a loopful of growth from the agar plate/slope 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 plates 48 ± 4 hours and examine for purity.
ANNEX IV

PREPARATION OF SOLUTIONS, REAGENTS AND MEDIA

General Remark: Use only reagent-grade chemicals.

**Cryoprotectant solution**

a. Cryoprotectant solution:

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<tr>
<th>Ingredient</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Beef extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Tryptone, pancreatic digest of casein</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Glycerol ((C_3H_8O_3)) [2]</td>
<td>150.0 g</td>
</tr>
<tr>
<td>Water</td>
<td>to 1000.0 g</td>
</tr>
</tbody>
</table>

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 20°C ± 1°C.

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

Polysorbate-80 solution, consisting of:

<table>
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<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polysorbate-80</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Water</td>
<td>to 1000.0 g</td>
</tr>
</tbody>
</table>

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**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
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<td>Tryptone-soy broth powder</td>
<td>30 g</td>
</tr>
<tr>
<td>Bacteriological agar</td>
<td>15 g</td>
</tr>
<tr>
<td>Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Add all to water; stir to dissolve on a hot plate. Sterilize by autoclaving at 121°C.
b. **Tryptone Soya Broth (TSB) for bacteria (Same as Soybean-Casein Digest Broth)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone, pancreatic digest of casein</td>
<td>17.0 g</td>
</tr>
<tr>
<td>Soya peptone, papaic digest of Soybean meal</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Water</td>
<td>800.0 mL</td>
</tr>
<tr>
<td>Dipotassium phosphate ($\text{K}_2\text{HPO}_4$)</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Water</td>
<td>to 1000.0 mL</td>
</tr>
</tbody>
</table>

Sterilise by autoclaving. After sterilisation the pH of the medium should be equivalent to $7.2 \pm 0.2$ when measured at $20 \pm 1^\circ$C.

**Neutraliser in eluent**

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

**Phosphate buffer saline (PBS)**

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

**Phosphate buffer (PB) stock solution**

Dissolve 34.0 g of potassium dihydrogen phosphate ($\text{KH}_2\text{PO}_4$) in 500 mL of water. Adjust pH to $7.2 \pm 0.2$ with 0.1 N NaOH or 0.1 N HCl and bring to 1000 mL with deionised water. Alternative phosphate buffers with the same pH may be used.

**Soil load**

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

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

b. Add 0.5 g bovine serum albumin (BSA) to 10 mL of PBS (*high* molecular weight component), mix and pass through a 0.45 µm pore diameter membrane filter, aliquot and store at either $4 \pm 2^\circ$C or $-20 \pm 2^\circ$C.

c. Add 0.04 g mucin (bovine or porcine) to 10 mL of PBS (*mucoid substance*), mix, and autoclave (15-20 minutes at 121°C), aliquot. Store at either $4 \pm 2^\circ$C or $-20 \pm 2^\circ$C.
The stock solutions of the soil load have a self-life of at least one year when stored between 4 ± 2°C and –20 ± 2°C.

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

**Test substance**

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

**Test substance diluent**

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

a. Preparation of Solution A: dissolve 19.84 g magnesium chloride (MgCl₂) and 46.24 g calcium chloride (CaCl₂) in water and dilute to 1000 mL. Sterilise by membrane filtration or in the autoclave. Store the solution in the refrigerator for no longer than one month;

b. Preparation of Solution B: dissolve 35.02 g sodium bicarbonate (NaHCO₃) in water and dilute to 1000 mL. Sterilise by membrane filtration. Store the solution in the refrigerator for no longer than one week;

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

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

Note: The final hardness expressed as calcium carbonate (CaCO₃) is 375 mg/L. Other levels of water hardness may be used as appropriate.

**Water**

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

MATERIAL AND EQUIPMENT

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

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

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

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

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 identical in their topography and finish.

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.

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°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. Membranes with either 0.22 µm or 0.45 µm pore diameter may be used as appropriate for the test organism. Reusable or disposable filtration systems may be used.

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

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

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

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

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

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

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

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

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

**Spectrophotometer:** for measuring turbidity of microbial suspensions.

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

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

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

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

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

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

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

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

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.

![Diagram of Desiccator](image)

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 ± 10 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.