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GUIDANCE DOCUMENT FOR QUANTITATIVE METHOD FOR EVALUATING ANTIBACTERIAL ACTIVITY OF POROUS AND NON-POROUS ANTIBACTERIAL TREATED MATERIALS

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IOMC
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FOREWORD

The work on the development of guidance for evaluating antibacterial activity of porous and non-porous biocide treated materials started in 2008 within the Task Force on Biocides (TFB). The project was included in the work plan of the Test Guidelines Programme in January 2011.

The proposed test method is based on existing basic efficacy tests, including ISO 22196 (antibacterial activity on plastics/non-porous surfaces) and ISO 20743 (antibacterial activity on textiles/ porous surfaces). The work has first consisted of checking that the ring test data obtained and validation studies conducted in the framework of the development of the ISO test methods are sufficient for an OECD test method. Additional testing and comparison of test conditions between the two ISO methods have been carried out by the International Biodeterioration Research Group (IBRG); a validation report will be further available.

A draft Guidance Document for a *Quantitative Method for Evaluating Antibacterial Activity of Porous and non-Porous Antibacterial Treated Materials* was approved by the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) at its 26th meeting in April 2014. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 7th July, 2014.

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

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GUIDANCE DOCUMENT FOR QUANTITATIVE METHOD FOR EVALUATING ANTIBACTERIAL ACTIVITY OF POROUS AND NON-POROUS ANTIBACTERIAL TREATED MATERIALS

INTRODUCTION

1. The scope of this Guidance Document is to measure the basic antibacterial performance of porous (textile) and non-porous (plastic) materials that have been treated with a biocide with the intention of introducing antibacterial/ hygienic properties into that material. The method can also be used for materials which claim to have inherent antibacterial properties (e.g. certain metals and certain natural fibres). It is intended to function as a Tier 1 basic activity test to illustrate 'proof of principle', *i.e.* an active substance incorporated into a model matrix demonstrates activity against target micro-organisms under standardised conditions (1) (2). It has been designed to harmonise the methods employed in a number of existing basic activity tests, typified by those described in ISO 22196 (2) and the 'germ count' part of ISO 20743 (4), and to be suitable for as wide a range of active substances as possible. The primary manner in which the methods have been harmonised is to utilise the cell preparation method and suspending medium described in ISO 22196 for all types of materials. The validity of this was determined through a series of international ring tests that demonstrated that using such an approach for porous materials did not compromise the outcome of the test and actually reduced the within laboratory and between laboratory variability (5).

2. The method provides only a basic foundation for conducting tests on antimicrobial treated articles, and a second tier method must be developed to ensure an accurate assessment of antimicrobial activity. A guidance document is currently under development for tier 2 testing, i.e. laboratory-based tests to substantiate claims made for the article with test conditions that simulate intended use, durability and compatibility of the article – provided that the protocol describes the claim being supported in an adequate manner. Further, Tier 2 testing protocols will also accommodate use of shorter contact times (e.g. 2h) and inoculum dried on treated surfaces.

3. Typical modifications include the use of additional test species and strains such as Methicillin resistant *Staphylococcus aureus* for demonstrating basic activity against such clinically important strains. In some instances the performance intended may require the use of elevated concentrations of nutrients (e.g. 1/20 nutrient broth in place of the 1/500 specified) or an alternative suspending medium for a specific purpose (e.g. bovine serum albumin, blood or yeast extract). Similarly, it may be useful to explore the function of the materials when incubated at either lower or higher temperatures than specified in the guideline. The Guidance Document can also be used as a bioassay to determine the impact of other factors on the performance of treated materials such as the impact of laundering, surface cleaning, abrasion or soiling, etc. These would normally be performed as a pre-test.

SUMMARY

4. To determine the antibacterial properties of materials treated with a biocide, samples of porous or non-porous antibacterial treated materials – such as textiles, paper, plastics, metals, surface coatings and ceramics, etc. – are inoculated with defined suspensions of bacteria and then incubated. The changes over

time in the size of populations on treated compared with untreated materials are used to measure antibacterial properties of the treated materials.

BACKGROUND AND SCOPE

5. This method is used for measuring the antibacterial performance of porous and non-porous materials that have been treated with a biocide so that antibacterial-like activity or activity that inhibits growth or metabolism is introduced into that material. It is not intended for use in determining the ability of a biocide to protect a material against biodeterioration or fouling. The method was developed based on two ISO standards; ISO 22196 and the 'germ count' part of ISO 20743. Modifications were made and validated by two, statistically designed international ring tests conducted by the International Biodeterioration Research Group (IBRG) under guidance of the Expert Group on Efficacy of Biocides Treated Articles (EBTA) of the OECD Task Force on Biocides (5).

6. A liquid suspension of bacteria is applied to materials both treated and untreated with antibacterial finishes: replicate sub-samples of each treated or untreated material are inoculated with a specified number of bacteria suspended in a solution containing a low concentration of nutrients. The inoculated materials are then incubated under conditions of controlled temperature and humidity for a specified period of time. After this interval the sub-samples are immersed in a neutraliser suitable for deactivating the active substance(s) used to produce the intended antimicrobial effect, and agitated to remove surviving organisms. The number of colony forming units present in the resulting suspension is then determined using standard, dilution plate count techniques. Changes in the sizes of the populations of the test organisms are calculated in relation to the numbers present on the untreated variants and/or in relation to the numbers applied.

7. This test method is appropriate for both porous and non-porous materials such as textiles, paper, plastics, metals, surface coatings and ceramics etc. It is intended to measure the antibacterial properties of such materials. In most instances further studies will be required to support and substantiate actual claims being made for the performance of treated materials in practice as part of a regulatory process (see paragraph 2).

TEST METHOD

Basic Requirements

8. Definitions and abbreviations used in this document are given in Annex 1. Details of relevant materials are found in Annexes 2 and 3.

Prerequisites for the Test Materials

9. The following information on the treated material should be known before the start of the test:

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

b) chemical name and concentrations of active substances used; In case the article is coated or treated with an active layer, the concentration in that layer (weight/weight) and its thickness should be indicated.

c) conditions and duration (shelf-life) for storage of the treated materials as specified by the manufacturer; depending on the label claim and jurisdiction.

d) susceptibility of the treated materials to physical manipulations and/or sterilisation techniques.

Test Bacteria

10. *Escherichia coli* and *Staphylococcus aureus* are employed in this method. However, other species can be used in addition to these two species. In some instances the additional species will require different methods to cultivate, expose and recover them that are not described in this Guidance Document; however, the conditions for a valid test (see paragraph 35) apply to all microorganism species used. Any additional steps have to be fully documented in the final report. Any deviations have to be justified and it must be demonstrated that they do not alter the fundamental performance of the test materials in any significant manner.

11. The standard test species are:

- Escherichia coli (ATCC 8739, CIP 53.126, DSM 1576, NBRC 3972, NCIB 8454)
- *Staphylococcus aureus* (ATCC 6538, CIP 53.156, DSM 346, NBRC 12732, NCIB 8625)

12. For the standard test species, stock cultures are stored at 5ºC ± 3ºC on Trypticase Soya Agar (TSA) and transferred monthly (see Annex 2). After five transfers or if more than one month has passed between transfers, the stock culture is discarded and replaced with a fresh culture, obtained from either the institute or culture collection concerned or from frozen or lyophilised long term stocks held by the testing institute.

13. Two days prior to testing, bacteria are transferred from the stock cultures using a sterile inoculating loop to an agar plate of TSA and incubated at 35ºC ± 1°C for 16 h to 24 hours. From this culture, bacteria are transferred onto a fresh agar plate of TSA and incubated at 35ºC ± 1°C for 16 h to 24 hours. This plate will be used to prepare the inoculum used in the test. When conducting testing on numerous lots or materials multiple plates of an organism can be prepared.

Preparation of Test Materials (including untreated controls)

14. Testing is performed on at least six (three per test species per lot of test material) sub-samples from each treated test material. At least twelve samples of the untreated material (controls) – *i.e.* the same material ideally, without biocidal treatment – are required. Half of the untreated test materials (three for each test species) are used to measure colony forming units present immediately after inoculation and the other half (three for each test species) are used to measure colony forming units present after incubation for 24 hours. Note: if additional incubation times – e.g. 1, 3, 6, and/or 12 hours – are analysed, replicate sub-samples should be added as appropriate.

15. The use of more than three replicates per test species of the treated test material may help reduce variability, especially for materials that show smaller antibacterial effects. When testing a series of antibacterial treatments for a single material, each antibacterial treatment may be compared to a single set of untreated materials if all the tests are conducted at the same time using the same test inoculum.

Non-Porous Materials

16. Samples of test material should be no more than 10 mm in thickness, flat and measure 50 ± 2 mm × 50 ± 2 mm. During the exposure phase, the test material samples are placed in sterile Petri dishes (90 mm diameter – see paragraph 20). If it is difficult or impossible to cut the test material into a square of this size, then test samples of different sizes and shapes may be used, as long as they can be covered with a covering film with a surface area between 400 mm^2 and 1600 mm^2. It is preferable to prepare the test samples from the material that will be used *in fine*. However, if the shape of the material prevents this, the test samples may be prepared in a format suitable for testing, using the same raw materials and processing methods as normally used for the 'final' material. If the test samples differ from the 50 mm × 50 mm square dimensions, the actual dimensions used will be stated in the test report.

17. When preparing the samples, care should be taken to avoid contamination with microorganisms or extraneous organic debris. Similarly, the test samples should not be allowed to come into contact with each other. If metal foil is used to avoid cross-contamination between samples during transport and storage, it is necessary to ensure that the metal does not have any antibacterial effect. If necessary, test samples can be cleaned/ disinfected/ sterilised prior to testing (*e.g.* by wiping with a solution of 70% v/v ethanol in water). It should be noted that the cleaning of test samples can cause changes such as softening, dissolution of the surface coating or elution of components and so should be avoided. If cleaning is required due to cross contamination, the cleaning method will be stated in the final report.

Porous Materials

18. Test samples with a mass of 0.40 g ± 0.05 g, cut to a size suitable for the sterile containers used during the exposure phase of the test, will be used. When preparing samples, care should be taken to avoid contamination with microorganisms or extraneous organic debris. Similarly, the test samples should not be allowed to come into contact with each other. If metal foil is used to avoid cross-contamination between samples during transport and storage, it is necessary to ensure that the metal does not have any antibacterial effect. If necessary (for example, where they have been found to be contaminated with bacterial endospores that cause interference in the test), test samples can be disinfected/sterilised prior to testing (*e.g.* by steam sterilisation using an autoclave). It should be noted that the pre-treatment of test samples can cause changes to some materials and so should be avoided. If such pre-treatment is required the reason and method used must be detailed in the final report. Similarly, if durability after laundering is part of the intended process the details of this will be included in the study and both treated and untreated material must be processed in an identical manner. It may be necessary to compare unlaundered untreated material with laundered treated material to ensure that laundering agents have not introduced antibacterial properties.

Preparation of the Test Inoculum

19. Bacteria are transferred using a sterile inoculating loop from the pre-incubated plates described in paragraph 13 into a small amount of $^1/_{500}$ nutrient broth (Annex 2). It is important to ensure that the test bacteria are evenly dispersed and sterile glass beads plus a vortex mixer can be used to achieve this if necessary. The number of bacteria in the resulting suspension is then estimated using direct microscopic observation and a counting chamber or another appropriate method (e.g. spectro-photometrically). One suspension is prepared for each test species. These suspensions are then diluted with $^1/_{500}$ nutrient broth, as appropriate for the estimated bacterial concentration, to obtain a bacterial concentration that is between 2.5×10^5 cells/ml and 10×10^5 cells/ml, with a target concentration of 6×10^5 cells/ml. These suspensions are used as the test inoculum. The test inoculum

is used within 2 hours of preparation and held at room temperature. It should be mixed thoroughly by hand immediately prior to use. The number of colony forming units in the individual inocula is verified by the same dilution plate count method as used for all other stages of the test.

Inoculation of Test Materials (including controls)

Non-Porous Materials

20. The surface to be tested is the exposed outer surface of the material. Cross-sections of the material should not be tested. Each test sample prepared as in paragraphs 16-17 is placed into a separate sterile Petri dish with the test surface uppermost. An aliquot (0.4 ml) of the appropriate test inoculum as prepared in paragraph 19 is transferred onto the test surface using a sterile pipette. The test inoculum is then covered with a piece of sterile covering film (Annex 3) that measures 40 mm × 40 mm and gently press down on the film such that the test inoculum spreads to the edges and is held in intimate contact with the surface of the test sample. It is important to ensure that the test inoculum does not leak beyond the edges of the film. After the samples have been inoculated and the cover films applied, the lids of the Petri dishes are replaced (see Figure 1 in Annex 5).

21. Unless otherwise specified, the standard size of the cover film is a square of (40 ± 2) mm × (40 ± 2) mm for a test sample measuring 50 mm × 50 mm. If the test sample is not of a standard size, the size of the film will be reduced in direct proportion. However, the size of film will not be reduced to less than 400 mm^2 and the edges of the cover film always are no less than 2.5 mm inside the edge of the test sample on all sides. If the size of the cover film differs from 40 mm × 40 mm, the actual size used will be stated in the test report. When a different sized film is employed, the volume of inoculum used should also be adjusted to be proportional to the area of the cover film used and recorded in the test report (see paragraph 22).

22. It is essential that the test inoculum does not leak beyond the edges of the cover film. For some surfaces (*e.g.* those that are very hydrophilic) this might be difficult to achieve. Should this occur, the volume of test inoculum applied to the test surface may be reduced but should never be less than 0.1 ml. However, when the volume of the test inoculum is decreased, the concentration of the bacterial cells in the inoculum is to be increased to provide the same number of bacterial cells as when the normal volume of test inoculum is applied. The objective is to achieve a similar number of cells *per* mm^2 as would be achieved using the standard procedure.

Porous Materials

23. Place each of the test samples prepared as in paragraph 18 into separate sterile containers (e.g vials with a screw closure and a volume of *ca* 30 ml) by selecting the following method as appropriate to the nature of the test material (see also Figure 2 in Annex 5).

a) If the test material tends to curl easily, or if it contains wadding or down, place a glass rod (Annex 2) onto the material in the container. Alternatively, secure both ends of the test material with thread.

b) If the test material is a yarn, arrange the yarn in a bundle and place a glass rod onto the material in the container.

c) If the test material is a carpet or of similar construction, cut the pile and place a glass rod onto the material in the container.

24. An aliquot (0.2 ml) of the appropriate test inoculum as prepared in paragraph 19 is transferred onto each sub-sample of the test materials at several points using a sterile pipette taking care to ensure that no inoculum touches the surface of the container. After the test samples have been inoculated the containers are closed.

Incubation

25. Unless otherwise specified, incubate the inoculated treated test samples and half of the untreated test samples at a temperature of 35ºC ± 1°C. For non-porous materials the inoculated sub-samples (see paragraphs 20-22) for inoculation of test materials shall be incubated at a relative humidity of not less than 90% for 24h ± 1h. This achieved either through the use of humid chambers or a humidity controlled incubator. In the case of the inoculated porous materials (see paragraph 23 for inoculation of test material), the sub-samples are incubated in closed containers and this results in a high level of humidity and conditions under which the inoculum does not dry out. The relative humidity is therefore not controlled specifically. Other temperatures may be used if agreed upon by all parties or if they are more appropriate to an alternative microbial species. If a temperature other than 35ºC ± 1°C is used, it will be recorded in the test report and its use justified.

Recovery of Bacteria from the Test Samples

26. Half of the untreated (control) samples are analysed for the number of colony forming units present immediately after inoculation. The remaining half, plus the treated samples, will be analysed after incubation (see paragraph 25).

27. An aliquot (10 ml for non-porous material and 20 ml for porous materials) of a neutraliser (e.g. Annexes 2 and 4) that has been validated for the active substance(s) employed in the treated material (Annex 4) is added to either each of the Petri dishes (non-porous materials – see paragraph 16) or each of the containers (porous materials – see paragraph 18) containing the test sub-samples. An alternative method is to transfer the inoculated sub-samples to another container (e.g. a sterile Stomacher bag) and then add an aliquot (10 ml for non-porous material and 20 ml for porous materials) of the neutraliser to facilitate recovery of any surviving bacteria by agitation / manual manipulation (see Figure 1 in Annex 5).

28. In the case of non-porous materials it is important to ensure that the neutraliser completely washes the samples by using a pipette to collect and release it at least four times. If, due to the nature of the test material, an alternative method or a different volume of neutraliser has to be employed, the suitability of the recovery method will be demonstrated, the recovery rate will be quantified. The deviation and its justification must be included in the test report and taken into account in the calculation of any antibacterial effects.

29. In the case of porous materials the contents of the containers are mixed vigorously by hand to ensure that the bacteria have been released from the test samples. Sterile glass beads and a vortex mixer may be employed if necessary (Annex 3).

Measurement of Colony Forming Units

30. The number of colony forming units present in the suspension produced in paragraphs 26-29 is determined by dilution plate count onto TSA. Any validated method (e.g. pour plate, surface plate and spiral dilution) may be employed. The suspensions are diluted in the neutraliser employed such that accurate counts of colonies can be performed. The undiluted neutraliser solution obtained from the treated samples will also be analysed by the pour plate technique using TSA. This must be performed irrespective of the method used to analyse the suspensions from the untreated samples and

the diluted suspensions from the treated samples. This is to increase the limit of detection of the method (a membrane filtration method could also be performed if a lower limit of detection was considered necessary for the material under test).

31. For the pour plate technique, transfer an aliquot (1 ml) of each undiluted neutraliser suspension into individual sterile Petri dishes. Molten (at approximately 45°C ± 1ºC TSA (approximately 15 ml *per* dish) is then placed into each Petri dish and swirled gently to disperse the bacteria. Once set the Petri dishes are incubated at 35°C ± 1°C for 24 to 48 hours depending on the species. The same conditions shall be employed for the plates derived from the other dilutions / method.

32. After incubation the number of colonies present on the plates are counted and recorded.

RESULTS

Presentation of Results and Interpretation of Data

33. The results are expressed as colony forming units *per* cm^2 (based on the area of the covering film employed) for non-porous materials and as colony forming units *per* g for porous materials quoted as the geometric mean of the data. Microbiological effects should be interpreted based on the difference between populations exposed to the treated and untreated materials as well as the size of the populations prior to exposure / incubation and after. If differences need to be calculated they should be expressed as the true difference and not as percentage differences. Any differences between treated and untreated samples shall be tested for statistical significance ($p < 0.05$) using a recognised technique. The data will almost certainly require transformation (probably base-10 logarithm) before analysis by parametric statistical techniques such as the t-test or ANOVA in order to satisfy the Gaussian distribution requirement of these tests. Non parametric methods can also be used (e.g. Mann-Whitney). The relative power and power-efficiency of the various tests is outside the scope of this protocol and is discussed in standard texts. With the small sample sizes and the distributions found in this biological testing there is often only small differences between parametric and non-parametric methods.

34. The major determinant of an antibacterial effect is the difference between the populations exposed to the treated material and the untreated material. In many instances the populations exposed to the untreated material will either remain relatively constant in size or will increase whereas the population exposed to the treated material will decrease. The reduction attributed to the treatment is calculated from the populations present on both the treated material and the untreated material after exposure. Some materials possess intrinsic antimicrobial properties and the population exposed to the untreated material will decline during the exposure phase. Again, the important difference is between the populations present on the treated and untreated material and this is used to describe the impact of the treatment on the population. On some materials the population exposed to the untreated material will increase significantly during the exposure phase (this is often observed with textiles). An increase may also be observed in the populations exposed to the treated material. The important difference is between the populations present on the treated and untreated materials and this can be used to describe an effect related to a reduction in growth.

Conditions for a Valid Test

35. When the conditions given in (a) and (b) respectively, are satisfied, the test is deemed valid. If all conditions are not met, the test is not considered valid and the samples shall be re-tested.

a) The average number of colony forming units recovered immediately after inoculation from the untreated test samples, is within the range 6.2×10^3 CFU/cm^2 to 2.5×10^4 CFU /cm^2 for non-porous materials and between 1.2×10^5 CFU/g to 4.5×10^5 CFU/g for porous materials.

b) The number of colony forming units recovered from each untreated test sample after incubation for 24 hours will not be less than 6.2×10^1 CFU/cm^2 for non-porous materials and 1.2×10^3 CFU/g for porous materials.

TEST REPORT

36. The test report includes the following information:

a) active substances and their corresponding concentrations as described in paragraph 9, section "Prerequisites for the Test Materials";

b) interfering substances and their concentrations used during the test;

c) the details of the materials tested including their size/weight and the details listed as items a) and b) in paragraph 9, section "Prerequisites for the Test Materials";

d) for non-porous materials the type of polymer used for the cover film and its size, shape and thickness;

e) the species of bacteria used and their strain numbers, indicating the reason if other species of bacteria were used;

f) the volume of test inoculum used;

g) the number of viable bacteria in the test inoculum (CFU/ml);

h) the details of the neutraliser employed and the data validating it;

i) the results of the validity check described in section "Conditions for a Valid Test";

j) the results as either CFU/cm^2 or CFU/g and data describing the variance of the data and the statistical significance of any differences detected;

k) details of any deviation from this Guidance Document as well as details of any alternative procedures, if used, including cleaning/sterilisation of the test samples, the use of an alternative recovery method and the use of an alternative incubation temperature. These deviations should be supported by relevant scientific explanations and supporting data;

l) identification of the test laboratory, and the name and signature of the head of the laboratory;

m) the date of commencement of the experiments;

n) the date of the test report; and

o) a reference to this Guidance Document and explanation for any deviation from the test method and whether these deviations affected the results.

LITERATURE

(1) OECD (2008). Guidance Document on the Evaluation of the Efficacy of Antimicrobial Treated Articles with Claims for External Effects. ENV/JM/MONO(2008)27. Environment, Health and Safety Publications, series on Biocides, No. 1, OECD, Paris, 2008.

(2) OECD (2007). Analysis and assessment of current protocols to develop harmonised test methods and relevant performance standards for the efficacy testing of treated articles/treated materials. ENV/JM/MONO(2007)4. Environment, Health and Safety Publications, series on Pesticides, No. 36, OECD, Paris, 2007.

(3) ISO 22196 (2011). Plastics – Measurement of antibacterial activity on plastic surfaces and other non-porous materials, developed by TC 61 (Plastics).

(4) ISO 20743 (2007). Textiles – Determination of antibacterial activity of antibacterial finished products, developed by TC 38 (Textiles) WG 23 (Testing of antimicrobial activity).

(5) Report of international ring-tests conducted by the International Biodeterioration Research Group (IBRG) (2012): under development.

ANNEX 1

DEFINITIONS AND ABBREVIATIONS

TSA: trypticase soya agar

CFU: colony forming unit.

inoculum: test organism in the suspension media as applied to the test material.

neutralisation is a process to quench microbicidal or microbistatic activity of a test substance remaining at the end of the contact time

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

active substance is a compound or formulation that is used in the treated material/article under evaluation for its microbicidal/antimicrobial activity.

test organism is one selected for testing; an organism that has characteristics that allows it to be readily identified. It also may be referred to as a *surrogate, simulant, target* or *marker microbe.* Ideally, it should be easy and safe to handle.

working culture is the culture of the test organism which has undergone the required number of passages from the stock culture and suitable for preparing the *inoculum*.

ANNEX 2

PREPARATION OF SOLUTIONS, REAGENTS AND MEDIA

General remark

Use only reagent-grade chemicals.

Growth and recovery media and supplements

The 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 system. Sterility tests should be conducted on each new batch of semi-solid media, suspending medium or neutraliser used by incubating at least two randomly selected tubes / agar plates for at least five days at both 20°C ± 1°C and 35°C ± 1°C. The ability of each new batch of medium to support the growth of the target species should be determined and recorded.

Trypticase Soya Agar (TSA)

Tryptone, pancreatic digest of casein	15.0 g
Soya peptone, papaic digest of soybean meal	5.0 g
Sodium chloride (NaCl)	5.0 g
Agar	15.0 g
Water	to 1000 ml

Sterilise by autoclaving at 121°C ± 2ºC for 15 minutes. After sterilisation the pH of the medium should be equivalent to 7.3 $\pm$ 0.2 when measured at 20°C ± 1ºC.

Suspension medium – 1/500 nutrient broth (1/500 NB)

Prepare nutrient broth by dissolving 3.0 g of meat extract, 10.0 g of peptone and 5.0 g of sodium chloride in 1000 ml of distilled or deionised water. Dilute the nutrient broth 1:499 with distilled or deionised water and then adjust the pH to a value between 6.8 and 7.2 with either sodium hydroxide or hydrochloric acid. Sterilise by autoclaving at a minimum of 121ºC for 15 minutes. If it is not used immediately after preparation, store it at 5°C ± 3°C. Never use a solution of 1/500 nutrient broth that has been kept for one week or longer after preparation.

Neutraliser

The choice of chemical neutraliser will be dependent on the active substance employed within the treated material. The neutraliser employed should be manufacturer-specified whenever possible, however, an example, general neutraliser, is given below, and a neutraliser suitable for use with silver ions (SCDLP) is given in ISO 22196 (2) and ISO 20743 (3). All neutralisers shall be validated as described in Annex 4 and recorded, along with the validation data, in the final report.

Peptone, digest of casein	20 g
Histidine hydrochloride	1 g
Sodium thiosulphate	5 g
Glucose	2.5 g
Soya lecithin	30 g
Polysorbate 80	20 g
Water	1000 ml (final volume)
pH before sterilisation	7.2 ± 0.2

Sterilise by autoclaving at a minimum of 121 ºC for 15 minutes and record the final pH.

Water

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

ANNEX 3

MATERIALS, EQUIPMENT, FACILITIES AND PERSONNEL

All work shall be performed by experienced microbiologists in facilities suitable for the handling of the microorganisms under test.

Sterilise all laboratory ware 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. Many of the consumable items used in this guideline can be purchased pre-sterilised and ready for use.

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 re-certification.

bunsen burner: with a gas source and flame igniter.

colony counter (optional)

covering film: that does not affect bacterial growth or absorb water (made of polyethylene, polypropylene or polyester [poly(ethylene terephthalate)]). Film that is 0.05 mm to 0.10 mm thick is recommended.

dispenser: for dispensing sterile 10 ml aliquots of diluent/neutraliser.

forceps, straight or curved a) with smooth flat tips to handle samples. 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. Caution should be exercised to avoid contamination and any fire hazards from igniting the alcohol.

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

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

glass rods: for use in holding porous samples in place.

gloves: sterile, disposable, for handling test items.

hot air oven: an oven at 60°C ± 2°C to dry clean glassware.

incubators: an incubator to maintain a temperature of 35°C ± 1°C.

inoculating loops: Sterile plastic or sterilisable metal inoculating loops (10 µl).

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 marking pens.

miscellaneous laboratory-ware: pipette tips, plastic vials for storing stocks of microbes, dilution tubes.

petri dishes (plastic): 90 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): 10-1000 µl with disposable tips – to measure test substance, eluents and diluents as appropriate.

pipette and tips (electronic or non-electronic positive displacement): 10-100 µl pipette and appropriate pipette tips fitted with "plungers" that can dispense accurately 10 µl.

refrigerator: 5ºC ± 3 °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.

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 organisms directly from either a recognised culture collection or other commercial sources.

vials or tubes for dilution: wide-mouthed and suitable to hold 30 ml easily.

vortex mixer: to mix the cell suspensions and neutralised suspensions to ensure efficient recovery of the test organism(s).

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

ANNEX 4

VALIDATION OF THE NEUTRALISER

The neutraliser shall be validated to demonstrate its suitability using the species to be tested against.

Preparation of the Samples and Pre-Incubation

For non-porous materials, (six) replicate test samples containing the highest concentration of the active substance under test and prepared as in "Preparation of Test Materials – Non-porous Materials" are placed into individual sterile Petri dishes with the test surface uppermost. For porous materials, (six) replicate test samples containing the highest concentration of the active substance under test and prepared as in "Preparation of Test Materials – Porous Materials" are placed into separate containers.

For non-porous materials, an aliquot (0.4 ml) of sterile distilled water is placed onto the test surface using a sterile pipette. This is then covered with a piece of sterile covering film (Annex 3) that measures 40 mm × 40 mm and gently press down on the film such that the liquid spreads to the edges and is held in intimate contact with the surface of the test sample. Other sizes of test material should be adapted as described in "Inoculation of Test Materials (including controls)". The samples should then be incubated at 5ºC ± 3 °C in a refrigerator for 23 hours in a closed container. A reduced temperature is used to prevent the growth of adventitious microorganisms under conditions that would allow the migration / release of any active substance to occur for the same contact intervals as is employed in the test.

For porous materials, an aliquot (0.2 ml) of sterile distilled water is transferred onto each sub-sample of the test materials at several points using a sterile pipette taking care to ensure that none touches the surface of the container. If necessary, one of the methods described for accommodating hydrophobic or curled materials etc in “Inoculation of Test Materials (including controls) should be applied. After the test samples have been inoculated the containers are closed and then held at 5ºC ± 3 °C in a refrigerator for 23 hours. A reduced temperature is used to prevent the growth of adventitious microorganisms under conditions that would allow the migration / release of any active substance to occur for the same contact intervals as is employed in the test.

The samples should be removed from the refrigerator for 1 hour and then an aliquot (10 ml) of the neutraliser solution (*e.g.* Annex 2) is added to either each of the containers/petri dishes containing the test samples.

In the case of non-porous materials it is important to ensure that the neutraliser completely washes the samples by using a pipette to collect and release it at least four times. If, due to the nature of the test materials, an alternative method or a different volume of neutraliser has to be employed then this shall be employed in the validation process and documented fully.

In the case of porous materials the contents of the containers will be mixed vigorously by hand. Sterile glass beads and a vortex mixer may be employed if they will be used in the final test (Annex 3).

Preparation of the Inocula

This step should be performed prior to the addition of neutraliser to the test samples (see above) but no more than one hour before removing the samples from the refrigerator. Bacteria shall be transferred using a sterile inoculating loop from pre-incubated plates as described in "Test Microorganisms" into a small amount of $^{1}/_{500}$ nutrient broth (Annex 2). It is important to ensure that the test bacteria are evenly dispersed and sterile glass beads plus a vortex mixer can be used to achieve this if necessary. The number of bacteria in the resulting suspension is then estimated using direct microscopic observation and a counting chamber or another appropriate method (*e.g.* spectrophotometrically). One suspension is prepared for each test species. These suspensions are then diluted with $^{1}/_{500}$ nutrient broth, as appropriate for the estimated bacterial concentration, to obtain a bacterial concentration that is between 2.5×10^3 cells/ml and 10×10^3 cells/ml, with a target concentration of 6×10^3 cells/ml. These suspensions are used as the test inoculum for the validation of the neutraliser. The test inoculum is used within two hours of preparation. The number of colony forming units in the individual inocula will be verified by dilution plate count in triplicate.

Inoculation of the Neutraliser

After a contact interval of 5 minutes (5 min ± 10 seconds) between the neutraliser and the test materials (see above), a sub-sample (5 ml) of each of the replicates of neutraliser is decanted into individual sterile containers. Three of these are inoculated with an aliquot (0.1 ml) of a suspension containing cells of *Escherichia coli* and the remaining three are inoculated with an aliquot (0.1 ml) of a suspension containing cells of *Staphylococcus aureus* as prepared above (or the alternative test species) and then mixed thoroughly by hand. The resulting mixtures are allowed to stand at ambient room temperature for 15 minutes (± 10 seconds) and then re-mixed. After this interval the number of colony forming units is determined by dilution plate count onto TSA. Any validated method (e.g. pour plate, surface plate and spiral dilution) may be employed but it must be the same as the one employed in the main test. The plates are incubated at 35°C ± 1ºC for 24-48 hours. After incubation, the number of colonies present on the plates is counted and recorded.

The log-average (geometric mean) of the number of colony forming units *per* ml of the inocula and the neutraliser solutions are calculated from the plate counts. To be considered suitable for use as a neutraliser, the log-average value of the number of colony forming units recovered from the neutraliser should not be less than half of the number that would be achieved by calculating the number that should be present based on the log-average value of the number of colony forming units present in the inoculum.

ANNEX 5

ADDITIONAL GUIDANCE

Figure 1: Non-Porous Materials – One Test Bacteria

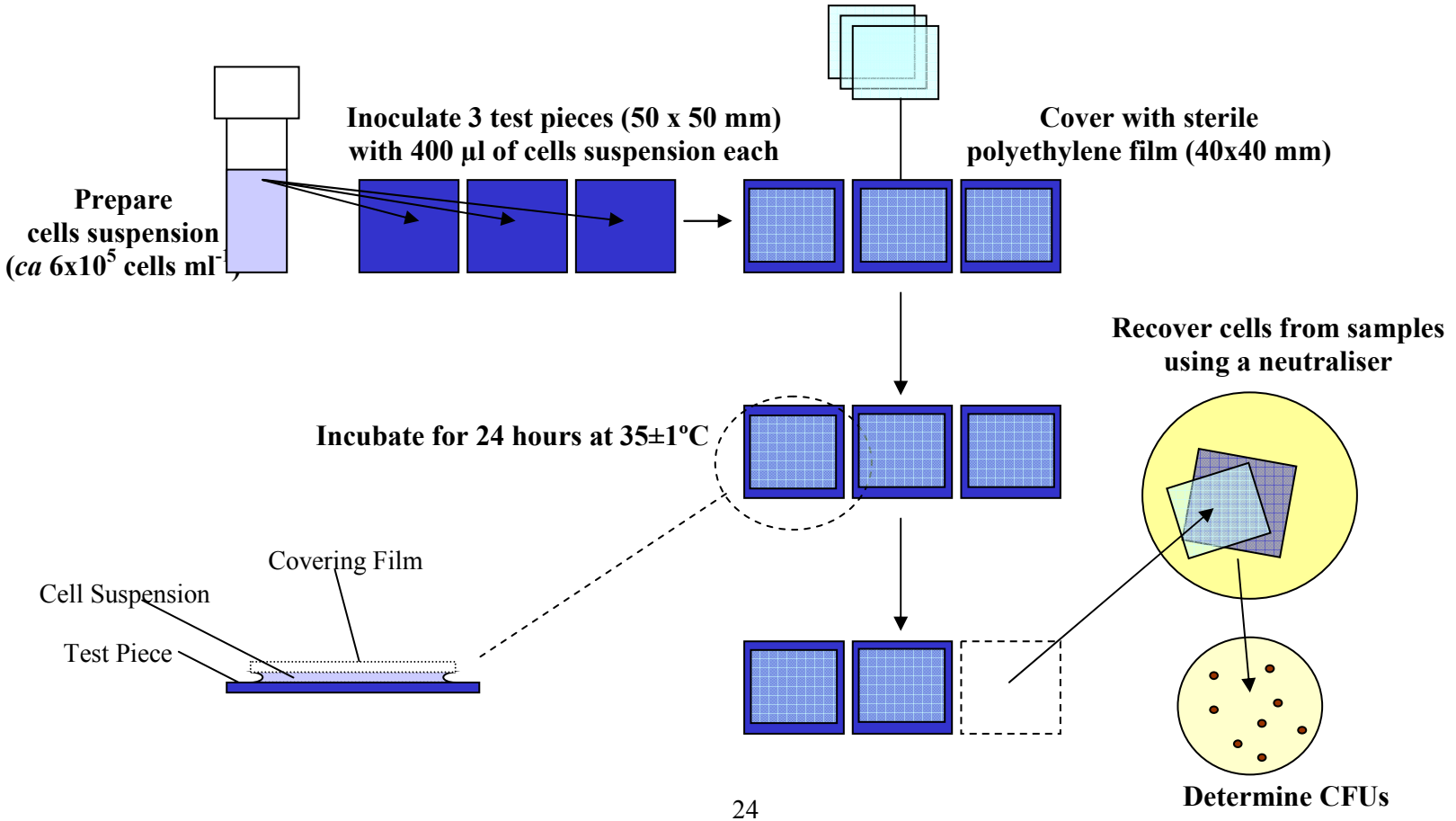

Figure 2: Porous Materials – One Test Bacteria