PAS 2424:2014

BSI Standards Publication

Quantitative surface test for the evaluation of residual antimicrobial (bactericidal and/or yeasticidal) efficacy of liquid chemical disinfectants on hard non-porous surfaces – Test method

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Foreword

This PAS was sponsored by Byotrol Technology Limited. Its development was facilitated by BSI Standards Limited and it was published under licence from The British Standards Institution. It came into effect on 30 November 2014.

Acknowledgement is given to the following organizations that were involved in the development of this PAS as members of the steering group:

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Acknowledgement is also given to the members of a wider review panel who were consulted in the development of this PAS.

This PAS is not to be regarded as a British Standard. It will be withdrawn upon publication of its content in, or as, a British Standard.

The PAS process enables a specification to be developed rapidly in order to fulfil an immediate need in industry. A PAS may be considered for further development as a British Standard, or constitute part of the UK input into the development of a European or International Standard.

Use of this document

It has been assumed in the preparation of this PAS that the execution of its provisions will be entrusted to appropriately qualified and experienced people, for whose use it has been produced.

Presentational conventions

The provisions of this PAS are presented in roman (i.e. upright) type. Its methods are expressed as a set of instructions, a description, or in sentences in which the principal auxiliary verb is "shall".

Commentary, explanation and general informative material is presented in italic type, and does not constitute a normative element.

Spelling conforms to The Shorter Oxford English Dictionary. If a word has more than one spelling, the first spelling is used.

Contractual and legal considerations

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

Compliance with this PAS does not itself confer immunity from legal obligations.

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Introduction

Background

Increasingly, numerous biocidal products are displaying claims of residual antimicrobial activity (e.g. long-lasting protection, 24-h protection and residual action). With these emerging product claims and the impending Biocidal Products Regulation (BPR) which requires the submission of product dossiers to substantiate product claims, there is a need for a test method by which residual antimicrobial activity can be measured and assessed.

At present there is no European Standard test methodology for assessing the residual antimicrobial activity of a chemical disinfectant/antimicrobial product, therefore researchers and companies have designed test methods in an attempt to demonstrate residual antimicrobial efficacy and support these claims. However, these methods largely involve applying a product to a surface and leaving it for a defined period of time before challenging with micro-organisms. The limitation of such methods is that the surface remains undisturbed following application. In reality, consumer research (*Cleaning Behaviours in the Home* [1]) shows that when observing behavioural habits in the domestic environment or workplace, once a product has been applied to a surface, the surface is continually exposed to abrasion such as touching and wiping. This results in potential re-soiling and re-contamination of the surface before the next time a product is applied. Consumer research (*Cleaning Behaviours in the Home* [1]) also shows that the application of a product typically occurs every 24 h.

The test method in PAS 2424 has therefore been designed to reflect within a laboratory test method the actual conditions in which a product is designed to be used. It takes into consideration abrasion and re-contamination by including abrasion cycles and re-inoculations over a 24-h period and remains as close as possible to the practical conditions that are outlined in the current European Standards (e.g. test surface, contact times, micro-organisms, organic load, etc).

Overview of the test method in this PAS

a) An initial inoculum of bacteria or yeast is applied to a steel disc and allowed to dry. This initial step simulates a contaminated surface before the application of a disinfectant product.

b) A prepared sample of the test product (chemical disinfectant) is applied to the inoculated disc and allowed to dry. This gives a dried treatment which represents a surface that is likely to be exposed to abrasion and re-contamination before the next time it is treated with a disinfectant product.

c) Over a period of 24 h the disc undergoes a series of abrasion cycles and re-inoculations which are designed to simulate the abrasion and re-contamination (via touch and exposure) of a surface in between treatment with a disinfectant product.

d) The disc is exposed to a final inoculum challenge (24 h after product application) under defined conditions for a specified contact time. The final inoculum challenge simulates an event that will promote the application of a disinfectant product.

e) After the specified contact time the disc is transferred to a validated neutralizer solution so that the action of the residual disinfectant is quenched. The number of surviving organisms which can be recovered from the disc is determined quantitatively.

f) The number of micro-organisms on a disc treated with hard water in place of disinfectant is also determined. The reduction in viable counts as a result of the residual product is calculated by the difference. In order to demonstrate residual efficacy the product shall give a ≥3 log reduction when challenged 24 h after product application.

Relationship with other publications

PAS 2424 has been designed to take into consideration the experimental conditions set out in BS EN 1276:2009, *Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas – Test method and requirements (phase 2/step 1)* and BS EN 13697:2001, *Chemical disinfectants and antiseptics – Quantitative non-porous surface test for the evaluation of bactericidal and/or fungicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas – Test method and requirements without mechanical action (phase 2/step 2)*. These standards have been chosen because they are recognized test methods that contain experimental conditions representative of the conditions of use of chemical disinfectant products.

BS EN 13697:2001 involves applying an inoculum to a surface and allowing it to dry before applying a test product to the dried film for a specified contact time and transferring the surface to neutralization medium to quench the antimicrobial reaction. PAS 2424 is not designed to test a product at this initial stage but is intended to assess the residual efficacy of a product that has dried onto a surface which is subsequently exposed to abrasion.

Therefore, PAS 2424 has been designed as an extension of BS EN 13697:2001 and is intended to be used in conjunction with this recognized standard allowing BS EN 13697:2001 to assess the initial antimicrobial action of a product and PAS 2424 to assess the residual antimicrobial action.

It is for this reason that the results from PAS 2424 will only be considered if the product tested also achieves a pass according to BS EN 13697:2001 (≥4 log reduction) under conditions required for the product's application of use (e.g. specified contact times, micro-organisms, organic load, etc.).

Conclusion

PAS 2424 is regarded as a suitable solution for screening disinfectant products for their residual antimicrobial efficacy on a surface, and takes into consideration that surfaces are subjected to a degree of abrasion post-treatment. Ultimately, the test is designed to show how a disinfectant product will most likely perform in terms of its real usage, and will enable residual antimicrobial efficacy claims to be made about the product.

This document specifies a test method for determining whether a product does or does not have residual antimicrobial (bactericidal and/or yeasticidal) properties on hard, non-porous surfaces over a 24-h time period with abrasive action.

This laboratory test simulates practical conditions of application and therefore takes into account factors such as contact time, temperature, test organisms, test surface and interfering substance, i.e. conditions which may influence its action in practical situations.

The conditions are intended to cover general purposes and to allow reference between laboratories and product types. However, for some applications, the recommendations of use of a product may differ and therefore additional test conditions need to be used.

1 Scope

This PAS specifies a test method for residual bactericidal and/or yeasticidal activity of liquid, chemical disinfectant products that are applied to hard, non-porous surfaces which are likely to undergo abrasive action. The method described is intended to determine the activity of commercial formulations or active substances under the conditions in which they are used in accordance with BS EN 13697:2001.

It has been designed as an extension of BS EN 13697:2001 and is intended to be used in conjunction with this recognized standard allowing BS EN 13697:2001 to assess the initial antimicrobial action of a product and PAS 2424 to assess the residual antimicrobial action. Therefore the results from this PAS will only be considered if the product tested also achieves a pass according to BS EN 13697:2001 (≥4 log reduction) under conditions required for the test product's application of use (e.g. specified contact times, micro-organisms, organic load, etc.).

It is applicable to ready-to-use products or dilute-to-use products that form a homogeneous, physically stable preparation when diluted with hard water.

It is applicable to products that are used in areas that are covered in BS EN 13697:2001. This includes but is not limited to hard surfaces in the food industry, institutional areas such as schools, hospitals and nursing homes, in the workplace and in the home/domestic environment.

It is not applicable to thickened or viscous products such as toilet bleaches or gels, wash-off products and products that are permanently bound to a surface.

This PAS is designed for laboratories that perform antimicrobial testing in order to test the residual antimicrobial properties of liquid, chemical disinfectant products.

NOTE 1 The sticky nature of thickened products and gels make the abrasion regime difficult to perform and therefore could lead to unreliable results.

NOTE 2 The testing of wash-off products would require a method with the inclusion of a rinsing step.

NOTE 3 Intrinsically antimicrobial surfaces are designed to remain active for longer than the 24 h covered in this test method.

2 Normative references

The following referenced documents are necessary for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

BS EN 10088-1, *Stainless steels – Part 1: List of stainless steels*

BS EN 10088-2, *Stainless steels – Technical delivery conditions for sheet/plate and strip of corrosion resisting steels for general purposes*

BS EN 12353, *Chemical disinfectants and antiseptics – Preservation of test organisms used for the determination of bactericidal (including Legionella), mycobactericidal, sporicidal, fungicidal and virucidal (including bacteriophages) activity*

BS EN 1276:2009, *Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas – Test method and requirements (phase 2/step 1)*

BS EN 13697:2001, *Chemical disinfectants and antiseptics – Quantitative non-porous surface test for the evaluation of bactericidal and/or fungicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas – Test method and requirements without mechanical action (phase 2/step 2)*

BS EN 14885:2006, *Chemical disinfectants and antiseptics – Application of European Standards for chemical disinfectants and antiseptics*

3 Terms, definitions and abbreviations

For the purpose of this PAS, where possible, the terms and definitions given in BS EN 14885:2006, *Chemical disinfectants and antiseptics – Application of European Standards for chemical disinfectants and antiseptics* have been selected.

3.1 Terms and definitions

For the purpose of this PAS, the following terms and definitions apply.

3.1.1 bactericide
product which kills vegetative bacteria under defined conditions

[SOURCE: BS EN 14885:2006, **3.2.1**]

NOTE The adjective derived from "bactericide" is "bactericidal".

3.1.2 chemical disinfectant
product that is capable of chemical disinfection

[SOURCE: BS EN 14885:2006, **3.1.3**]

NOTE "Chemical disinfection" is defined in BS EN 14885:2006 as a "reduction of the number of micro-organisms in or on an inanimate matrix, achieved by the irreversible action of a product on their structure or metabolism, to a level judged to be appropriate for a defined purpose".

3.1.3 neutralizer
chemical agent or formulation that suppresses the residual microbicidal activity of a product within a specific test but does not kill, inactivate or inhibit the test organisms

[SOURCE: BS EN 14885:2006, **3.3.5**]

3.1.4 product
chemical agent or formulation used as a chemical disinfectant or antiseptic

[SOURCE: BS EN 14885:2006, **3.3.6**]

3.1.5 residual bactericidal activity
capability of a product to continue to produce a reduction in the number of viable bacteria cells of relevant test organisms under conditions defined in this PAS

3.1.6 residual yeasticidal activity
capability of a product to continue to produce a reduction in the number of viable yeast cells of relevant test organisms under conditions defined in this PAS

3.1.7 test organism
strain of a micro-organism selected for testing products within a standardised test

[SOURCE: BS EN 14885:2006, **3.3.7**]

NOTE For the purpose of this PAS the term micro-organism includes vegetative bacteria and yeast.

3.1.8 yeasticide
product that kills yeasts under defined conditions

[SOURCE: BS EN 14885:2006, **3.2.16**]

NOTE The adjective derived from "yeasticide" is "yeasticidal".

3.2 Abbreviations

ATCC – American Type Culture Collection

NCPF – National Collection of Pathogenic Fungi

NCTC – National Collection of Type Cultures

4 Principle

A prepared sample of the test product (chemical disinfectant) is applied to a steel disc that has been inoculated with bacteria or yeast and allowed to dry under defined conditions. Over a period of 24 h, the disc undergoes a series of abrasion cycles and inoculations designed to simulate the abrasion and re-contamination of a surface in between product application. The disc is exposed to a final inoculum challenge (24 h after product application) under defined conditions for a specified contact time. After this contact time the disc is transferred to a validated neutralizer solution so that the action of the disinfectant is quenched. The number of surviving organisms which can be recovered from the disc is determined quantitatively.

The number of bacteria or yeast on a steel disc treated with hard water in place of disinfectant is also determined. The reduction in viable counts as a result of the residual product is calculated by the difference. In order to demonstrate residual efficacy the product shall give a ≥3 log reduction 24 h after product application.

NOTE A 24-h time period has been applied for the purpose of this test method as a result of consumer research: Cleaning Behaviours in the Home *[1], for the areas to which this PAS applies. Should other areas be explored, further research would need to be performed into the number of abrasions a specific surface is exposed to over the desired time period.*

5 Performance requirements

5.1 General

When tested according to the obligatory test conditions set out in this PAS, the product shall achieve a ≥3 log (99.9 %) reduction in viable counts in the presence of 3.0 g/l bovine albumin fraction V.

Products that are tested using this method shall also achieve a pass according to the criteria specified in BS EN 13697:2001. The results from PAS 2424 shall only be considered if the product tested has also achieved a pass according to BS EN 13697:2001 (≥4 log reduction) under conditions required for the product's application of use (e.g. specified contact times, micro-organisms, organic load, etc.).

5.2 Requirements for residual bactericidal activity

For a claim of residual bactericidal activity, activity shall be evaluated using the following strains: *Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus hirae* and *Escherichia coli* at a temperature of 20 °C ± 2 °C and a final contact time of 5 min ± 10 s.

5.3 Requirements for residual yeasticidal activity

For a claim of residual yeasticidal activity, activity shall be evaluated using the following strain: *Candida albicans* at a temperature of 20 °C ± 2 °C and a final contact time of 15 min ± 10 s.

Obligatory test conditions shall be passed in order to claim residual performance using the test method in this PAS.

NOTE Where appropriate (for specific purposes) additional bactericidal/yeasticidal activity should be determined under other conditions (e.g. time, temperature, additional strains, interfering substances and number of abrasion cycles) in order to take into account intended specific use conditions.

6 Materials and reagents

6.1 Test organisms

The residual bactericidal activity shall be evaluated using the following strains as test organisms:

- *Pseudomonas aeruginosa* ATCC 15442/NCTC 13359;
- *Escherichia coli* ATCC 10536/NCTC 10418;
- *Staphylococcus aureus* ATCC 6538/NCTC 10788;
- *Enterococcus hirae* ATCC 10541/NCTC 13383.

The residual yeasticidal activity shall be evaluated using the following strain as a test organism:

- *Candida albicans* ATCC 10231/NCPF 3179.

For certain product applications, additional test organisms may be used. For example, this may include, but is not limited to:

- *Listeria monocytogenes* ATCC 35152/NCTC 7973;
- *Salmonella typhimurium* ATCC 13311/NCTC 74;
- (Methicillin resistant) *Staphylococcus aureus* MRSA/NCTC 13277.

NOTE If additional organisms are used, they should be incubated under optimal growth conditions (e.g. temperature, time, culture media and atmosphere) and noted in the test report.

6.2 Culture media and reagents

6.2.1 General

All reagents shall be of analytical grade and/or appropriate for microbiological purposes.

All media may be sourced from external suppliers (either dehydrated or pre-prepared) and shall be of analytical grade and suitable for microbiological use.

NOTE 1 To improve reproducibility, commercially available dehydrated material should be used for the preparation of culture media and the manufacturer's instructions should be rigorously followed.

NOTE 2 All weights of chemical substances given in the test method in this PAS refer to the anhydrous salts.

6.2.2 Water

The water shall be freshly distilled or de-ionized water, sterilized by autoclave (**7.24**) or membrane filtration at a maximum of 0.45 µm pore size (**7.18**). The water shall be free from substances that are toxic or inhibiting to micro-organisms.

NOTE If the water is sterilized during sterilization of the reagents, this is not necessary, e.g. if the water is used for preparation of culture media which is subsequently sterilized.

6.2.3 Tryptone soya agar

Tryptone soya agar (TSA) consisting of:

- Tryptone, pancreatic digest of casein 15.0 g
- Soya peptone, papaic digest of soyabean meal 5.0 g
- Sodium chloride (NaCl) 5.0 g
- Agar 15.0 g
- Water (see **6.2.2**) to 1000.0 ml

Sterilize in the autoclave (see **7.24**). After autoclaving the pH of the medium shall be equivalent to 7.2 ± 0.2 when measured at 20 °C.

6.2.4 Malt extract agar

Malt extract agar (MEA), consisting of:

- Malt extract (technical grade) 30.0 g
- Soya peptone 3.0 g
- Agar 15.0 g
- Water (see **6.2.2**) to 1000.0 ml

Sterilize in the autoclave (see **7.24**). After autoclaving the pH of the medium shall be equivalent to 5.6 ± 0.2 when measured at 20 °C.

6.2.5 Diluent (tryptone sodium chloride solution)

A diluent, consisting of:

- Tryptone, pancreatic digest of casein 1.0 g
- NaCl 8.5 g
- Water (see **6.2.2**) to 1 000.0 ml

Sterilize in the autoclave (see **7.24**). After autoclaving the pH of the medium shall be equivalent to 7.0 ± 0.2 when measured at 20 °C.

6.2.6 Neutralizer solution

The selected neutralizer shall be validated for the product being tested in accordance with **9.5.2** and **9.5.3**.

Sterilize in the autoclave (see **7.24**).

NOTE 1 A list of neutralizers that may be used can be found in Annex A: this list is not exhaustive and other validated neutralizers may also be used.

NOTE 2 For autoclaving of the neutralizer solution it may be ideal to make up smaller volumes in a larger vessel to prevent overflow (e.g. 250.0 ml of neutralizer solution in a 500.0 ml Duran bottle).

6.2.7 Hard water for dilution of products and for use in wet wears

For the preparation of 1000.0 ml of hard water, the procedure is as follows.

a) Prepare solution A: dissolve 19.84 g magnesium chloride ($MgCl_2$) and 46.24 g calcium chloride ($CaCl_2$) in water (see **6.2.2**) and dilute to 1 000.0 ml. Sterilize by membrane filtration with a maximum 0.45 µm pore size (see **7.18**).

b) Store the solution at 4 to 8 °C for no longer than one month.

c) Prepare solution B: dissolve 35.02 g sodium bicarbonate ($NaHCO_3$) in water (see **6.2.2**) and dilute to 1 000.0 ml. Sterilize by membrane filtration with a maximum 0.45 µm pore size (see **7.18**).

d) Store the solution at 4 to 8 °C for no longer than one week.

e) Place ~600.0 ml of water (see **6.2.2**) in a 1 000.0 ml volumetric flask and add 6.0 ml of solution A followed by 8.0 ml of solution B. Mix and dilute to 1 000.0 ml with water (see **6.2.2**). The pH of the hard water shall be 7.0 ± 0.2. If necessary, adjust the pH by using approximately 1 mol/l sodium hydroxide (NaOH) or approximately 1 mol/l hydrochloric acid (HCl) as required.

f) Sterilize by membrane filtration with a maximum 0.45 µm pore size (see **7.18**).

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

6.2.8 Interfering substance

The interfering substance shall be made at 2 × the required concentration to account for dilution encountered during the test.

Obligatory conditions are 3.0 g/l bovine albumin fraction V.

Dissolve 0.6 g of bovine albumin in 100.0 ml of water (see **6.2.2**). The final concentration of bovine albumin in the test procedure shall be 3.0 g/l. Sterilize by membrane filtration with a maximum 0.45 µm pore size (see **7.18**).

Additional interfering substances may be chosen according to the product's intended use. For example, this may include, but is not limited to the following:

a) Milk (dairies, etc.) – skimmed milk, guaranteed free of antibiotics and additives and reconstituted at a rate of 10.0 g powder per litre of water (**6.2.2**), shall be prepared as follows: prepare a solution of 200 g milk-powder in 1000 ml water. Heat for 30 min at (105 ± 3) °C [or 5 min at (121 ± 3) °C]. The final concentration of reconstituted milk in the test procedure is 10.0 g/l.

b) Yeast extract (breweries, etc.) – dehydrated yeast extract for bacteriology, shall be prepared as follows: prepare a 20.0 g/l solution in water (**6.2.2**), adjust to pH 7.0 ± 0.2 with sodium hydroxide (NaOH); sterilize in the autoclave (**7.24**). The final concentration of yeast extract in the test procedure is 10.0 g/l.

c) Sucrose (beverage industry) – prepare a 20.0 g/l solution of sucrose in water (**6.2.2**), sterilize by membrane filtration (**7.18**). The final concentration of sucrose in the test procedure is 10.0 g/l.

d) Sodium dodecyl sulphate (SDS) (cosmetic industry) – prepare a 10.0 g/l solution of sodium dodecyl sulphate ($C_{12}H_{25}NaO_4S$) in water (**6.2.2**). Sterilize in the autoclave (**7.24**). The final concentration of sodium dodecyl sulphate in the test procedure is 5.0 g/l.

e) Buffer solutions (cleaning) – the buffer solution used shall be described in the test report and pH values shall be recorded. The final pH in the test tubes (together with test organisms and product) shall be controlled and found equal to 5.0 ± 0.2 or 9.0 ± 0.2.

NOTE Alternative interfering substances have been sourced from BS EN 1276:2009 and BS EN 13697:2001, as examples only.

6.3 Test surface

These shall be stainless steel discs of type 1.4301, 2.0 cm in diameter with a Grade 2B finish on both sides used in accordance with EN 10088-1 and EN 10088-2. The surfaces shall be of approximately 1.5 mm in thickness. The discs shall be used once and subsequently discarded.

7 Apparatus

7.1 *Water bath*, capable of being controlled at 20 °C ± 1 °C and 48 °C ± 1 °C (for tempering melted media if used).

7.2 *Boiling water bath*, for melting solid media.

7.3 *Incubator (for bactericidal activity)*, capable of being controlled at 37 °C ± 1 °C.

7.4 *Incubator (for yeasticidal activity)*, capable of being controlled at 30 °C ± 1 °C.

7.5 *Suitable apparatus for drying the test product at 30 °C* (e.g. incubator or dry heat oven).

7.6 *pH meter*, having an inaccuracy of no more than 0.1 pH units at 20 °C.

7.7 *Colorimeter*, capable of measuring at 590 nm (or other suitable apparatus for determining cell density).

7.8 *Cuvettes*.

7.9 *Stopwatch*.

7.10 *Vortex mixer*.

7.11 *Containers*, such as test tubes, culture bottles, etc.

7.12 *Centrifuge tubes*, of diameter ~3.5 cm.

7.13 *Pipettes.*

7.14 *Pipette tips*.

7.15 *Petri dishes*, of diameter 90 mm.

7.16 *Forceps*, capable of lifting the test surface without rotation and without disrupting the test product.

7.17 *Laminar air flow cabinet*, for use in preparing the steel discs.

7.18 *Membrane filters*, of maximum pore size 0.45 µm.

7.19 *100 % polypropylene hard surface wipes*, 25 gsm, non-treated, non-patterned for use in performing abrasion cycles.

7.20 *Inoculation loops*, 10 µl.

7.21 *Trigger spray bottle*, G shaped with plastic on/off nozzle, 3-finger, standard spray.

7.22 *Weights*, capable of adding up to ~210.0 ± 2g, of diameter to fit inside a 3.5 cm centrifuge tube.

7.23 *Glass beads,* of diameter 3 mm to 4 mm.

7.24 *Autoclave*.

7.25 *Elastic bands*, capable of securing a polypropylene wipe to the lid of a centrifuge tube for use in performing abrasions.

Sterilize all equipment that will come into contact with culture media, reagents and products by autoclave at 121 °C + 3 °C for a minimum of 15 min unless bought pre-sterilized or unless otherwise stated in the manufacturer's instructions

NOTE 1 Optional apparatus could include a steel disc holder (capable of securing a steel disc in place without movement during abrasions). See Annex B.

NOTE 2 The number of each consumable used during a test is dependent on the number of products being tested, the size of each of the consumables, individual lab/operator preference and equipment availability.

8 Preparation

8.1 Organism test suspensions

8.1.1 Stock cultures of test organisms

Stock cultures shall be maintained in accordance with the requirements of BS EN 12353.

8.1.2 Working cultures of test organisms

a) Bacteria

In order to prepare the working culture of the test organism, subculture from the stock culture (see **8.1.1**) by using a sterile inoculating loop (**7.20**) to streak onto 90.0 mm TSA plates (see **6.2.3**) and incubate at 37 °C ± 1 °C (see **7.3**). This shall be labelled the master culture.

After 18 to 24 h prepare a second subculture in the same way taken from the master culture and incubate at 37 °C ± 1 °C (see **7.3**). This shall be labelled the 1A culture.

After 18 to 24 h prepare a third subculture in the same way taken from the 1A culture and incubate at 37 °C ± 1 °C (see **7.3**). This shall be labelled the 2A culture. No further subcultures shall be taken.

The 1A or 2A culture shall be the working culture.

b) Yeast

In order to prepare the working culture of *Candida albicans*, subculture from the stock culture (see **8.1.1**) by using a sterile loop (**7.20**) to streak onto 90.0 mm MEA plates (see **6.2.4**) and incubate at 30 °C ± 1 °C (see **7.4**). This shall be labelled the master culture.

After 42 to 48 h prepare a second subculture in the same way taken from the master culture and incubate at 30 °C ± 1 °C (see **7.4**). This shall be labelled the 1A culture.

After 42 to 48 h prepare a third subculture in the same way taken from the 1A culture and incubate at 30 °C ± 1 °C (see **7.4**). This shall be labelled the 2A culture. No further subcultures shall be taken.

The 1A or 2A culture shall be the working culture.

8.1.3 Test suspensions

8.1.3.1 Bacterial test suspension

Take ~10.0 ml of tryptone saline diluent (see **6.2.5**) and place into a sterile tube with ~5g of glass beads (**7.23**). Take the working culture (see **8.1.2**) and using a sterile inoculating loop (**7.20**), transfer loopfuls of the cells into the diluent by immersing the loop in the diluent and rubbing it against the side of the tube to dislodge the cells. Shake the tube gently to suspend the cells. Aspirate the suspension from the glass beads and transfer to another tube. Check for a homogeneous suspension. Adjust the number of cells in the suspension to between 1.5×10^8 cfu/ml and 5.0×10^8 cfu/ml using the diluent (**6.2.5**) and a colorimeter (**7.7**) or any other suitable means.

8.1.3.2 Yeast test suspension

Take ~10 ml of tryptone saline diluent (see **6.2.5**) and place into a sterile tube with ~5 g of glass beads (**7.23**). Take the working culture (see **8.1.2**) and using a sterile inoculating loop (**7.20**), transfer loopfuls of the cells into the diluent by immersing the loop in the diluent and rubbing it against the side of the tube to dislodge the cells. Shake the tube gently to suspend the cells. Aspirate the suspension from the glass beads and transfer to another tube. Check for a homogeneous suspension. Adjust the number of cells in the suspension to between 1.5 x 10^7 cfu/ml and 5.0 x 10^7 cfu/ml using the diluent (**6.2.5**) and a colorimeter (**7.7**) or any other suitable means.

NOTE Each laboratory should produce calibration data for each test organism for the method of adjusting cells that is used.

8.1.4 Enumeration of test suspension

To enumerate the initial test suspensions, use diluent (see **6.2.5**) to perform a series of dilutions. For the bacterial suspension (see **8.1.3.1**) dilute to 10^{-7} and for the yeast suspension (see **8.1.3.2**) dilute to 10^{-6}. Using a suitable means of enumerating micro-organisms (e.g. pour plates or spread plates), enumerate the number of micro-organisms present in 1 ml. Plate out in duplicate.

Use TSA (see **6.2.3**) for bacterial enumeration and MEA (see **6.2.4**) for yeast enumeration. Incubate TSA plates for 18 to 24 h at 37 °C ± 1 °C (**7.3**) and incubate MEA plates for 42 to 48 h at 30 °C ± 1 °C (**7.4**). Count the dilution plate with between 15 and 300 cfu.

Incubate the plates for a further 20 to 24 h. Do not recount plates that no longer show well separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.

Take duplicate plate values (x, x^1) and calculate the $\log_{10}$ number of cfu per 0.01 ml of test suspension using the following calculation where d is the dilution factor taken into account.

$$N = \log_{10} \frac{\left(x + x^1\right)}{2} \times \frac{0.01}{d}$$

N = $\log_{10}$ number of cfu per 0.01ml of test suspension

x and x^1 = cfu/ml from duplicate plates

d = dilution of plate dilution counted from e.g. 10^{-3} or 0.001

2 = number of replicate plates

0.01 = volume in ml of test suspension enumerated

EXAMPLE

For each test suspension, record the number of cfu/ml and calculate the decimal log per 0.01 ml using the following calculation:

$$N = \log_{10} \frac{\left(x + x^1\right)}{2} \times \frac{0.01}{d}$$

Duplicate x values counted on the 10^{-6} plate: 40, 46

$$N = \log_{10} \frac{(40+46)}{2} \times \frac{0.01}{10^{-6}}$$

$N = \log_{10} \{43 \times 10\,000\}$

$N = \log_{10} \{430\,000\}$

$N = 5.63$

8.1.5 Preparation of inocula

8.1.5.1 Re-inoculation culture

Prepare a test suspension (see **8.1.3**) and prepare a series of dilutions in diluent (**6.2.5**) to give a suspension of ~10^6 for bacteria and ~10^5 for yeast. Add 1.0 ml of the suspension to 1.0 ml of interfering substance (see **6.2.8**) to give the required conditions and mix well before use. Cultures used for further re-inoculations may be kept at approximately 2 to 8 °C for no more than 8 h and equilibrated to room temperature before use.

NOTE The re-inoculation culture is intended to represent low levels of contamination encountered through abrasion and is therefore lower than the initial and final inoculation cultures.

8.1.5.2 Initial and final inoculum cultures

Prepare a test suspension (see **8.1.3**) and add 1.0 ml of the suspension to 1.0 ml of interfering substance (see **6.2.8**) to give the required conditions and mix well before use.

NOTE The initial and final inoculation cultures are intended to represent a high level of contamination that would usually promote the application of a product and is therefore higher than the re-inoculation culture.

8.2 Preparation of test product

Ready to use products shall be in date however they do not require any further preparation. All dilutable products shall be in date and prepared as per manufacturer's directions of use using sterile hard water (see **6.2.7**). Therefore all products shall be tested at their in-use concentration. Once diluted in hard water (**6.2.7**) the products shall give a physically homogeneous, stable solution and shall be applied within 60 min.

8.3 Preparation of test surface

Prepare 5 replicate steel discs (see **6.3**) per product being tested and 5 replicate steel discs to be treated with hard water (see **6.2.7**).

Prior to use, the steel discs (**6.3**) shall be cleaned thoroughly and sterilized. In order to remove any dirt or grease from the manufacturing process, rinse the discs (**6.3**) thoroughly with running freshly-distilled water for at least 5 min before placing in 70 % (V/V) iso-propanol for 15 min. Dry by evaporation on both sides under laminar flow (**7.17**).

Clean and dry the discs thoroughly before autoclaving. Sterilize in the autoclave (**7.24**). Ensure the discs are thoroughly dry before use.

NOTE The discs should not be cleaned with any product that might inhibit the action of the test product e.g. surfactant.

Place the steel discs (**6.3**) aseptically into separate Petri dishes (**7.15**) ensuring the dish is in a horizontal position. Prepare 5 steel discs (**6.3**) per product and 5 steel

discs (**6.3**) for the water control by inoculating 0.01 ml of the initial inoculum culture (**8.1.5.2**) onto the centre of each disc. Spread the inoculum to within 0.5 cm from the edge of the disc using a sterile inoculating loop (**7.20**). Dry the discs at 30 °C (**7.5**) until the inoculum is visibly dry (≤ 30 min).

For one disc per test product, permanently mark one side of the disc before application of the initial inoculum. This is to be the untreated side of the disc and should be identifiable after final inoculation to aid in the recovery for Validation A (**9.5.1**).

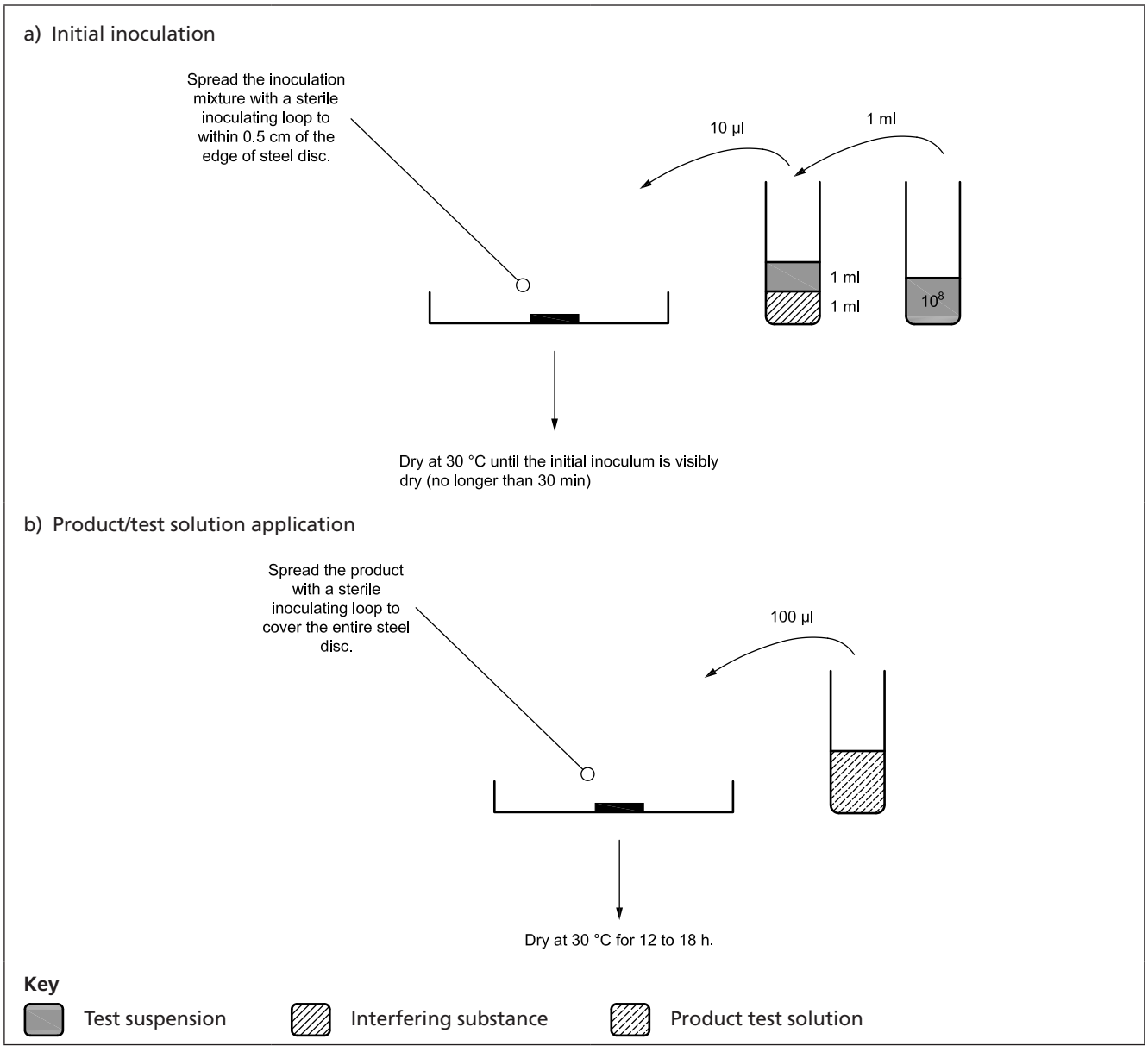
Pipette 0.1 ml of test product (see **8.2**) onto 5 separate steel discs (**6.3**) that have been inoculated with the initial inoculum culture (**8.1.5.2**) ensuring that the entire surface is covered. Pipette 0.1 ml of hard water (see **6.2.7**) onto a separate 5 inoculated steel discs (**6.3**). Dry the discs at 30 °C (**7.5**) for 12 to 18 h.

NOTE The test product may be left to dry overnight providing the entire test regime is completed within 24 h.

Repeat this procedure for additional test suspensions, contact times, temperatures and interfering substances required.

NOTE See Figure 1 for a graphical representation of the preparation of the test surface.

Figure 1 **Preparation of test surface**



9 Procedure

9.1 Experimental conditions

9.1.1 General

The selection of experimental conditions such as temperature, contact time and interfering substance shall be carried out according to the practical use conditions considered for the product as follows below.

9.1.2 Test temperature and humidity

The abrasion cycles and final challenge shall be carried out at 20 °C ± 2 °C at 40 to 70% relative humidity. Additional temperatures may be selected depending on the product's application of use.

9.1.3 Contact time

The contact time shall be 5 min ± 10 s for bacteria and 15 min ± 10 s for yeast. Additional contact times may be selected depending on the product's application of use.

9.1.4 Strains

The strains shall be selected according to **6.1**. Additional strains may be selected depending on the product's application of use.

9.1.5 Interfering substances

The interfering substance shall be bovine albumin fraction V (see **6.2.8**) under obligatory dirty conditions (3.0 g/l). Additional interfering substances may be selected depending on the product's application of use.

NOTE The test conditions above indicate obligatory test conditions, however if additional conditions are used this should be acknowledged in the test report.

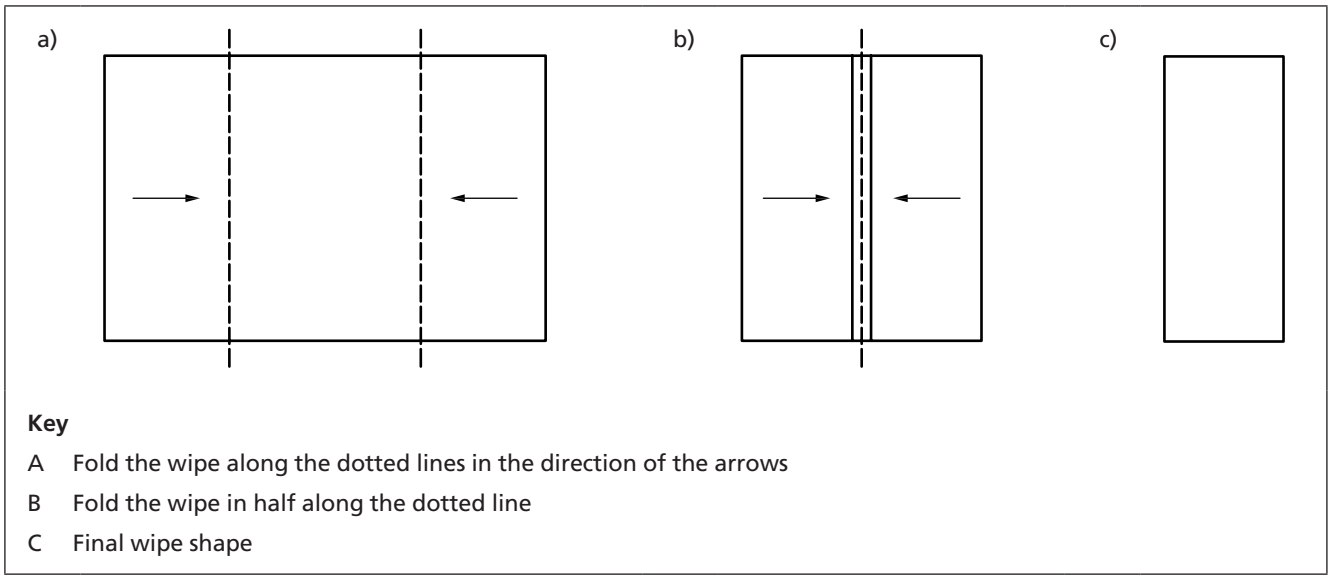
9.2 Abrasion regime

9.2.1 General

Weight a 3.5 cm in diameter centrifuge tube (**7.12**) to 210.0 g ± 2 g (**7.22**). Take a polypropylene wipe (see **7.19**) and fold in half lengthways and then in half lengthways again following the process shown in Figure 2 so that the final wipe is 4 sheets in thickness.

*NOTE Additional steel discs (**6.3**) may be used as weights to weight the centrifuge tube.*

Figure 2 **Folding of polypropylene wipe**



Key

A Fold the wipe along the dotted lines in the direction of the arrows

B Fold the wipe in half along the dotted line

C Final wipe shape

9.2.2 Dry abrasion

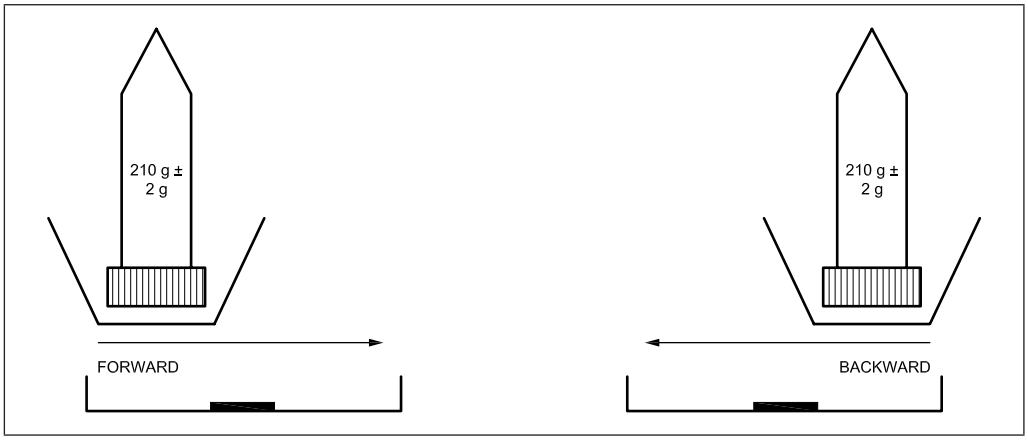
Wrap the folded (see Figure 2) polypropylene wipe (**7.19**) smoothly around the lid end of the weighted centrifuge tube and secure using an elastic band (**7.25**) so that the wipe does not move. Hold the prepared steel disc (see **8.3**) in place to prevent movement and pass the weighted centrifuge tube across the surface of the disc in a forward motion followed by a backward motion making sure that the wipe comes into contact with the entire surface of the disc (see Figure 3). Ensure that no pressure is exerted onto the disc as all weight exerted onto the disc shall be provided by the weights inside the centrifuge tube.

Repeat for all 5 replicates ensuring that a separate wipe is used for each replicate.

A steel disc holder can be used to hold the steel disc securely in place (see Annex B). If used, the holder should be manufactured at the same time as the discs so that the holder is the same thickness, allowing the discs to sit flush. If a holder is used it should be treated with 70 % (V/V) iso-propanol in between each abrasion and only one abrasion should be performed at any one time, using a separate polypropylene wipe for each abrasion. This is to avoid cross contamination of the discs.

Alternatively the discs can be held in place using forceps or a sterile pipette tip to the side of the disc to prevent movement.

Figure 3 **Abrasion**

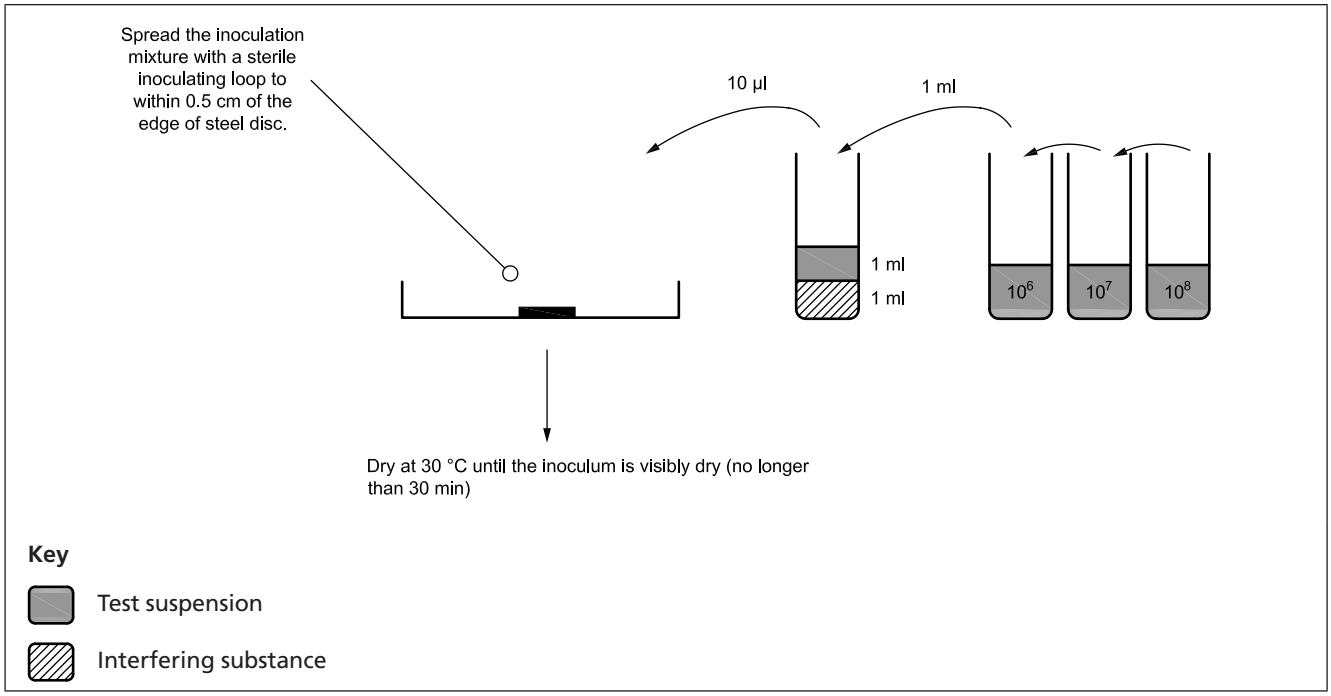


9.2.3 Re-inoculation

Pipette 0.01 ml of the re-inoculation culture (see **8.1.5.1**) onto the centre of each steel disc and spread to within 0.5 cm from the edge of the disc using a sterile inoculating loop (**7.20**). Leave the lid of the Petri dish ajar and allow the inoculum to dry at 30 °C ± 1 °C (**7.5**) until visibly dry (≤ 30 min).

NOTE This is a re-inoculation as shown in Figure 4.

Figure 4 **Re-inoculation**



9.2.4 Wet abrasion

Wrap a new folded (see Figure 2) polypropylene wipe (**7.19**) smoothly around the lid end of the weighted centrifuge tube and secure using an elastic band (**7.25**). Take the tube and from a distance of ~75.0 cm, wet the wipe with sterile standardized hard water (see **6.2.7**) by spraying it twice from a trigger spray bottle (see **7.21**) that has been rinsed through once with 70 % (V/V) iso-propanol and once with sterile hard water (see **6.2.7**). If the trigger head has multiple settings, the trigger spray shall be set to the spray position in which a fine mist is dispensed.

As performed in a dry abrasion, hold the prepared steel disc (see **8.3**) in place to prevent movement and take the weighted centrifuge tube and pass it across the surface of the disc in a forward motion followed by a backward motion (see Figure 3) making sure that the wipe comes into contact with the entire surface of the disc. Ensure that no pressure is exerted onto the disc as all weight exerted onto the disc shall be provided by the weights inside the centrifuge tube. After a wet abrasion has been performed, dry the discs at 30 °C (**7.5**) until they are visibly dry (≤15 min) before performing a re-inoculation (**9.2.3**).

Repeat the processes described in **9.2** until a total of 3 dry abrasions, 3 wet abrasions and 5 re-inoculations have been achieved for each steel disc following the regime detailed in Table 1.

Table 1 **Summary of abrasion regime**

24 h	Initial inoculation/dry (**8.3**)	
	Product application/dry (**8.3**)	
	Dry abrasion (**9.2.2**)	**Abrasion cycle 1**
	Re-inoculation/dry (**9.2.3**)	
	Wet abrasion/dry (**9.2.4**)	
	Re-inoculation/dry (**9.2.3**)	
	Dry abrasion (**9.2.2**)	**Abrasion cycle 2**
	Re-inoculation/dry (**9.2.3**)	
	Wet abrasion/dry (**9.2.4**)	
	Re-inoculation/dry (**9.2.3**)	
	Dry abrasion (**9.2.2**)	**Abrasion cycle 3**
	Re-inoculation/dry (**9.2.3**)	
	Wet abrasion/dry (**9.2.4**)	
	Final inoculation (**9.3**)	
	Incubation	
	Count plates	
	Re-incubation	
	Record final results	

9.3 Final inoculation and residual antimicrobial challenge

Once the abrasion regime (see **9.2**) has been completed and 24 h after product application, pipette 0.01 ml of the final inoculation culture (see **8.1.5.2**) onto the centre of each steel disc at appropriate intervals and spread the inoculum to within 0.5 cm from the edge of the disc using a sterile inoculating loop. After the specified period of exposure (see **9.1.3**), use sterile forceps (**7.16**) to transfer the steel discs into separate tubes containing 10 ml of neutralizer solution (**6.2.6**) and ~5 g of glass beads ensuring that the disc is completely submerged in the neutralizer solution. (see **7.23**). Vortex the tubes for ~30 s to remove any viable cells from the surface of the disc.

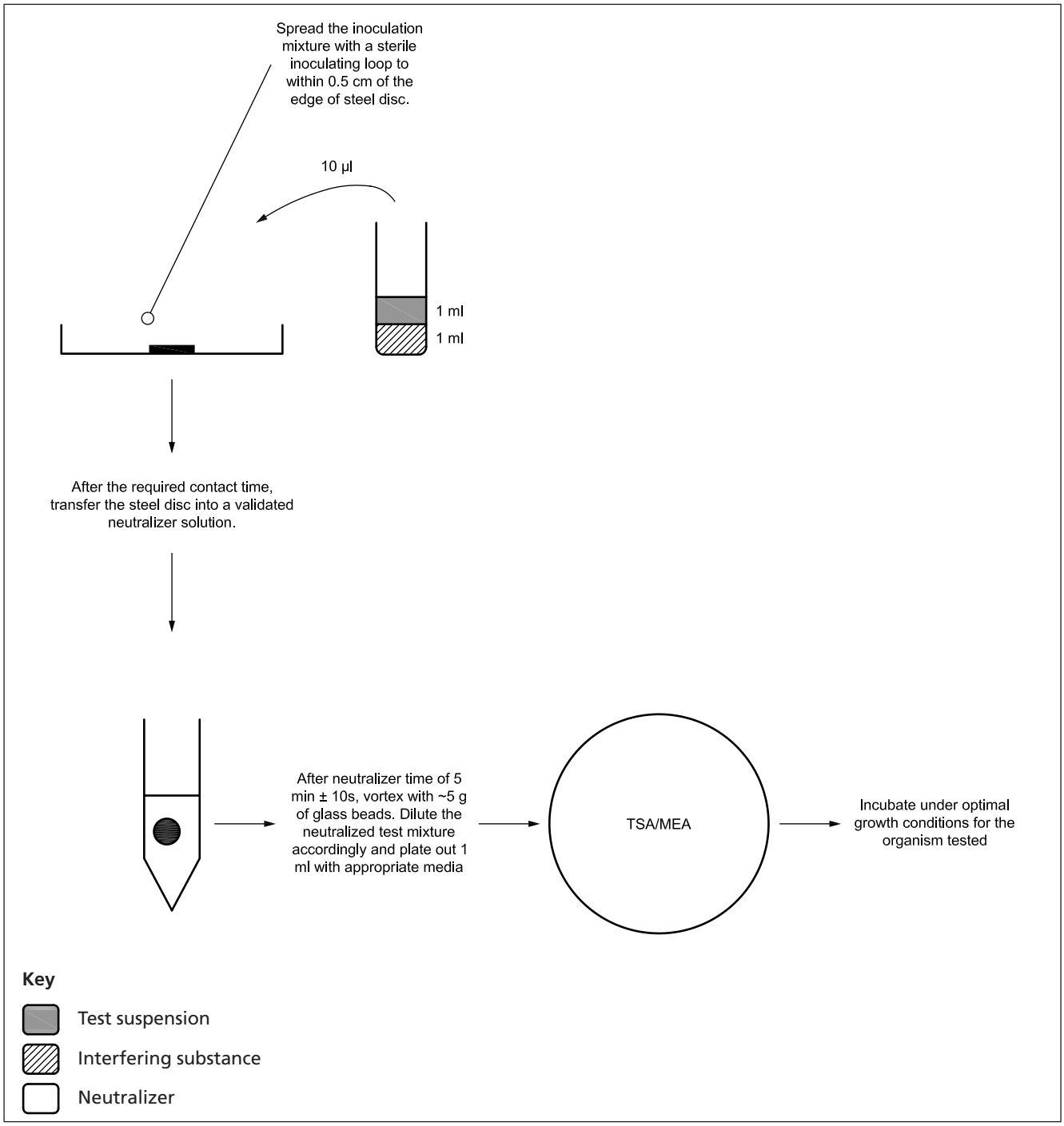
For the disinfectant test, after a neutralization time of 5 min ± 10 s, prepare a series of dilutions (to 10^{-2}) of the neutralized mixture in diluent (see **6.2.5**) or neutralizer solution (see **6.2.6**). For the water control after a neutralization time of 5 min ± 10 s, prepare a series of dilutions (to 10^{-4}) of the neutralized mixture in diluent (see **6.2.5**) or neutralizer solution (see **6.2.6**).

Using a suitable means of enumerating micro-organisms (e.g. pour plates or spread plates), enumerate the number of micro-organisms present in 1 ml.

Use TSA (see **6.2.3**) for bacterial enumeration and MEA (see **6.2.4**) for yeast enumeration. Incubate TSA plates for 24 h at 37 °C ± 1 °C (**7.3**) and incubate MEA plates for 42 to 48 h at 30 °C ± 1 °C (**7.4**). Count the dilution plate with between 15 and 300 cfu. Incubate the plates for a further 20 to 24 h. Do not recount plates that no longer show well separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.

NOTE See Figure 5 for a graphical representation of the final inoculation and residual antimicrobial challenge.

Figure 5 **Final inoculation and residual antimicrobial challenge**



9.4 Counting of the test mixtures and calculation of log reduction

For both the disinfectant test and the water control, count the dilution plates with between 15 and 300 cfu for each of the 5 replicates. Multiply the plate count by the dilution factor and log these values. Take an average of the 5 replicate log values and minus the disinfectant test result from the water control result to give the microbicidal effect.

$$a = b \times \frac{10}{d}$$

$$N_w\,(\text{or } N_d) = \frac{\log_{10}\left(a^1\right) + \log_{10}\left(a^2\right) + \log_{10}\left(a^3\right) + \log_{10}\left(a^4\right) + \log_{10}\left(a^5\right)}{5}$$

$[ME = N_w - N_d]$

When:

$a^{1\text{-}5}$ = cfu/disc for the neutralized test mixture/water control mixture

$b^{1\text{-}5}$ = cfu/ml of the neutralized test mixture/water control mixture

5 = number of replicates

10 = volume in ml of neutralizer solution used

d = dilution factor from plate counted from e.g. 10^{-3} or 0.001

N_d = average log of cfu per test surface for the disinfectant test

N_w = average log of cfu per test surface for the water control

ME = microbicidal effect ($\log_{10}$ reduction)

EXAMPLE For each test condition record the number of cfu/ml in the test mixture for the test product and the water control and calculate the decimal log using the following calculations:

$$a^{(1\text{–}5)} = b^{(1\text{–}5)} \times \frac{10}{d}$$

$$N_w\,(\text{or } N_d) = \frac{\log_{10}\left(a^1\right) + \log_{10}\left(a^2\right) + \log_{10}\left(a^3\right) + \log_{10}\left(a^4\right) + \log_{10}\left(a^5\right)}{5}$$

Replicate values for $b^{1\text{-}5}$ for the water control counted on the 10^{-3} plate: 95, 95, 80, 93, 95

$$a^{(1\text{–}5)} = b^{(1\text{–}5)} \times \frac{10}{d}$$

$$a^1 = 95 \times \frac{10}{10^{-3}}$$

$$a^1 = 950\,000$$

$$a^2 = 95 \times \frac{10}{10^{-3}}$$

$$a^2 = 950\,000$$

$$a^3 = 80 \times \frac{10}{10^{-3}}$$

$$a^3 = 800\,000$$

$$a^4 = 93 \times \frac{10}{10^{-3}}$$

$$a^4 = 930\,000$$

$$a^5 = 95 \times \frac{10}{10^{-3}}$$

$$a^5 = 950\,000$$

Replicate values $a^{(1\text{-}5)}$ for the water control: 950 000, 950 000, 800 000, 930 000, 950 000

$$N_w = \frac{\log_{10}(950\,000) + \log_{10}(950\,000) + \log_{10}(800\,000) + \log_{10}(930\,000) + \log_{10}(950\,000)}{5}$$

$N_w = 5.96$

Replicate values for $b^{(1\text{-}5)}$ for the neutralized test mixture counted on the 10^{-0} plate = 13, 12, 11, 1, 2.

$$a^{(1\text{–}5)} = b^{(1\text{–}5)} \times \frac{10}{d}$$

$$a^1 = < 15 \times \frac{10}{10^{-0}}$$

$a^1 = 150$

$$a^2 = < 15 \times \frac{10}{10^{-0}}$$

$a^2 = 150$

$$a^3 = < 15 \times \frac{10}{10^{-0}}$$

$a^3 = 150$

$$a^4 = < 15 \times \frac{10}{10^{-0}}$$

$a^4 = 150$

$$a^5 = < 15 \times \frac{10}{10^{-0}}$$

$a^5 = 150$

Replicate values $a^{(1\text{-}5)}$ for the neutralized test mixture: 150, 150, 150, 150 ,150

$$N_d = \frac{\log_{10}(150) + \log_{10}(150) + \log_{10}(150) + \log_{10}(150) + \log_{10}(150)}{5}$$

$N_d = 2.18$

For each test condition calculate the microbicidal effect by calculating the log reduction using the following calculation.

$[ME = N_w – N_d]$

Using the previously calculated N_w and N_d values: $N_w = 5.96$, $N_d = 2.18$

$ME = 5.96 – 2.18$

$ME = >3.79$

9.5 Validation experiments

9.5.1 Organism recovery (A)

9.5.1.1 Principle of organism recovery validation experiment

The organism recovery validation experiment ensures that the test procedure (see **9.3**) is capable of successfully recovering all viable organisms from the surface of the steel disc (see **8.3**).

9.5.1.2 Procedure for organism recovery validation experiment

After performing the final inoculation and residual antimicrobial challenge (see **9.3**) and after 1.0 ml of the neutralized test mixture has been sampled, recover one steel disc (see **8.3**) per product which has been previously marked (see **8.3**), drain off the neutralizer solution and gently rinse with 10.0 ml of water (see **6.2.2**). Use sterile forceps (**7.16**) to transfer the disc to a Petri dish containing ~10.0 ml of solidified TSA (see **6.2.3**) for bacteria or 10.0 ml of solidified MEA (see **6.2.4**) for yeast, ensuring that the surface is orientated test side up. Add 0.1 ml of water (see **6.2.2**) to the surface of the disc and use a sterile inoculating loop (**7.20**) to scrape the surface for ~1 min. Pour ~10.0 ml of the same melted counting medium, tempered at 48 °C ± 1 °C (**7.1**) over the surface of the steel disc.

Repeat this procedure for additional test suspensions, contact times, temperatures and interfering substances.

Incubate TSA plates for 18 to 24 h at 37 °C ± 1 °C (**7.3**) and incubate MEA plates for 42 to 48 h at 30 °C ± 1 °C (**7.4**). Record number of colony forming units (A). Incubate the plates for a further 20 to 24 h. Do not recount plates that no longer show well separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.

The organism recovery should be <100 cfu for active substances. For non-active substances (e.g. water control), the number of colony forming units may not be countable.

9.5.2 Neutralizer validation (B)

9.5.2.1 Principle of neutralizer validation experiment

The neutralizer validation experiment ensures that the chosen neutralizer solution (see **6.2.6**) sequesters the residual antimicrobial properties of the product being tested within the appropriate contact time.

9.5.2.2 Procedure for neutralizer validation experiment

Pipette 10.0 ml of neutralizer solution (see **6.2.6**) into two tubes. Into one tube add 0.1 ml of test product (see **8.2**). This shall be the neutralization test (NT). Into the other tube add 0.1 ml of hard water (see **6.2.7**). This shall be the neutralization control (NC). Mix and leave for a contact time of 5 min ± 10 s.

Prepare a test suspension (**8.3.1**) and prepare a series of dilutions to give approximately 10^5 cfu/ml. Mix 1.0 ml of the diluted test suspension with 1.0 ml of interfering substance (**6.2.8**) and apply 0.01 ml of the mixture to duplicate steel discs (see **6.3**). Using sterile forceps (**7.16**) transfer one inoculated disc to the neutralizer test tube (NT) and one to the neutralizer control tube (NC). After the required contact time (see **9.1.3**) vortex the tubes for ~30 s with ~5.0 g of glass beads (**7.23**).

Using a suitable means of enumerating micro-organisms (e.g. pour plates or spread plates), enumerate the number of micro-organisms present in 1.0 ml. Plate out in duplicate.

Repeat this procedure for all products being tested.

Incubate TSA plates for 18 to 24 h at 37 °C ± 1 °C (**7.3**) and incubate MEA plates for 42 to 48 h at 30 °C ± 1 °C (**7.4**). Record number of colony forming units. Incubate the plates for a further 20 to 24 h. Do not recount plates that no longer show well separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.

Recoveries from the neutralization test (NT) should be similar to the number of cells recovered from the neutralization control (NC) such that:

$B = NC - NT$ which is not greater than ± 0.3

When:

$$\text{NC or NT} = \log_{10} \frac{\left(y + y^1\right)}{2} \times 10$$

When:

NC = $\log_{10}$ number of cfu per test surface of the neutralization control

NT = $\log_{10}$ number of cfu per test surface of the neutralization test

y and y^1 = cfu/ml from duplicate plates

10 = volume in ml of neutralizer solution used

2 = number of replicate plates

EXAMPLE

For each test product tested, record the number of cfu/ml and calculate the decimal log using the following calculation:

Duplicate y values for calculating NC counted on the 10^{-0} plate: 50, 60

$$\text{NC} = \log_{10} \frac{\left(y + y^1\right)}{2} \times 10$$

$$\text{NC} = \log_{10} \frac{(50 + 60)}{2} \times 10$$

$$\text{NC} = \log_{10} \frac{110}{2} \times 10$$

NC = $\log_{10}$[55 x 10]

NC = $\log_{10}$[550]

NC = 2.74

Duplicate y values for calculating NT counted on the 10^{-0} plate: 51, 55

$$\text{NT} = \log_{10} \frac{\left(y + y^1\right)}{2} \times 10$$

$$\text{NT} = \log_{10} \frac{(51 + 55)}{2} \times 10$$

$$\text{NT} = \log_{10} \frac{106}{2} \times 10$$

NT = $\log_{10}$[53 x 10]

NT = $\log_{10}$[530]

NT = 2.72

Calculate the difference in decimal log between NC and NT to ensure NC – NT is not greater than ± 0.3 using the previously calculated NC and NT values: NC = 2.74, NT = 2.72.

B = NC-NT

B = 2.74 – 2.72

B = 0.02

9.5.3 Neutralizer toxicity (C)

9.5.3.1 Principle of neutralizer toxicity validation experiment

The neutralizer toxicity experiment ensures that neutralizer solution (see **6.2.6**) is not toxic to the organisms tested (see **6.1**) and therefore that the neutralizer solution itself does not have any antimicrobial activity.

9.5.3.2 Procedure for neutralizer toxicity validation experiment

Pipette 10.0 ml of the neutralizer solution (see **6.2.6**) into a tube and into a separate tube, pipette 10.0 ml of sterile hard water (see **6.2.7**). Prepare a test suspension (**8.1.3.1**) and prepare a series of dilutions to give approximately 10^5 cfu/ml. Mix 1.0 ml of the diluted test suspension with 1.0 ml of interfering substance (**6.2.8**) and pipette 0.01 ml into each tube. Mix well and leave for 30 min contact time. After the contact time, vortex the tubes for ~30 s.

Using a suitable means of enumerating micro-organisms (e.g. pour plates or spread plates), enumerate the number of micro-organisms present in 1 ml. Plate out in duplicate.

Incubate TSA plates for 18 to 24 h at 37 °C ± 1 °C (**7.3**) and incubate MEA plates for 42 to 48 h at 30 °C ± 1 °C (**7.4**). Count the dilution plate with between 15 and 300 cfu. Incubate the plates for a further 20 to 24 h. Do not recount plates that no longer show well separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.

Recoveries from the neutralization test (NT) should be similar to the number of cells recovered from the neutralization control (NC) such that:

C = NC – NT which is not greater than ± 0.3

When:

$$\text{NC or NT} = \log_{10} \frac{\left(z + z^1\right)}{2} \times 10$$

When:

NC = $\log_{10}$ number of cfu per test surface of the neutralization control

NT = $\log_{10}$ number of cfu per test surface of the neutralization test

z and z^1 = cfu/ml from duplicate plates

10 = volume in ml of neutralizer solution used

2 = number of replicate plates

EXAMPLE

For each test suspension, record the number of cfu/ml and calculate the decimal log using the following calculation:

Duplicate z values for calculating NC counted on the 10^{-0} plate: 60, 60

$$\text{NC} = \log_{10} \frac{\left(z + z^1\right)}{2} \times 10$$

$$\text{NC} = \log_{10} \frac{(60 + 60)}{2} \times 10$$

$$\text{NC} = \log_{10} \frac{120}{2} \times 10$$

$NC = \log_{10}[60 \times 10]$

$NC = \log_{10}[600]$

$NC = 2.78$

Duplicate z values for calculating NT counted on the 10^{-0} plate: 53, 55

$$NT = \log_{10} \frac{\left(z + z^{1}\right)}{2} \times 10$$

$$NT = \log_{10} \frac{(53 + 55)}{2} \times 10$$

$$NT = \log_{10} \frac{108}{2} \times 10$$

$NT = \log_{10}[54 \times 10]$

$NT = \log_{10}[540]$

$NT = 2.73$

Calculate the difference in decimal log between NC and NT to ensure NC – NT is not greater than ± 0.3 using the previously calculated NC and NT values: NC = 2.78, NT = 2.73.

$C = NC\text{-}NT$

$C = 2.78 - 2.73$

$C = 0.05$

10 Calculation and expression of results

10.1 Basic limits and verification of methodology

For each test check that:

a) $N\text{-}N_w$ is not greater than 2 $\log_{10}$ orders.

b) N-NC is not greater than 2 $\log_{10}$ orders.

c) NC-NT is not greater than ± 0.3 for the neutralizer toxicity test (B) and the neutralizer validation test (C).

d) A is <100 cfu/ml for active concentrations.

e) Log_{10} values are expressed to 2 dp.

f) Plate counts for the calculation of ME, N, N_w, N_d, NC, and NT are 15-300 cfu.

10.2 Residual activity on non-porous surfaces for general purposes

Residual bactericidal activity on surfaces for general purposes is characterized by the concentration of tested product for which the criteria in **10.1** are met and for which a ≥3 log (99.9 %) reduction in viable counts is achieved under the required conditions: at a temperature of 20 °C ± 2 °C with a final contact time of 5 min ± 10 s (24 h after product application) when the test organisms are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus hirae* and *Escherichia coli*.

Residual yeasticidal activity on surfaces for general purposes is characterized by the concentration of tested product for which the criteria in **10.1** are met and for which a ≥3 log (99.9%) reduction in viable counts is achieved under the

required conditions: at a temperature between of 20 °C ± 2 °C (24 h after product application) with a final contact time of 15 min ± 10 s when the test organism is *Candida albicans*.

The test product shall have also achieved a pass (≥4 log reduction) according to the limits set out in BS EN 13697:2001 under conditions required for the product's application of use (e.g. specified contact times, micro-organisms, organic load, etc.).

10.3 Residual activity on non-porous surfaces for specific purposes

For specific purposes, additional conditions such as temperature, contact time, organisms and interfering substances may be included.

The residual bactericidal concentration for a specific purpose is the concentration of the test product for which a ≥3 log reduction is observed. The product shall have met the criteria in (**10.1**) and passed the PAS 2424 standard under the obligatory test conditions.

The residual yeasticidal concentration for a specific purpose is the concentration of the test product for which a ≥3 log reduction is observed. The product shall have met the criteria in (**10.1**) and passed the PAS 2424 standard under the obligatory test conditions.

The test product shall have also achieved a pass (≥4 log reduction) according to the limits set out in BS EN 13697:2001 under conditions required for the product's application of use (e.g. specified contact times, micro-organisms, organic load, etc.).

11 Test report

The test report shall refer to this PAS.

The test report shall state at least the following:

a) identification of the laboratory;

b) identification of the sample:
   1) name of the product;
   2) batch number;
   3) manufacturer;
   4) date of delivery;
   5) storage conditions;

c) experimental conditions:
   1) period of analysis;
   2) product diluents used in the test;
   3) product test concentrations;
   4) appearance of product dilutions;
   5) contact time(s);
   6) test temperature;
   7) interfering substance;
   8) stability of the test mixture (e.g. presence of any precipitate, etc.);
   9) temperature of incubation;
   10) neutralizer used;
   11) organism strains;
   12) test surface;

d) operating procedure:
   - full details for the test for validation of the neutralization medium shall be given;

e) test results:
   - validation tests;
   - evaluation of bactericidal and/or yeasticidal activity;

f) conclusion;

g) Locality, date and identified signature(s).

NOTE An example test report is given in Figure 6 with example test results given in Table 2. All details provided are fictional.

Figure 6 **Example test report – Residual antimicrobial activity on surfaces in general use conditions**

a) Identification of the test laboratory ..... Test House A

b) Identification of the sample

Name of Product	Test Product B
Batch number	C
Manufacturer	XYZ Limited
Date of delivery	02 Oct 13
Storage conditions	Room temperature, out of direct sunlight
Product diluent (if any)	None
Active substance/ concentration (if known)	Unknown

c) Test method and its validation

Method	Dilution neutralization
Neutralizer	

11.67 g/l Lecithin, 100.0 ml/l Tween 80, 1.0 g/l Tryptone, 8.52 g/l sodium chloride, 10 g/l Sodium dodecyl sulphate, 5.0 g/l Sodium thiosulphate, sterilized in the autoclave

d) Experimental Conditions

Period of analysis	07 Oct 13 to 21 Oct 13
Product diluents used during the test	None
Product test concentrations	Neat
Product appearance	Colourless, clear solution
Interfering substances	3.0 g/l of bovine albumin
Test temperature	20 °C ± 2 °C
Contact time	5 min ± 10 s
Temperature of incubation	37 °C ± 1 °C
Identification of organism strains used	

Pseudomonas aeruginosa ATCC 15442/NCTC 13359

e) Test results

See Table 2.

f) Conclusion

In accordance with PAS 2424:2014, batch C (02 Oct 13), when tested neat, possesses residual bactericidal activity with abrasive action on surfaces in 5 min at 20 °C under dirty conditions (3.0 g/l bovine albumin) for reference strain *Pseudomonas aeruginosa* by exhibiting a ≥3 log reduction 24 h after application.

Table 2 **Example test results**

			Residual antimicrobial activity of Test Product B, batch C in 5 minutes contact time at 20 °C in the presence of 3g/l bovine albumin						
			Dilution (*d*)	**cfu/ml (b^1-b^5)**	**cfu/disc (a^1-a^5)**	**Log_{10}cfu**	**Average Log (N_w/N_d)**	**Log reduction (ME)**	**Status**
***Pseudomonas aeruginosa* NCTC 13359**	**Test product at dilution of use (N_d)**	Rep 1	10^{-0}	<15	<150	2.18	**N_d** = 2.18	**ME** = >3.79	PASS
		Rep 2	10^{-0}	<15	<150	2.18			
		Rep 3	10^{-0}	<15	<150	2.18			
		Rep 4	10^{-0}	<15	<150	2.18			
		Rep 5	10^{-0}	<15	<150	2.18			
	Water control (N_w)	Rep 1	10^{-3}	95	950000	5.98	**N_w** = 5.96	N/A	
		Rep 2	10^{-3}	95	950000	5.98			
		Rep 3	10^{-3}	80	800000	5.90			
		Rep 4	10^{-3}	93	930000	5.97			
		Rep 5	10^{-3}	95	950000	5.98			

		Neutralizer toxicity (C)						
		z	***z^1***	**Average cfu/ml**	**Average cfu**	**Average log (NC/NT)**	**C**	**Status**
	NC (Neutralizer control)	60	60	60	600	**NC** = 2.78	**C** = 0.05	PASS
	NT (Neutralizer test)	53	55	54	540	**NT** = 2.73		

		Neutralizer validation (B)						
		y	***y^1***	**Average cfu/ml**	**Average cfu**	**Average log (NC/NT)**	**B**	**Status**
	NC (Neutralizer control)	50	60	55	550	**NC** = 2.78	**B** = 0.02	PASS
	NT (Neutralizer test)	55	51	53	530	**NT** = 2.73		

	Organism recovery (A)	
	cfu	**Status**
Product B, batch C	**A** = 12	PASS
Water control	**A** = >300	N/A

Table 2 **Example test results** *(continued)*

KEY:

cfu = colony forming units

y and y^1 = cfu/ml from duplicate plates for neutralizer validation

z and z^2 = cfu/ml from duplicate plates for neutralizer toxicity

$a^{1\text{-}5}$ = cfu/disc for the neutralized test mixture/ water control mixture

$b^{1\text{-}5}$ = cfu/ml of the neutralized test mixture/water control mixture

N_d = average log of cfu per test surface for the disinfectant test

N_w = average log of cfu per test surface for water control

NT = neutralization test ($\log_{10}$ of cfu per test surface of the neutralization test)

NC = neutralization control ($\log_{10}$ of cfu per test surface of the neutralization control)

ME = microbicidal effect ($\log_{10}$ reduction)

Annex A (informative)

Neutralizers

Any of the following neutralizers may be used.

Table A.1 **Neutralizers**

Antimicrobial agent	Chemical compounds able to neutralize residual antimicrobial activity	Examples of suitable neutralizers
Quaternary ammonium compounds and fatty amines Amphoteric compounds	• Lecithin • Saponin • Polysorbate 80 • Sodium dodecyl sulphate • Ethylene oxide condensate of fatty alcohol (non-ionic surfactants)	• Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l • Polysorbate 80, 30 g/l + sodium dodecyl sulphate, 4 g/l + lecithin, 3 g/l • Ethylene oxide condensate of fatty alcohol, 3 g/l + lecithin, 20 g/l + polysorbate 80, 5 g/l
Biguanides and similar compounds	• Lecithin • Saponin • Polysorbate 80	• Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l
Oxidizing compounds (chlorine, iodine, hydrogen peroxide, peracetic acid, hypochlorites, etc.)	• Sodium thiosulphate • Catalase [for hydrogen peroxide or products releasing hydrogen peroxide]	• Sodium thiosulphate, 3 g/l to 20 g/l + polysorbate 80, 30 g/l + lecithin, 3 g/l • Polysorbate 80, 50 g/l + catalase 0.25 g/l + lecithin 10 g/l
Aldehydes	• L–histidine • Glycine	• Polysorbate 80, 30 g/l + lecithin, 3 g/l + L-histidine, 1 g/l (or + glycine, 1 g/l) • Polysorbate 80, 30 g/l + saponin, 30 g/l + L-histidine, 1 g/l (or + glycine, 1 g/l)
Phenolic and related compounds (orthophenylphenol, phenoxyethanol, triclosan, phenylethanol, etc.) Anilides	• Lecithin • Polysorbate 80 • Ethylene oxide condensate of fatty alcohol	• Polysorbate 80, 30 g/l + lecithin, 3 g/l • Ethylene oxide condensate of fatty alcohol, 7 g/l + lecithin, 20 g/l, + polysorbate 80, 4 g/l
Alcohols	• Lecithin • Saponin • Polysorbate 80	• Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l

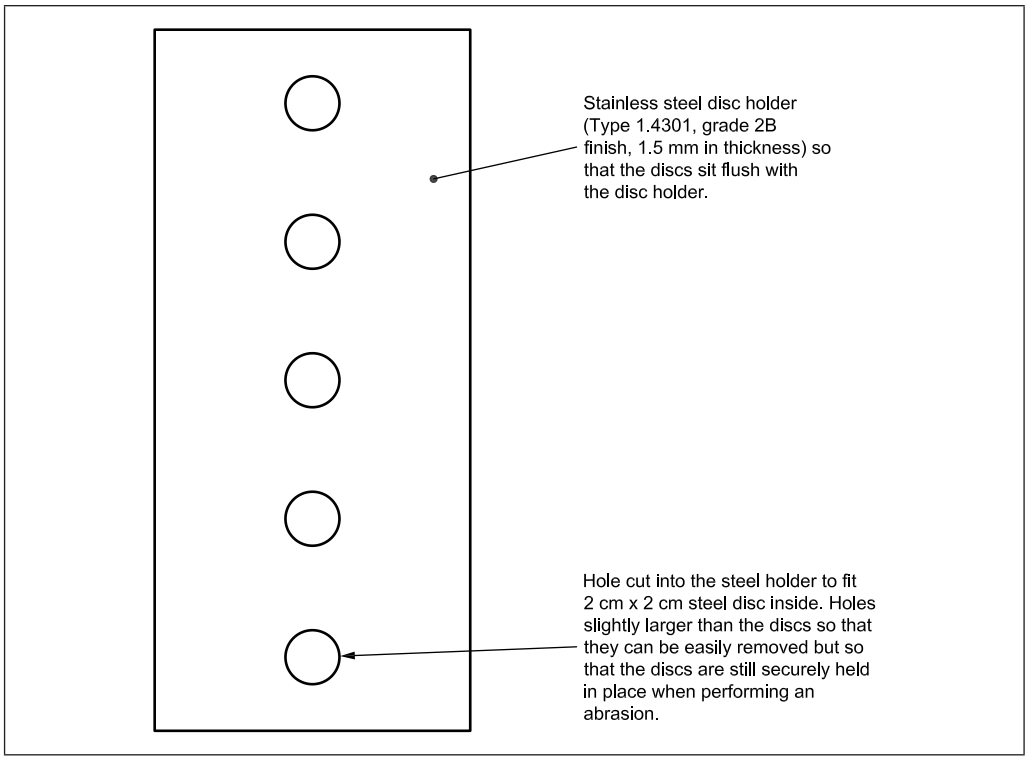
NOTE 1 Other neutralizer mixtures may be required for products containing more than one antimicrobial agent.

NOTE 2 The concentrations of the various neutralizing compounds or of the neutralizer as such may not be adequate to neutralize high concentrations of the products.

NOTE 3 Neutralizer solutions have been sourced from BS EN 1276:2009 as examples only. This list is not exhaustive and other neutralizers may be used however a neutralizer validation test should be performed.

Annex B (informative) Steel disc holder

Figure B.1 **Steel disc holder**



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