EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

DRAFT
prEN 13697

September 2022

ICS 71.100.35

Will supersede EN 13697:2015+A1:2019

English Version

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

Antiseptiques et désinfectants chimiques - Essai quantitatif de surface non-poreuse pour l'évaluation de l'activité bactéricide, levuricide et/ou fongicide des désinfectants chimiques utilisés sans action mécanique dans le domaine de l'agro-alimentaire, dans l'industrie, dans les domaines domestiques et en collectivité - Méthode d'essai sans action mécanique et prescriptions (phase 2, étape 2)

Chemische Desinfektionsmittel und Antiseptika - Quantitativer Oberflächen-Versuch zur Bestimmung der bakteriziden und levuroziden und/oder fungiziden Wirkung chemischer Desinfektionsmittel auf nicht porösen Oberflächen in den Bereichen Lebensmittel, Industrie, Haushalt und öffentliche Einrichtungen ohne mechanische Behandlung - Prüfverfahren und Anforderungen ohne mechanische Behandlung (Phase 2, Stufe 2)

This draft European Standard is submitted to CEN members for enquiry. It has been drawn up by the Technical Committee CEN/TC 216.

If this draft becomes a European Standard, CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration.

This draft European Standard was established by CEN in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the CEN-CENELEC Management Centre has the same status as the official versions.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Republic of North Macedonia, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Türkiye and United Kingdom.

Recipients of this draft are invited to submit, with their comments, notification of any relevant patent rights of which they are aware and to provide supporting documentation.

Warning : This document is not a European Standard. It is distributed for review and comments. It is subject to change without notice and shall not be referred to as a European Standard.

EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Rue de la Science 23, B-1040 Brussels

Contents

Page

European foreword ... 3
Introduction ... 4
1 Scope ... 5
2 Normative references ... 6
3 Terms and definitions ... 7
4 Requirements ... 7
5 Test methods ... 8
5.1 Principle ... 8
5.2 Materials and reagents ... 9
5.2.1 Test organisms ... 9
5.2.2 Culture media and reagents ... 9
5.2.3 Test surface ... 12
5.3 Apparatus and glassware ... 13
5.4 Preparation of test organism suspensions and product test solutions ... 14
5.4.1 Test organism suspensions ... 14
5.4.2 Product test solutions ... 17
5.5 Procedure ... 18
5.5.1 Choice of experimental conditions ... 18
5.5.2 Test procedure ... 18
5.5.3 Counting of the test mixtures ... 23
5.6 Calculation and expression of results ... 24
5.6.1 Elaboration of data: counting of weighed average values ... 24
5.6.2 Verification of methodology ... 25
5.6.3 Expression of results ... 25
5.6.4 Conclusion ... 25
5.7 Test report ... 26
Annex A (informative) Corresponding referenced strains ... 28
Annex B (informative) Neutralizers ... 30
Annex C (informative) Expression of results with the dilution-neutralization method ... 32
Annex D (informative) Bactericidal activity in surfaces in general use conditions (for clean conditions) ... 34
Annex E (informative) Precision of the test result ... 36
Annex F (informative) Alternative drying end point ... 37
Bibliography ... 39

European foreword

This document (prEN 13697:2022) has been prepared by Technical Committee CEN/TC 216 “Chemical disinfectants and antiseptics”, the secretariat of which is held by AFNOR.

This document is currently submitted to the CEN Enquiry.

This document supersedes EN 13697:2015+A1:2019.

In comparison with the previous version EN 13697:2015+A1:2019 of edition EN 13697:2015, the following modifications have been made:

— the inoculum under clean conditions for *Pseudomonas aeruginosa* and *Candida albicans* has been reduced to the previous level as in EN 13697:2015;

— a more exact monitoring of the drying process has been included as Annex F (informative) in order to support achieving sufficient levels of surviving cells for valid results;

— inclusion of yeasticidal activity in the title of the document;

— clarification that 1 % reconstituted milk can be used as sole soiling in dairy industry (no need to test BSA);

— the designation of variables Nd, Nc, NC and NT have been renamed to Na, A, B and C for harmonization with other CEN TC 216 standards;

— clarification of the counting procedure in 5.5.3;

— correction of the Formulae (2) and (3) in 5.5.3.

The following changes in version EN 13697:2015+A1:2019 as compared to edition EN 13697:2015 were maintained:

— deletion of obligatory and additional conditions (see Table 1 and 5.5.1);

— update of Bovine albumin and skimmed solutions preparations (see 5.2.2.8.2);

— add of instruction for using vacuum desiccator;

— clarification to the determination of microbicidal concentrations by updating 5.5.2.1 b) and adding pictures of carriers.

Data obtained from EN 13697:2015 and from EN 13697:2015+A1:2019 are still valid.

Introduction

This document describes a surface test method for establishing whether a product proposed as a disinfectant in the fields described in Clause 1 has or does not have bactericidal and/or fungicidal or yeasticidal activity on non-porous surfaces.

This document has been revised in order to modify the test parameters under “clean conditions” adopted for *P. aeruginosa* and *C. albicans*; in order to harmonize the designation of test variables N, B, C, A, Na with the other recent CEN TC 216 standards and to clarify and/or correct details in colony counting and calculation.

The laboratory test closely simulates practical conditions of application. Chosen conditions (contact time, temperature, organisms on surfaces ...) reflect parameters which are found in practical situations including conditions which may influence the action of disinfectants. Each use concentration found from this test corresponds to defined experimental conditions.

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 can differ and therefore additional test conditions need to be used.

1 Scope

This document specifies a test method (phase 2/step 2) and the minimum requirements for bactericidal and/or fungicidal or yeasticidal activity of chemical disinfectants that form a homogeneous physically stable preparation in hard water or – in the case of ready-to-use products – with water in food, industrial, domestic and institutional areas, excluding areas and situations where disinfection is medically indicated and excluding products used on living tissues.

The scope of this document applies at least to the following:

a) processing, distribution and retailing of:

    1) food of animal origin:

        i) milk and milk products;

        ii) meat and meat products;

        iii) fish, seafood and products;

        iv) eggs and egg products;

        v) animal feeds;

        vi) etc.

    2) food of vegetable origin:

        i) beverages;

        ii) fruits, vegetables and derivatives (including sugar distillery);

        iii) flour, milling and backing;

        iv) animal feeds;

        v) etc.

b) institutional and domestic areas:

    1) catering establishments;

    2) public areas;

    3) public transports;

    4) schools;

    5) nurseries;

    6) shops;

    7) sports rooms;

    8) waste container (bins);

9) hotels;

10) dwellings;

11) clinically non sensitive areas of hospitals;

12) offices;

13) etc.

c) other industrial areas:

1) packaging material;

2) biotechnology (yeast, proteins, enzymes...);

3) pharmaceutical;

4) cosmetics and toiletries;

5) textiles;

6) space industry, computer industry;

7) etc.

Using this document, it is possible to determine the bactericidal or fungicidal or yeasticidal activity of the undiluted product. As three concentrations are tested, in the active to non-active range, dilution of the product is required and, therefore, the product forms a homogeneous stable preparation in hard water.

EN 14885 specifies in detail the relationship of the various tests to one another and to use recommendations.

NOTE 1 The method described is intended to determine the activity of commercial formulations or active substances on bacteria and/or fungi in the conditions in which they are used.

NOTE 2 This method cannot be used to evaluate the activity of products against mycobacteria.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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.

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*

EN 14885, *Chemical disinfectants and antiseptics — Application of European Standards for chemical disinfectants and antiseptics*

ISO 4793, *Laboratory sintered (fritted) filters — Porosity grading, classification and designation*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in EN 14885 apply.

4 Requirements

The product shall demonstrate at least a 4 decimal log (lg) reduction for bacteria and at least a 3 decimal log (lg) reduction for fungi, when tested in accordance with Table 1 and 5.5.1.

Table 1 — Experimental conditions

Test conditions	**Bactericidal activity on non-porous surfaces without mechanical action**	**Yeasticidal activity on non-porous surfaces without mechanical action**	**Fungicidal activity on non-porous surfaces without mechanical action**
Test organisms (see 5.2.1) **minimum spectrum of test organisms**	*Enterococcus hirae* *Escherichia coli* *Pseudomonas aeruginosa* *Staphylococcus aureus*	*Candida albicans*	*Candida albicans* *Aspergillus brasiliensis*
Test organisms **additional** (examples)	*Salmonella typhimurium* *Lactobacillus brevis* *Enterobacter cloacae*	*Saccharomyces cerevisiae* (for breweries) *Saccharomyces cerevisiae* var. *diastaticus* (for breweries)	any relevant test organism
Test temperature	In a range from (4 ± 1) °C to (40 ± 1) °C For tests performed at room temperature, the range shall be between 18 °C and 25 °C	In a range from (4 ± 1) °C to (40 ± 1) °C For tests performed at room temperature, the range shall be between 18 °C and 25 °C	In a range from (4 ± 1) °C to (40 ± 1) °C For tests performed at room temperature, the range shall be between 18 °C and 25 °C
Contact time	in a range from 1 min to 60 min (from 1 min to 5 min at intervals of 1 min and from 5 min to 60 min at intervals of 5 min)	in a range from 1 min to 60 min (from 1 min to 5 min at intervals of 1 min and from 5 min to 60 min at intervals of 5 min)	in a range from 1 min to 60 min (from 1 min to 5 min at intervals of 1 min and from 5 min to 60 min at intervals of 5 min)
Interfering substance clean conditions	0,3 g/l bovine albumin for *Staphylococcus aureus*, *Enterococcus hirae, Escherichia coli* and *Pseudomonas aeruginosa*	0,3 g/l bovine albumin for *C. albicans*	0,3 g/l bovine albumin for *C. albicans* and *A. brasiliensis*

Test conditions	Bactericidal activity on non-porous surfaces without mechanical action	Yeasticidal activity on non-porous surfaces without mechanical action	Fungicidal activity on non-porous surfaces without mechanical action
Interfering substance dirty conditions	3,0 g/l bovine albumin for *Staphylococcus aureus*, *Enterococcus hirae, Pseudomonas aeruginosa* and *Escherichia coli*	3,0 g/l bovine albumin for *C. albicans*	3,0 g/l bovine albumin for *C. albicans* and *A. brasiliensis*
Interfering substance in dairy industry	1,0 % reconstituted milk (= 1,0 g/l milk powder)	1,0 % reconstituted milk (= 1,0 g/l milk powder)	1,0 % reconstituted milk (= 1,0 g/l milk powder)
Interfering substance **additional**	any relevant substance	any relevant substance	any relevant substance
Log reduction from a water control (decimal lg)	≥ 4 lg	≥ 3 lg	≥ 3 lg
The referenced test conditions (General purposes) are by no means intended as requirements for the use of a product, nor as requirements for the evaluation and acceptance of products by regulatory authorities. The application time for the product is specified by the manufacturer. If specific applications have to be considered, the bactericidal/yeasticidal/fungicidal activity has to be determined additionally under relevant conditions concerning application time, temperature, strains and Interfering Substances.			

5 Test methods

5.1 Principle

A test suspension of bacteria or fungi in a solution of interfering substances is inoculated onto a test stainless steel surface and dried. A prepared sample of the product under test is applied in a manner which covers the dried film. The surface is maintained at a specified temperature for a defined period of time. The surface is transferred to a previously validated neutralization medium so that the action of the disinfectant is immediately neutralized. The number of surviving organisms which can be recovered from the surface is determined quantitatively.

The number of bacteria or fungi on a surface treated with hard water in place of the disinfectant is also determined and the reduction in viable counts attributed to the product is calculated by difference.

5.2 Materials and reagents

5.2.1 Test organisms

The bactericidal activity shall be evaluated using the following four strains:

— *Pseudomonas aeruginosa* ATCC 15 442[1)];

— *Staphylococcus aureus* ATCC 6 538;

— *Enterococcus hirae* ATCC 10 541;

— *Escherichia coli* ATCC 10 536.

The fungicidal or yeasticidal activity shall be evaluated using the following two strains:

— *Candida albicans* ATCC 10 231;

— *Aspergillus brasiliensis (ex A. niger)* ATCC 16 404.

If required for specific applications, additional strains may be chosen from, for example:

— *Salmonella typhimurium* ATCC 13 311;

— *Lactobacillus brevis* DSM 6 235;

— *Enterobacter cloacae* DSM 6 234;

— *Saccharomyces cerevisiae* (for breweries) or ATCC 9 763 or DSM 1 333;

— *Saccharomyces cerevisiae* var. *diastaticus* (for breweries) DSM 70 487.

NOTE See Annex A for corresponding strain numbers in some other culture collections.

If additional strains are used, they shall be incubated under optimum growth conditions (temperature, time, atmosphere) and noted in the test report.

If the additional strains selected do not correspond to the specified strains, their suitability for supplying inocula of sufficient concentration shall be verified. If the additional strains tested are not classified at a reference centre, their identification characteristics shall be stated. In addition, they shall be held by the testing laboratory or national culture under a reference for 5 years.

5.2.2 Culture media and reagents

5.2.2.1 General

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

5.2.2.2 Water

The water shall be free from substances that are toxic or inhibiting to bacteria and fungi. It shall be freshly glass distilled and not demineralized water.

Sterilize in the autoclave (see 5.3.2.1).

NOTE 1 If the water is sterilized during sterilization of the reagents, this is not necessary.

1) ATCC 15 442, ATCC 6 538, ATCC 10 541, ATCC 10 536, ATCC 10 231, ATCC 16 404 and ATCC 13311 are the collection numbers of strains supplied by the American Type Culture Collections. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of the product named. Equivalent products can be used if they can be shown to lead to the same results.

NOTE 2 If distilled water of adequate quality is not available, water for injectable preparation (see European Pharmacopoeia) can be used.

5.2.2.3 Tryptone Soya Agar (TSA)

For maintenance of bacterial strains and performance of viable counts.

Tryptone, pancreatic digest of casein	15,0 g
Soya peptone, papaic digest of Soybean meal	5,0 g
NaCl	5,0 g
Agar	15,0 g
Water (see 5.2.2.2)	1 000,0 ml

Sterilize in the autoclave (see 5.3.2.1). After sterilization, the pH of the medium shall be equivalent to 7,2 ± 0,2 when measured at 20 ° C.

5.2.2.4 Malt extract agar (MEA)

For maintenance of fungal strains, sporulation and performance of viable counts.

Malt extract (food grade, e.g. Cristomalt powder from Difal)	30,0 g
Agar	15,0 g
Water (see 5.2.2.2)	1 000,0 ml

The malt extract should be food grade (e.g. Cristomalt powder from Difal) or equivalent that is not highly purified and not only based on maltose (e.g. Malt extract from OXOID) [2)]. However, if there are problems producing at least 75 % spiny spores see 5.4.1.4.2.

Sterilize in the autoclave [5.3.2.1 a)]. After sterilization, the pH of the medium shall be equivalent to 5,6 ± 0,2 when measured at (20 ± 1) °C.

In case of encountering problems with neutralization (5.5.2.3 and 5.5.2.4), it may be necessary to add neutralizer to the MEA. Annex B gives guidance on the neutralizers that may be used.

5.2.2.5 Diluent

Tryptone sodium chloride solution:

Tryptone, pancreatic digest of casein	1,0 g
NaCl	8,5 g
Water (see 5.2.2.2)	1 000,0 ml

[2)] This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Sterilize in the autoclave (see 5.3.2.1). After sterilization the pH shall be equivalent to 7,0 ± 0,2 when measured at 20 °C.

5.2.2.6 Neutralizer

The neutralizer shall be validated for the product under test in accordance with 5.5.2.3 and 5.5.2.4. The neutralizer shall be sterile.

NOTE Information on neutralizers that have been found to be suitable for some categories of products is given in Annex B.

5.2.2.7 Hard water for dilution of the products

Hard water for dilution of products shall be prepared as follows:

— solution A: Dissolve 19,84 g anhydrous $MgCl_2$ and 46,24 g anhydrous $CaCl_2$ in water (see 5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration (5.3.2.19) or in the autoclave (5.3.2.1 a). Autoclaving – if used - may cause a loss of liquid. In this case, make up to 1 000 ml with water (5.2.2.2) under aseptic conditions. Store the solution in the refrigerator (5.3.2.15) for no longer than one month;

— solution B: Dissolve 35,02 g $NaHC0_3$ in water (see 5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration (5.3.2.19). Store the solution in the refrigerator (5.3.2.15) for no longer than one week.

Add at least 600 ml water (see 5.2.2.2) to 6,0 ml of solution A in a 1 000 ml volumetric flask, then add 8,0 ml solution B. Mix and dilute to 1 000 ml with water (see 5.2.2.2).

Sterilize by passing through a filter with a maximum effective pore size of 0,45 µm.

The pH of the hard water shall be 7,0 ± 0,2, when measured at (20 ± 1) °C (5.3.2.6). If necessary, adjust the pH by using a solution of approximately 40 g/l (about 1 mol/l) of sodium hydroxide (NaOH) or approximately 36,5 g/l (about 1 mol/l) of hydrochloric acid (HCl).

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

NOTE When preparing the test product solutions (5.4.2), the addition of the product to the hard water produces a different final water hardness expressed as calcium carbonate ($CaCO_3$) in each test tube. In any case, the final hardness is lower than 375 mg/l of calcium carbonate.

5.2.2.8 Interfering substances

5.2.2.8.1 General

The interfering substance shall be chosen according to the conditions of use laid down for the product.

The interfering substance shall be sterile and prepared at 2 times its final concentration in the test.

For the additional interfering substances, the ionic composition (e.g. pH, calcium and/or magnesium hardness) and chemical composition (e.g. mineral substances, protein, carbohydrates, lipids, detergents) shall be fully defined.

NOTE The term “interfering substance” is used even if it contains more than one substance.

The method of preparation and sterilization together with the composition shall be noted in the test report (see 5.7).

5.2.2.8.2 Bovine albumin solutions

Bovine albumin solutions for the test conditions shall be prepared as follows:

— Preparation for clean conditions:

  — dissolve 0,06 g of bovine albumin (Cohn fraction V for Dubos medium) in 100 ml of water (see 5.2.2.2);

  — sterilize by 0,45 µm membrane filtration (see 5.3.2.19), keep in the refrigerator and use within one month.

The final concentration of bovine albumin in the test procedure (see 5.5.2) is 0,3 g/l.

— Preparation for dirty conditions:

  — dissolve 0,6 g of bovine albumin (Cohn fraction V for Dubos medium) in 100 ml of water (see 5.2.2.2);

  — sterilize by 0,45 µm membrane filtration (see 5.3.2.19), keep in the refrigerator and use within one month.

The final concentration of bovine albumin in the test procedure (see 5.5.2) is 3,0 g/l.

5.2.2.8.3 Milk (dairies)

Skimmed milk, guaranteed free of antibiotics or additives, shall be reconstituted at a rate of 100 g powder per litre of water (see 5.2.2.2). The working solution shall be prepared as follows:

— prepare a solution of 2,0 % (*V/V*) in water (see 5.2.2.2) by adding 2 parts of reconstituted milk to 98 parts of water;

— sterilize for 30 min at 105 °C ± 3 °C (or 5 min at 121 °C ± 3 °C).

The final concentration of the reconstituted milk should be 1,0 % (v / v) in the test (see 5.5.2) or 1 g/l of milk powder during the test.

5.2.3 Test surface

Stainless steel discs (2 cm diameter discs) 304 with grade 2b finish on both sides. The surfaces should be flat.

The surfaces should be used only once.

Prior to use the surfaces should be placed in a beaker (minimum size: 50 ml) containing not less than 20 ml of 5 % (*V/V*) Decon® [3)] for 60 min. Immediately rinse the discs with running freshly distilled water for 10 s.

The surface shall not be allowed to dry to any extent. The discs shall only be handled with forceps. Rinse the discs with water (see 5.2.2.2) for a further 10 s to ensure complete removal of the surfactant. To supply a satisfactory flow of water, a sterilized fluid dispensing pressure vessel with suitable hose and connectors or other suitable method can be used and regulated to supply approximately 2 000 ml per min. To sterilize, place the clean disc in a bath containing 70 % (*V/V*) iso-propanol for 15 min. Remove the disc and dry by evaporation under laminar air flow.

3) Decon® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of this product.

5.3 Apparatus and glassware

5.3.1 General

Sterilize all glassware and parts of apparatus that will come into contact with the culture media and reagents or the sample, except those which are supplied sterile, by one of the following methods:

a) in the autoclave (see 5.3.2.1) by maintaining it at 121_{0}^{+3} °C for a minimum holding time of 15 min;

b) in the dry heat sterilizer (see 5.3.2.1) by maintaining it at 180 °C for a minimum holding time of 30 min, at 170 °C for a minimum holding time of 1 h or at 160 °C for a minimum holding time of 2 h.

5.3.2 Usual microbiological laboratory equipment [4]) and in particular, the following:

5.3.2.1 Apparatus for sterilization:

a) For moist heat sterilization, an autoclave capable of being maintained at 121_{0}^{+3} °C for 15 min;

b) for dry heat treatment, a hot air oven capable of being maintained at 180 °C for a minimum holding time of 30 min, at 170 °C for a minimum holding time of 1 h or at 160 °C for a minimum holding time of 2 h.

5.3.2.2 Temperature controlled cabinet capable of being controlled at test temperatures, (θ ± 1) °C.

5.3.2.3 Water baths capable of being controlled at 20 °C ± 1 °C, at 45 °C ± 1 °C and at additional test temperatures θ ± 1 °C (see 5.5.1).

5.3.2.4 Incubator (for bactericidal activity), capable of being controlled at either 36 °C ± 1 °C or 37 °C ± 1 °C. An incubator at 37 °C ± 1 °C may be used if an incubator at 36 °C ± 1 °C is not available.

5.3.2.5 Incubator (for fungicidal or yeasticidal activity), capable of being controlled at 30 °C ± 1 °C.

5.3.2.6 pH meter, having an accuracy of calibration of 0,1 pH units at 20 °C ± 1 °C.

5.3.2.7 Stopwatch.

5.3.2.8 Vortex mixer (mechanical shaker or electromechanical agitator, i.e. Vortex® mixer [5]).

5.3.2.9 Containers: Test tubes, culture bottles or flasks of suitable capacity.

5.3.2.10 Graduated pipettes of nominal capacities 10 ml, 1 ml, 0,1 ml and 0,05 ml, or calibrated automatic pipettes.

5.3.2.11 Petri dishes of size 90 mm to 100 mm.

5.3.2.12 Glass beads (Diameter: ≤ 5 mm).

---

[4]) Disposable equipment is an acceptable alternative to reusable glassware.

[5]) Vortex® in an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of this product.

5.3.2.13 Volumetric flasks.

5.3.2.14 Mechanical shaker.

5.3.2.15 Refrigerator capable of being controlled at 2 °C to 8 °C.

5.3.2.16 Forceps.

5.3.2.17 Microbiological filtered laminar air flow cabinet.

5.3.2.18 Fritted filter: Porosity of 40 µm to 100 µm according to ISO 4793.

5.3.2.19 Membrane filtration apparatus, constructed of a material compatible with the substances to be filtered.

The apparatus shall have a filter holder of at least 50 ml volume. It shall be suitable for use with filters of diameter 47 mm to 50 mm and 0,45 µm pore size for sterilization of hard water (5.2.2.7).

5.3.2.20 Vacuum desiccator, *Desiccator* with an active desiccant. Vacuum source may be a pump or central supply and should achieve a vacuum of 20 mmHg to 25 mmHg (508-635 torr; 677-847 mbar; 68000-85000 Pascal; conversion tables are readily available on the Internet for other units).

5.4 Preparation of test organism suspensions and product test solutions

5.4.1 Test organism suspensions

5.4.1.1 Stock cultures of test organisms

Stocks cultures shall be kept in accordance with the requirements of EN 12353.

5.4.1.2 Working culture of test organisms

a) Bacteria:

In order to prepare the working culture of the test organism, subculture from the stock culture (see 5.4.1.1) by streaking on TSA slopes (see 5.2.2.3) and incubate (see 5.3.2.4). After 18 h to 24 h, prepare a second subculture from the first subculture in the same way and incubate for 18 h to 24 h. From this second subculture, a third subculture may be produced in the same way.

NOTE 1 The second and/or third subculture are the working culture(s).

If it is not possible to prepare the second subculture on a particular day, a 48 h subculture may be used for subsequent subculturing, provided that the subculture has been kept in the incubator during the 48 h period. In these circumstances, prepare a further 24 h subculture after proceeding. Do not take a fourth subculture.

For additional strains (see 5.2.1), any departure from this method of culturing the bacteria or preparing the suspensions shall be noted, giving the reasons in the test report.

b) fungi:

In order to prepare the working culture of *Candida albicans,* subculture from the stock culture (see 5.4.1.1) by streaking onto MEA slopes (see 5.2.2.4) and incubate (see 5.3.2.5). After 42 h to 48 h, prepare a second subculture from the first subculture in the same way and incubate for 42 h to 48 h. From this second subculture, a third subculture may be produced in the same way.

NOTE 2 The second and/or third subculture are the working culture(s).

For *Aspergillus brasiliensis* (5.2.1), use only the first subculture grown on MEA (5.2.2.4) in Petri dishes or flasks with ventilated caps (5.3.2.17) and incubate at 30 °C ± 1 °C for 7 d to 9 d. No further subculturing is needed. Do not stack the Petri dishes during the incubation to improve the temperature homogenization.

At the end of incubation, all the cultures shall show a dark brown or black surface. Cultures with appearance of rare and small white or grey areas might be kept.

5.4.1.3 Test suspensions

a) Bacterial test suspension:

Take 10 ml of diluent (see 5.2.2.5) and place in a 100 ml flask with 5 g of glass beads (see 5.3.2.12). Take the working culture (see 5.4.1.2) and transfer loopfuls of the cells into the diluent. The cells should be suspended in the diluent by immersing the loop in the diluent and rubbing it against the side of the flask to dislodge the cells. Shake the flask for 3 min using a mechanical shaker (see 5.3.2.14). Aspirate the suspension from the glass beads and transfer to another flask. Adjust the number of cells in the suspension to $1{,}5 \times 10^8$ cfu/ml to $5{,}0 \times 10^8$ cfu/ml.

The numbers of units shall be estimated by means of spectrophotometer or any other suitable means. Maintain this suspension in the water bath at 20 °C ± 1 °C and use within 2 h.

b) Fungal test suspension:

1) *Candida albicans*:

Take 10 ml of diluent (see 5.2.2.5) and place in a 100 ml flask with 5 g of glass beads. Take the working culture (see 5.4.1.2) and transfer loopfuls of the cells into the diluent. The cells should be suspended in the diluent by immersing the loop in the diluent and rubbing it against the side of the flask to dislodge the cells. Shake the flask for 3 min using a mechanical shaker (5.3.2.14). Aspirate the suspension from the glass beads and transfer to another flask. Adjust the number of cells in the suspension to $1{,}5 \times 10^7$ cfu/ml to $5{,}0 \times 10^7$ cfu/ml using the diluent for tests to be performed under dirty conditions, estimating the numbers of units by means of a spectrophotometer or other suitable technique. Maintain this suspension in the water bath at 20 °C ± 1 °C and use within 2 h.

2) *Aspergillus brasiliensis*:

Take the working culture (see 5.4.1.2) and suspend the cells in 10 ml of sterile 0,05 % w/v polysorbate 80 [6)] solution in water (see 5.2.2.2). Using a sterile glass rod or spatula detach the conidiospores from the culture surface. Transfer the suspension into a flask and gently shake by hand for one minute together with glass beads (see 5.3.2.12). The suspension is filtered through a fritted filter (see 5.3.2.18).

Carry out a microscopic examination under × 400 magnification immediately after the preparation to show:

6) Analytical quality, non hydrolysed in accordance with European Pharmacopoeia, Volume 1. TWEEN 80® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of this product.

— the presence of a high concentration (at least 75 % of spiny spores) of characteristic mature spores, i.e. spiny spores (versus smooth spores);

— the absence of spore germination (check at least 10 fields of view);

— the absence of mycelia fragments (check at least 10 fields of view).

If germinated spores are present, discard the suspension.

If mycelia are present, proceed to a 2^{nd} fritted filtration.

If mycelia are still present, discard the suspension.

If 75 % spiny spores are not achieved it may be due to the *Aspergillus brasiliensis* culture or the media used to produce these spores. In this situation, it will be necessary to obtain the culture from another culture collection and/or use a MEA from a different supplier.

Adjust the number of spores in the suspension to $1{,}5 \times 10^7$ cfu/ml to $5{,}0 \times 10^7$ cfu/ml using the diluent (5.2.2.5), estimating the number of cfu by any suitable means. Use the suspension within 4 h in a water bath controlled at 20 °C ± 1 °C (5.3.2.3). In any case, adjust the temperature according to 5.5.1 only immediately before the start of the test (5.5.2).

The use of a cell counting device for adjusting the number of cells is highly recommended. When using a suitable counting chamber, follow the instructions explicitly.

For counting, prepare 10^{-5} and 10^{-6} dilutions of the test suspension using diluent (5.2.2.5). Mix (5.3.2.8).

Take a sample of 1,0 ml of each dilution in duplicate and inoculate using the pour plate or the spread plate technique.

a) When using the pour plate technique, transfer about half of each 1,0 ml sample into separate Petri dishes (i.e. in duplicate = four plates) and add 15 ml to 20 ml of melted MEA (5.2.2.4), cooled to (45 ± 1) °C.

b) When using the spread plate technique, spread about one quarter of each 1,0 ml sample on an appropriate number (at least four) of surface dried plates containing MEA (5.2.2.4) (i.e. in duplicate – at least eight plates).

This test suspension shall not be stored more than 2 d at 2 °C to 8 °C.

The test suspension shall be mixed (see 5.3.2.8) immediately before use to re-suspend the spores.

5.4.1.4 Counting of bacterial and fungal test suspensions

Dilute the adjusted bacterial suspensions (see 5.4.1.3) by 10^{-6} (serial dilutions) and 10^{-7} and dilute the adjusted yeast and mould spore suspension by 10^{-5} and 10^{-6} (see 5.4.1.3) using diluent (see 5.2.2.5). Mix the suspension (see 5.3.2.8).

Take a sample of 1,0 ml of each dilution in duplicate and inoculate pour plates. Pipette each 1,0 ml sample into separate Petri dishes (see 5.3.2.11) and add 15 ml to 20 ml melted TSA (see 5.2.2.2) for the bacteria and 15 ml to 20 ml melted MEA (see 5.2.2.3) for the fungi, cooled to 45 °C ± 1 °C. When using the spread plate technique, spread each 1,0 ml sample – divided into portions of approximately equal size – on an appropriate number (at least two) of surface dried plates containing TSA or MEA.

a) Counting of bacterial test suspensions

   For the bacterial strains, incubate the plates at (36 ± 1) °C or at (37 ± 1) °C for 18 h to 24 h. Discard any plates which are not countable for any reason. Count the plates and determine the number of

colony forming units. Incubate the plates for a further 18 h to 24 h. Do not recount plates which no longer show countable quantities of colonies. Recount the remaining plates.

b) Counting of fungal test suspensions

For the fungal strains, incubate the plates at (30 ± 1) °C for 18 h to 24 h (*Candida albicans*), or for 42 h to 48 h (*Aspergillus brasiliensis)*. Discard any plates which are not countable for any reason. Count the plates and determine the number of colony forming units. Incubate the plates for a further 18 h to 24 h. Do not recount plates which no longer show well-separated colonies. Recount the remaining plates. For *Aspergillus brasiliensis*, continue incubation for a further 20 h to 24 h and if necessary, a further 20 h to 24 h, provided the number of countable colonies (discrete colonies) is increasing.

Determine the highest number of colonies for each 1 ml sample.

For incubation and counting, see 5.4.1.5.

5.4.1.5 Counting the weighed mean of the test suspensions

a) Discard any plates that are not countable for any reason. Count the plates and determine the total number of cfu. 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.

b) Only the plates showing a number of colonies included in a 15-300 for bacteria and yeast and 15-150 for mould range were used to perform the result calculation. A deviation of 10 % is accepted, so the limits are 14 and 330 for bacteria and yeast and 14-165 for mould.

$$N = \mathrm{Log}\frac{0,025 \times c}{\left(\mathrm{n}_1 + 0,1\mathrm{n}_2\right) \times d} \quad (1)$$

where

c is the sum of the Vc values taken into account;

n_1 is the number of Vc values taken into account in the lower dilution, e.g. 10^{-6};

n_2 is the number of Vc values taken into account in the higher dilution, e.g. 10^{-7};

d is the dilution factor corresponding to the lower dilution e.g. 10^{-6}.

5.4.2 Product test solutions

Details of samples of the product as received shall be recorded.

Product test solutions shall be prepared in hard water (see 5.2.2.7) at minimum three different concentrations to include one concentration in the active range and one concentration in the non-active range.

For solid products, dissolve the product as received by weighing at least 1 g ± 10 mg of the product in a volumetric flask and dilute with hard water (see 5.2.2.7) Subsequent dilutions shall be prepared in volumetric flasks (see 5.3.2.13) on a volume/volume basis in hard water (see 5.2.2.7).

For liquid products, dilutions of the product shall be prepared in hard water (see 5.2.2.7) on a volume/volume basis using volumetric flasks (see 5.3.2.13).

The product as received may be used as one of the product test solutions. For products supplied in a ready to use state, water (see 5.2.2.2) shall be used to prepare dilutions.

When the product is diluted in hard water, it shall give a physically homogeneous stable preparation.

The product test solution and dilutions of it shall be prepared freshly and used within 2 h.

The concentration of the product stated in the test report shall be the test concentration. Record the test concentration in terms of volume per volume or weight per volume.

5.5 Procedure

5.5.1 Choice of experimental conditions

The selection of contact temperature, contact time and interfering substances shall be carried out according to the practical use considered for the product (see Clause 4, Table 1) as follows:

a) test temperature; θ (°C):

   1) test temperatures shall be chosen in a range from (4 ± 1) °C to (40 ± 1) °C. A temperature controlled chamber should be used for temperatures other than ambient. For tests performed at room temperature, the range shall be between 18 °C and 25 °C;

b) contact time; t (min):

   1) contact times shall be chosen in a range from 1 min to 5 min (±5 s) at intervals of 1 min and between 5 min to 60 min (±10 s) at intervals of 5 min;

c) strains:

   1) test organisms shall be as given in 5.2.1.

d) in case of interfering substances:

   1) the interfering substance to be tested is 0,3 g/l bovine albumin (5.2.2.8.2) for clean conditions (5.2.2.8.2) or 3,0 g/l bovine albumin (5.2.2.8.2) for dirty conditions according to Clause 4, Table 1 and practical applications;

   2) for dairy applications skim milk should be used (see 5.2.2.8.3).

The product shall not cause the formation of any observable precipitate in the experimental conditions used.

5.5.2 Test procedure

5.5.2.1 Test "*Na*" – determination of microbicidal concentrations

The procedure for determining microbicidal concentrations is as follows:

a) To prepare the microbial test suspension pipette 1,0 ml of the interfering substance (5.2.2.8) into a tube. Add 1,0 ml of the test suspension (5.4.1.3). Start the stopwatch immediately, mix and place the tube in a water bath or temperature controlled cabinet (5.3.2.2) at the at the chosen test temperature θ °C ± 1 °C for 2 min ± 10 s.

   Immediately before addition, the test suspension should be well mixed to fully re-suspend the organisms.

b) Place the test surfaces (5.2.3) in a sterile Petri dish and ensure that the dish is in a horizontal position. Prepare the test surfaces by inoculating 0,05 ml of the microbial test suspension (5.5.2.1 a) on to each test surface (see Figure 1). Dry the surfaces at 37 °C until they are visibly dry (see Figure 2). It is understood that drying of the test surfaces will occur at different rates due to the ambient conditions of the laboratory and the design of the incubator (e.g. with or without a fan). Alternatively, place surfaces in an open Petri dish to dry, inoculated side uppermost at 18 °C to 25 °C in a vacuum desiccator for not longer than 60 min.

Ensure that the Petri dish is partially open by placing its lid off centre. Check that desiccator is properly sealed. Attach the outlet of the desiccator to a vacuum source and start evacuation of the air to achieve a vacuum of 20 mmHg to 25 mmHg (508-635 torr; 677-847 mbar; 68000-85000 Pascal; conversion tables are readily available on the Internet for other units).

For this reason no time duration is given and the minimum required time for the surfaces to become visibly dry should be established for each laboratory. The drying time should not exceed 60 min. Allow the test surfaces to equilibrate with the chosen test temperature (θ ± 1) °C.

A specific procedure for monitoring the drying process of sensitive organisms (e.g. *P.aeruginosa*, *E. coli*, *C. albicans*) is given in Annex F (informative) which can be used in order to achieve a sufficient number of surviving cell counts.

Figure 1 — Inoculated carrier

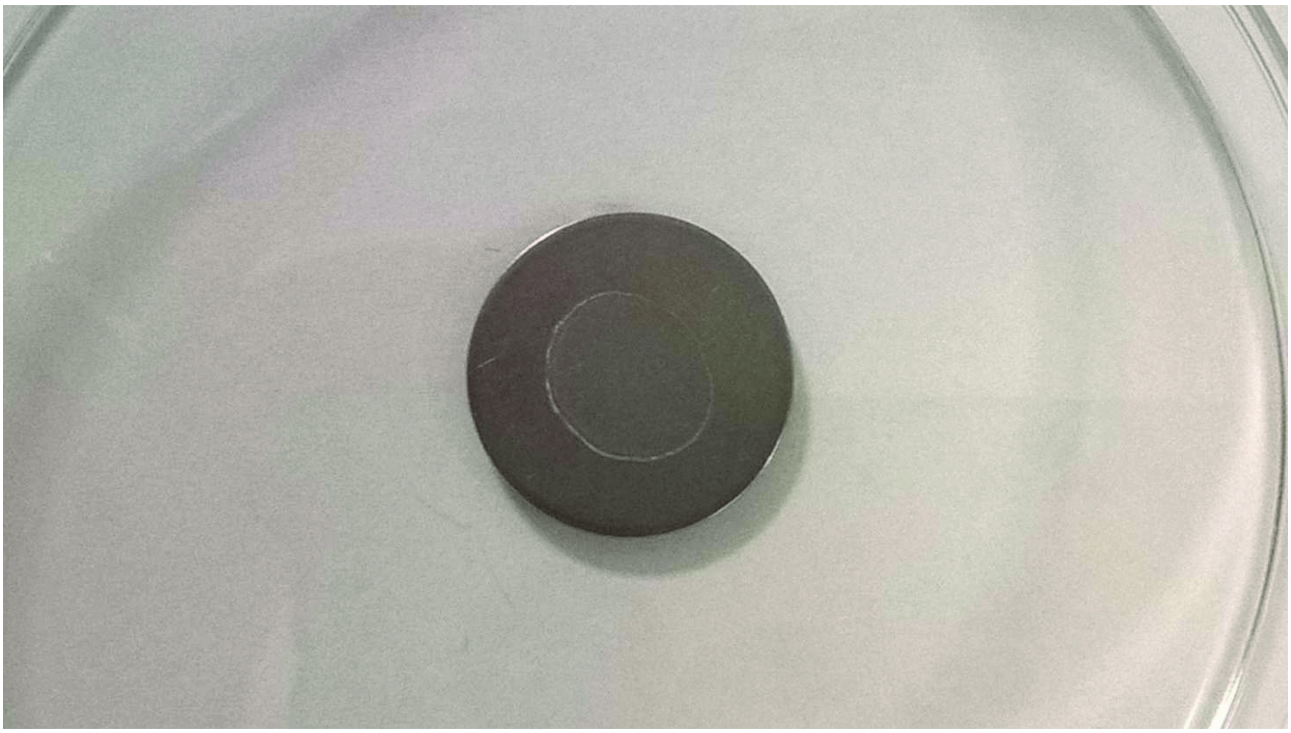


Figure 2 — Visibly dry inoculum

c) Pipette 0,1 ml of each product test solution (5.4.2) to be tested on to separate dried surfaces ensuring that the dried inoculum is totally covered by the test product. Place the surfaces in a temperature-controlled cabinet (5.3.2.2) at the chosen test temperature θ °C ± 1 °C and contact time t (see Figure 3).

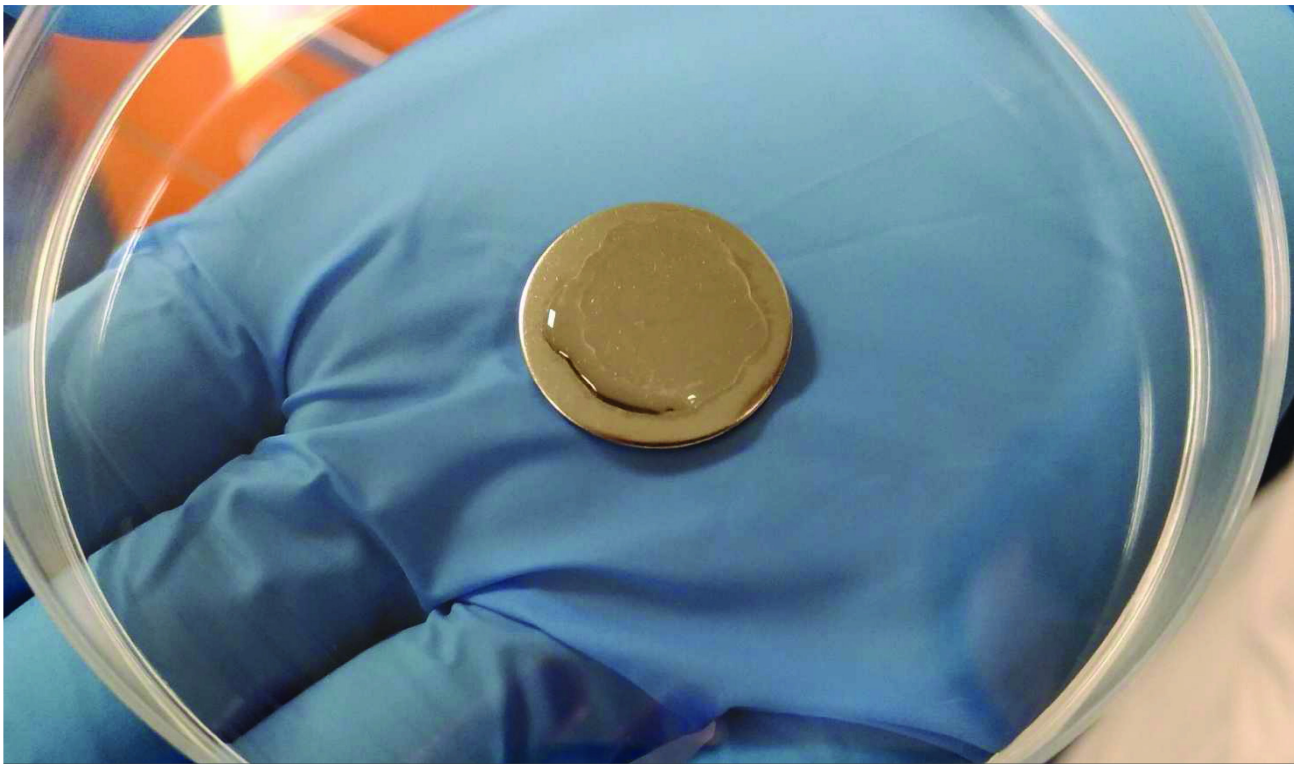


Figure 3 — Product applied onto inoculated carrier

d) At the end of *t*, transfer each of the surfaces (*Na*) to a separate container containing 10 ml of neutralizer (5.2.2.6) together with sufficient glass beads (for example 5 g) to support the surface. The surfaces should be placed with the inoculated surface downwards in contact with the beads. Shake the containers for minimum 1 min. The shaking should be sufficiently vigorous to ensure that the test surface moves constantly over the beads. After the neutralization time of 5 min ± 10 s prepare a series of 10-fold dilutions from 10^{-1} to 10^{-2} of the neutralized mixture in the diluent (5.2.2.5). Take a 1,0 ml sample of the neutralized mixture and each of the dilutions in duplicate and inoculate using pour plate or spread plate technique.

   1) When using the pour plate technique, pipette each 1,0 ml sample into separate Petri dishes and add 15 ml to 20 ml of melted TSA (5.2.2.3) for the bacteria and MEA (5.2.2.4) for the fungi, cooled to 45 °C ± 1 °C.

   2) When using the spread plate technique, spread each 1,0 ml sample – divided into portions of approximately equal size – on an appropriate number (at least two) of surface dried plates containing TSA (5.2.2.3).

For incubation and counting, see 5.5.3.

e) Recover the test surface (Nts), let the neutralizer drain off and rinse with 10 ml of water (5.2.2.2). Transfer to a Petri dish containing 10 ml of solidified TSA (5.2.2.3) for bacteria and Petri dish containing 10 ml of solidified MEA (5.2.2.4) for fungi and place on top of the agar, test side uppermost. Add 10 ml of TSA (5.2.2.3) and/or MEA (5.2.2.4) melted and cooled to 45 °C.

f) Perform the procedure a) to e) using the other product test solutions at the same time.

g) Perform the procedure a) to f) applying the other obligatory and if appropriate other additional experimental conditions (5.5.1).

5.5.2.2 Water control "*A*"

The procedure for determining the water control is as follows:

a) Place one test surface (5.2.3) in a sterile Petri dish and ensure that the dish is in a horizontal position. Inoculate 0,05 ml of the microbial test suspension [5.5.2.1 a)] on to the test surface. Dry the surface at 37 °C until it is visibly dry [5.5.2.1 b)].

b) For the water control (*A*), pipette 0,1 ml of hard water (5.2.2.7) or water (5.2.2.2) in the case of ready-to use products on to the test surface ensuring that the dried inoculum is totally covered by the water. Place the surface in a temperature controlled cabinet (5.3.2.2) at the chosen test temperature ($\theta \pm 1$) °C.

c) At the end of *t*, transfer the surface "*A*" into a container containing 10 ml of neutralizer (5.2.2.6) together with sufficient glass beads (for example 5 g) to support the surface. The surfaces should be placed with the inoculated surface downwards in contact with the beads. Shake the containers for minimum 1 min. The shaking should be sufficiently vigorous to ensure that the test surface moves constantly over the beads. After a neutralization time of 5 min ± 10 s prepare a series of 10-fold dilutions. For the bacterial strains prepare 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} dilutions and for the fungal strains 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}. Take a 1,0 ml sample of these dilutions in duplicate and inoculate using the pour plate or the spread plate technique.

d) Recover the test surface ("Nts"), let the neutralizer drain off and rinse with 10 ml of water (5.2.2.2). Transfer to a Petri dish containing 10 ml of solidified TSA (5.2.2.3) for bacteria and Petri dish containing 10 ml of solidified MEA (5.2.2.4) for fungi and place on top of the agar, test side uppermost. Add 10 ml of TSA (5.2.2.3) and/or MEA (5.2.2.4) melted and cooled to 45 °C. For incubation and counting, see 5.5.3.

5.5.2.3 Neutralizer control "B" - verification of the absence of toxicity of the neutralizer

To verify the absence of toxicity of the neutralizer, the procedure is as follows:

a) Prepare one inoculated test surface [5.5.2.1 a) and b)].

b) Pipette 10 ml of neutralizer (5.2.2.6) into a container with sufficient glass beads (for example 5 g) to support the surface. Then add 0,1 ml of hard water (5.2.2.7) or water (5.2.2.2) in the case of ready-to-use products. Mix and leave in contact for 5 min ± 10 s at 20 °C ± 1 °C.

c) At the end of the neutralization time transfer the inoculated and dried test surface into the container and place the inoculated surface own wards in contact with the beads (for example 5 g). Shake the containers for minimum 1 min. The shaking should be sufficiently vigorous to ensure that the test surface moves constantly over the beads.

d) For the bacterial strains prepare 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} dilutions and for the fungal strains 10^{-2}, 10^{-3}, 10^{-4}, 10^{-}. Take a sample of 1,0 ml of each of the dilutions in duplicate and inoculate using the pour plate or the spread plate technique [5.5.2.2 d)]. For incubation and counting, see 5.5.3.

5.5.2.4 Method validation "C" - dilution-neutralization validation

To validate the dilution neutralization method, the procedure is as follows:

a) Prepare one test surface [5.5.2.1 a) and b)].

b) Pipette 10 ml of neutralizer (5.2.2.7) into a container with sufficient glass beads to support the surface. Then add 0,1 ml of the highest product concentration used in the test (5.5.2.2). Mix and leave in contact for 5 min ± 10 s at 20 °C ± 1 °C.

c) At the end of the neutralization time transfer the inoculated test surface into the container and place the inoculated surface downwards in contact with the beads (for example 5 g). Shake the containers for minimum 1 min. The shaking should be sufficiently vigorous to ensure that the test surface moves constantly over the beads.

d) For the bacterial strains prepare 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} dilutions and for the fungal strains 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}. Take samples of 1,0 ml of each of the dilutions in duplicate and inoculate using pour plate or spread plate technique.

5.5.3 Counting of the test mixtures

For the bacterial strains, incubate the plates at 36 °C ± 1 °C or at 37 °C ± 1 °C for 18 to 24 h. Discard any plates which are not countable for any reason. Count the plates and determine the number of colony forming units. Incubate the plates for a further 18 h to 24 h. Do not recount plates which no longer show countable quantities of colonies. Recount the remaining plates.

For the fungal strains, incubate the plates at (30 ± 1) °C for 18 h to 24 h (*Candida albicans*), for 42 h to 48 h (*Aspergillus brasiliensis*). Discard any plates which are not countable for any reason. Count the plates and determine the number of colony forming units. Incubate the plates for a further 18 h to 24 h. Do not recount plates which no longer show well-separated colonies. Recount the remaining plates. For *Aspergillus brasiliensis*, continue incubation for a further 18 h to 24 h and if necessary, a further 18 h to 24 h, provided the number of countable colonies (discrete colonies) is increasing.

Record the number of colony forming units (Nts) remaining on the test surface.

Discard any plates that are not countable for any reason. Count the plates and determine the total number of cfu. 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.

Only the plates showing a number of colonies included in a 15-300 for bacteria and yeast and 15-150 for mould range were used to perform the result calculation. A deviation of 10 % is accepted, so the limits are 14 and 330 for bacteria and yeast and 14-165 for mould.

In the assay, where the number of cfu on every plate counted is < 14, the number of cfu/ml should be recorded as < $1{,}4 \times 10^2$ (<2,15 Log). Where the number of cfu on every plate counted is > 330 or 165, the number of cfu/ml should be recorded as > $3{,}3 \times 10^5$ (>5,52 Log) or > $1{,}6 \times 10^5$, (>5,22 Log).

Calculate *A* and *Na* as the log number of cfu recovered from the test surface using the following formula:

$$Na \text{ (or } A) = \log (c \times 10/(n \times d)) \quad (2)$$

where

c is the sum of *Vc* values taken into account;

n is the number of *Vc* values taken into account;

d is the dilution taken into account (e.g. 10^{-3}).

If one or both of the duplicate *Vc* values are either below the lower or above the upper limit, express the results as “less than” or “more than”. In particular, in the assay, where the number of cfu on every plate counted is < 14, the number of cfu/ml should be recorded as < $1{,}4 \times 10^2$ (<2,15 Log). Where the number

of cfu on every plate counted is > 330 or 165, the number of cfu/ml should be recorded as > $3,3 \times 10^5$ (>5,52 Log) or > $1,6 \times 10^5$, (>5,22 Log).

If both of the duplicate *Vc* values are zero, it is assumed that the count (in cfu) is less than 5/ml in the neutralization medium and an *Na* value of < 0,10 should be used.

Calculate *B* and *C* as the log number of cfu recovered from the test surface using the following formula:

$$B, C = \log (c \times 10/(n \times d)) \quad (3)$$

where

c is the sum of *Vc* values taken into account;

n is the number of *Vc* values taken into account;

d is the dilution taken into account (e.g. 10^{-3}).

5.6 Calculation and expression of results

5.6.1 Elaboration of data: counting of weighed average values

Rounding of the results shall be performed by rounding the exponential values to two significant ciphers.

When rounding ciphers: if the last cipher is higher than or equal to '5' the previous cipher is increased of a unit; if the last cipher is lower than '5' the previous cipher remains unchanged; proceed this way until reaching two significant ciphers.

Log values are rounded to two decimal significant ciphers as shown in Annex B and Annex C, Table C.1.

If observed counts from two consecutive dilutions fall within the 14-330 or 14-165 range, then a weighted average value is calculated as follows:

$$(m+m'+n+n')/2,2 \times V \times d \quad (4)$$

where

m, m' are the two replicas at the lower dilution expressed as cfu;

n, n' are the two replicas at the higher dilution expressed as cfu;

V is the volume of the inoculated into the plate expressed in ml;

d is the lower dilution factor.

In case not all the replicas at each dilution fall within the 14-330 or 14-165 range, the weighted average will take this into account as follows:

Case 1: only m and n fall within the 14-330 or 14-165 range, the formula will change as follows:

$$(m+n)/1,1 \times V \times d \quad (5)$$

Case 2: only m, m' and n fall within the 14-330 or 14-165 range, the formula will change as follows:

$$(m+m'+n)/2,1 \times V \times d \quad (6)$$

Case 3: only m, n and n' fall within the 14-330 or 14-165 range, the formula will change as follows:

$$(m+n+n')/1,2 \times V \times d \quad (7)$$

Case 4: only m falls within the 14-330 or 14-165 range, the formula will change as follows:

$$m/V \times d \quad (8)$$

5.6.2 Verification of methodology

For each test, check that:

a) the mean counts from duplicate plate used for calculation of N, A, Na, B, C are between 14 and 330 for bacteria and yeast strains and 14 and 165 for mould strains;

b) $6,57 \leq N \leq 7,10$ lg for bacteria

c) $5,57 \leq N \leq 6,10$ lg for *Candida albicans* and for *Aspergillus brasiliensis*;

d) B - A is not greater than ± 0,3 lg;

e) C - A is not greater than ± 0,3 lg;

f) Nts (number of residual cfu recovered from test surface) is less than 100 cfu for active concentrations. If not, the recovery of microorganisms has not been sufficient. For non-active concentrations, Nts may be not countable;

g) control of weighted mean counts: quotient is not lower than 5 and not higher than 15. It applies only to N calculation;

h) A shall be sufficiently high in order to demonstrate a 4 lg reduction for bacteria and a 3 lg reduction for fungi.

5.6.3 Expression of results

5.6.3.1 Reduction

The reduction (R) is expressed in logarithm.

For each test organism record the number of cfu/ml in the test procedure for microbicidal activity of the product (5.5.2.2) and the control procedure (5.5.2.3)." For each product concentration and each experimental condition, calculate and record the decimal log (lg) reduction separately using the formula:

$$R = A - Na \quad (9)$$

5.6.4 Conclusion

5.6.4.1 Activity on non-porous surfaces for general purposes

Bactericidal, fungicidal and/or yeasticidal activity on surfaces for general purposes is characterized by the concentration of the tested product for which criteria 5.6.1 and 5.6.2 are met and:

— for which a 4 lg or more reduction in viability is demonstrated, under clean or dirty conditions (see Clause 4), when the test organisms are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus hirae* and *Escherichia coli*;

and/or

— for which a 3 lg or more reduction in viability is demonstrated, under clean or dirty conditions (see Clause 4), when the test organisms are *Candida albicans* and *Aspergillus brasiliensis*;

and/or

— for which a 3 lg or more reduction in viability is demonstrated, under clean or dirty conditions (see Clause 4), when the test organisms are *Candida albicans*.

5.6.4.2 Activity on non-porous surfaces for dairies

Bactericidal and/or fungicidal or yeasticidal activity on surfaces for dairies is characterized by the concentration of the tested product for which criteria 5.6.1 and 5.6.2 are met and for which:

— a 4 log or more reduction in viability is demonstrated, when the test organisms are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus hirae* and *Escherichia coli*, and if required additional test organisms;

and/or for which:

— a 3 log or more reduction in viability is demonstrated, when the test organisms are *Candida albicans* and *Aspergillus brasiliensis*, and if required additional test organisms;

— a 3 log or more reduction in viability is demonstrated, when the test organisms are *Candida albicans*, and if required additional test organisms

under conditions: t in minutes, θ in degrees °C, and using milk as interfering substance (see 5.5.1).

5.6.4.3 Activity on non-porous surfaces for specific purposes

Bactericidal and/or fungicidal or yeasticidal activity on surfaces for specific purposes is characterized by the concentration of the tested product for which criteria 5.6.1 and 5.6.2 are met and for which:

— a 4 log or more reduction in viability is demonstrated, when the test organisms are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus hirae* and *Escherichia coli*, and if required additional test organisms;

and/or for which:

— a 3 log or more reduction in viability is demonstrated, when the test organisms are *Candida albicans* and *Aspergillus brasiliensis*, and if required additional test organisms;

— a 3 log or more reduction in viability is demonstrated, when the test organisms are *Candida albicans*, and if required additional test organisms

under additional conditions: t in minutes, θ in degrees °C, clean or dirty conditions and additional interfering substances (see 5.5.1).

5.7 Test report

The test report shall refer to this document.

The test report shall state, at least, the following information:

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;

6) active substance(s) and its/their concentration(s) (optional);

c) experimental conditions:

1) period of analysis;

2) product diluent used during the test;

3) product test concentrations;

4) appearance product dilutions;

5) contact time(s);

6) test temperature(s);

7) interfering substance;

8) reaction between the inoculum in the presence of interfering substances and product;

9) temperature of incubation;

10) neutralizer of the bacterial or fungal strains used;

11) identification of the bacterial and/or fungal strains used;

12) identification of the test surface;

d) operating procedure:

1) full details for the test for validation of the neutralization medium shall be given;

e) test results:

1) validation tests;

2) evaluation of bactericidal and/or fungicidal and/or yeasticidal activity;

f) conclusion;

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

NOTE An example of a typical test report is given in Table C.1.

Annex A
(informative)

Corresponding referenced strains

Pseudomonas aeruginosa :	ATCC	15 442
	CIP	103 467
	DSM	939
	NCIB	10 421
Staphylococcus aureus:	ATCC	6 538
	CIP	4.83
	DSM	799
	NCTC	10 788
	NCIB	9 518
Escherichia coli:	ATCC	10 536
	CIP	54 127
	DSM	682
	NCTC	10 418
	NCIMB	8 879
Enterococcus hirae:	ATCC	10 541
	CIP	5 855
	DSM	3320
	NCIMB	8 192
Salmonella typhimurium:	ATCC	13 311
	CIP	5 858
	NCTC	74
Lactobacillus brevis:	DSM	6 235
	CIP	103 474
Enterobacter cloacae:	DSM	6 234
	CIP	104 674

Saccharomyces cerevisiae:	ATCC	9 763
	IP	143 283
	DSM	1 333
	CBS	5 900
Candida albicans:	ATCC	10 231
	IP	4 872
	DSM	1386
	CBS	6 431
	NCTC	3 179
Aspergillus brasiliensis (ex A. niger):	ATCC	16 404
	DSM	1 988
	CBS	733.88
	IP	1 431.83
	NCTC	2 275
	CMI	149 007

Annex B
(informative)

Neutralizers

Any of the following neutralizers can be used:

— lecithin 3 g/l; polysorbate 80 [7] 30 g/l; sodium thiosulphate 5 g/l; L-histidine 1 g/l; saponine 30 g/l in diluent (see 5.2.2.4) or in phosphate buffer 0,25 mol/l at 1 % (*V/V*);

— phosphate buffer 0,25 mol/l:

  — KH_2PO_4 34 g;

  — water (see 5.2.2.2) 500 ml;

  — adjusted to pH 7,2 ± 0,2 with 1 mol/l NaOH;

  — water (see 5.2.2.2) up to 1 000 ml;

  — sterilized in an autoclave (see 5.3.1);

— fresh egg yolk diluted to 5 % (*V/V*) or 0,5 % (*V/V*);

— 30 g/l polysorbate 80[7]; 4 g/l sodium lauryl sulphate ; lecithin 3g/l;

— 5 % (*V/V*) fresh egg yolk; 40 g/l polysorbate 80[7];

— 7 % (*V/V*) ethylene oxide condensate of fatty alcohol; 20 g/l lecithin; 4 % (*V/V*) polysorbate 80[7];

— 4 % (*V/V*) ethylene oxide condensate of fatty alcohol; 4 g/l lecithin;

— 30 g/l polysorbate 80[7]; lecithin 3 g/l; L-histidine 1 g/l;

— glycine as a function of concentration of product;

— 30 g/l polysorbate 80[7]; lecithin 3 g/l;

— phospholipid emulsion (commercial) at 50 mg/ml (diluted 1 to 10);

— sodium thioglycollate at 0,5 g/l or 5 g/l;

— L cysteine at 0,8 g/l or 1,5 g/l;

— thiomalic acid at 0,075 % (*V/V*) (adjusted to pH 7 with NaOH);

---

[7] Analytical quality, non-hydrolysed in accordance with European Pharmacopoeia volume 1. TWEEN 80® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of this product.

— sodium thiosulphate at 5 g/l;

— catalase or peroxidase: One unit for of these enzymes catalyses the decomposition of 1 µmol of hydrogen peroxide per minutes at 25 °C and at pH 7;

— polysorbate 80[7)] 30 g/l ; saponin 30 g/l ; L-histidine 1 g/l ; L-cysteine 1g/l.

The above list is not exhaustive and other neutralizers may be tried.

Annex C
(informative)

Expression of results with the dilution-neutralization method

Table C.1 — Test results

Test organisms	Bacterial or fungal test suspension : N (See 5.4.1.3)	Validation test : C (See Annex A)	Validation test : B (See Annex A)	Water control : A (See 5.5.3)	Test procedure at concentrations % (*V/V*) (See 5.5.2.1) 0,5	0,75	1,00
Pseudomonas aeruginosa ATCC 15442	10^{-6} : 229 ;216 10^{-7} : 20 ;17 N : 6,74	10^{-3} : > 330 ; > 330 10^{-4} : 123 ;118 10^{-5} : 9 ;13 C : 7,08	10^{-3} : > 330 ; > 330 10^{-4} : 137 ;142 10^{-5} : 11 ;4 B : 7,14	10^{-3} : > 330 ; > 330 10^{-4} : 153 ;123 10^{-5} : 14 ;9 A : 7,14 Nts : > 100	10^{-0} : > 330 ; > 330 10^{-1} : 160 ;138 10^{-2} : 13 ;17 Na : 4,18 Nts : > 100 R : 2,96	10^{-0} : 0 ;0 10^{-1} : 0 ;0 10^{-2} : 0 ;0 Na : < 0,10 Nts : 12 R: > 6,04	10^{-0} : 0 ;0 10^{-1} : 0 ;0 10^{-2} : 0 ;0 Na : < 0,10 Nts : 0 R: > 7,04
Escherichia coli ATCC 10536	10^{-6} : 230 ;210 10^{-7} : 23 ;19 N : 6,74	10^{-3} : > 330 ; > 330 10^{-4} : 132 ;113 10^{-5} : 9 ;2 C : 7,09	10^{-3} : > 330 ; > 330 10^{-4} : 143 ;122 10^{-5} : 22 ;11 B : 7,14	10^{-3} : > 330 ; > 330 10^{-4} : 155 ;121 10^{-5} : 18 ;23 A : 7,15 Nts : > 100	10^{-0} : > 330 ; > 330 10^{-1} : 166 ;144 10^{-2} : 22 ;18 Na : 4,20 Nts : > 100 R: 2,95	10^{-0} : 210 ;198 10^{-1} : 27 ;19 10^{-2} : 5 ;2 Na : 3,65 Nts : 0 R: 3,50	10^{-0} : 0 ;0 10^{-1} : 0 ;0 10^{-2} : 0 ;0 Na : < 0,10 Nts : 0 R: > 7,05

Test organisms	Bacterial or fungal test suspension : N (See 5.4.1.3)	Validation test : C (See Annex A)	Validation test : B (See Annex A)	Water control : A (See 5.5.3)	Test procedure at concentrations % (*V/V*) (See 5.5.2.1) 0,5	0,75	1,00
Staphylococcus aureus ATCC 6538	10^{-6} : 227 ;202 10^{-7} : 18 ;23 N : 6,72	10^{-3} : > 330 ; > 330 10^{-4} : 166 ;134 10^{-5} : 15 ;11 C : 7,18	10^{-3} : > 330 ; > 330 10^{-4} : 158 ;144 10^{-5} : 13 ;8 B : 7,18	10^{-3} : > 330 ; > 330 10^{-4} : 162 ;146 10^{-5} : 12 ;16 A : 7,19 Nts : > 100	10^{-0} : > 330 ; > 330 10^{-1} : 265 ;240 10^{-2} : 33 ;28 Na : 4,41 Nts : > 100 R: 2,78	10^{-0} : > 330 ; > 330 10^{-1} : 63 ;52 10^{-2} : 4 ;7 Na : 3,76 Nts : 23 R: 3,43	10^{-0} : 0 ;0 10^{-1} : 0 ;0 10^{-2} : 0 ;0 Na : < 0,10 Nts : 0 R: > 7,09
Enterococcus hirae ATCC 10541	10^{-6} : 235 ;255 10^{-7} : 29 ;31 N : 7,09	10^{-3} : > 330 ; > 330 10^{-4} : 198 ;178 10^{-5} : 27 ;18 C : 7,28	10^{-3} : > 330 ; > 330 10^{-4} : 201 ;187 10^{-5} : 17 ;24 B : 7,29	10^{-3} : > 330 ; > 330 10^{-4} : 194 ;179 10^{-5} : 23 ;19 A : 7,28 Nts : > 100	10^{-0} : > 330 ; > 330 10^{-1} : > 300 ; > 300 10^{-2} : 127 ;132 Na : 5,11 Nts : > 100 R: …	10^{-0} : > 330 ; > 330 10^{-1} : 128 ;145 10^{-2} : 9 ;13 Na : 4,14 Nts : 8 R: …	10^{-0} : 0 ;0 10^{-1} : 0 ;0 10^{-2} : 0 ;0 Na : < 0,10 Nts : 0 R: > …

Annex D
(informative)

Bactericidal activity in surfaces in general use conditions (for clean conditions)

a)	Identification of the test laboratory	Besson test house;
b)	Identification of the sample:	
	Name of the product	Z
	Batch number	94–71–51
	Manufacturer	Centipede Formulations Inc
	Date of delivery	1994–02–11
	Storage conditions	room temperature and darkness
	Product diluent recommended by the manufacturer for use.....	Potable water
	Active substance(s) and its (their) concentration(s) (optional).....	Not indicated
c)	Test method and its validation:	
	Method	Dilution neutralization;
	Neutralizer	30 g/l, lecithin, sterilized in the autoclave ;
d)	Experimental conditions:	
	Period of analysis	1994–02–20 to 1994–03–12
	Product diluent used during the test.....	sterile hard water 300 mg/kg $CaCO_3$
	Product test concentrations	2 g/l, 4 g/l and 8 g/l
	Appearance product dilutions.....	colourless, clear solution
	Interfering substances.....	0,3 g/l of bovine albumin (0,85 % skimmed milk fo Pseudomonas aeruginosa)
	Test temperature	θ = between 18 °C and 25 °C
	Contact time	t = 5 min ± 10 s
	Temperature of incubation	37 °C ± 1 °C
	Identification of the bacterial strains used.....	*Pseudomonas aeruginosa* ATCC 15 442
		Escherichia coli ATCC 10 536
		Staphylococcus aureus ATCC 6 538
		Enterococcus hirae ATCC 10 541
e)	Test results:	
	See Table C.1.	
f)	Conclusion:	

In accordance with this document the batch 94–71–51 of the product Z, when diluted at 1 % (*V*/*V*), in hard water, possesses bactericidal activity on surfaces in 5 min at 20 °C under clean conditions 0,3 g/l bovine albumin for referenced strains *Pseudomonas aeruginosa, Escherichia coli*, *Staphylococcus aureus* and *Enterococcus hirae.*

g) Locality, date and identified signature.

NOTE The test product, batch N° and manufacturer are given as imaginary examples only.

Annex E
(informative)

Precision of the test result

The formula used for the calculation of the precision of the test results can be found in EN 1040:2005, Annex E. The number of repetitions which give a precision of the reduction factor need to be established by appropriate collaborative studies. A guidance to the interpretation of the test results concerning their precision and the number of test repetitions is given in the EN 14885.

Annex F
(informative)

Alternative drying end point

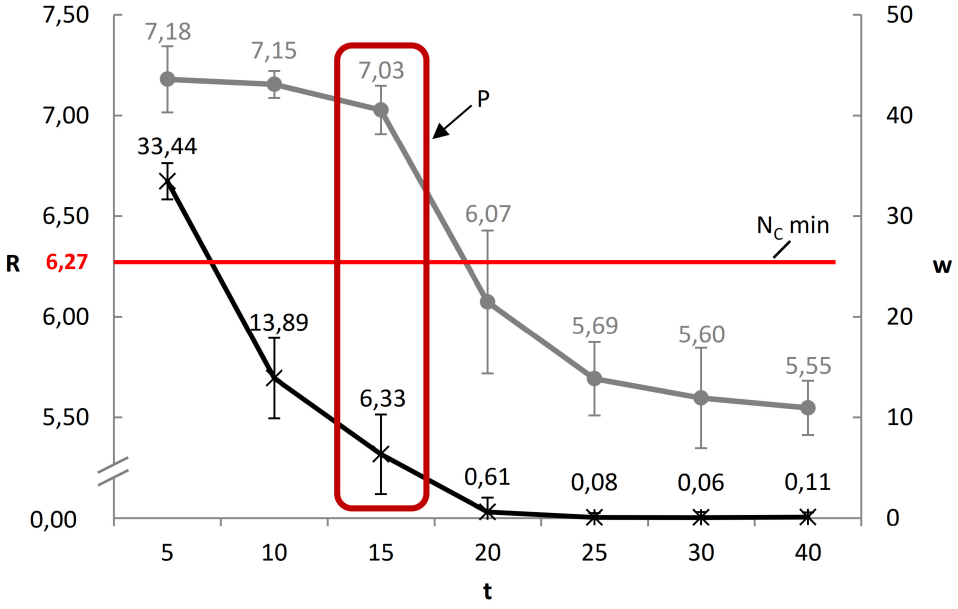
To ensure sufficiently high A for drying sensitive test organisms the test can be performed with inoculated stainless-steel carriers that were dried until a specific, fixed drying end point before the carriers are visibly dry. To determine the fixed drying end point a drying curve for these test organisms is to be recorded under the respective conditions in the laboratory where the tests are carried out.

For the creation of the drying curve, weigh several carriers on an analytical balance (readability: 0,000 1 g), inoculate every carrier with 0,05 mL test suspension [see Figure F.2 a)] and let the inoculum dry in an incubator or desiccator.

Take one carrier out of the incubator/desiccator every 5 min until the carriers are visibly dry [see Figure F.2 c)]. Weigh the carriers again immediately to determine the amount of remaining liquid and transfer them into separate containers with 10 mL neutralizer or diluent and glass beads.

Proceed according to 5.5.2.1 d) and 5.5.3 to determine the number of recovered cfu/mL for every carrier.

Create the drying curve with data from at least three parallels such as shown in Figure F.1.

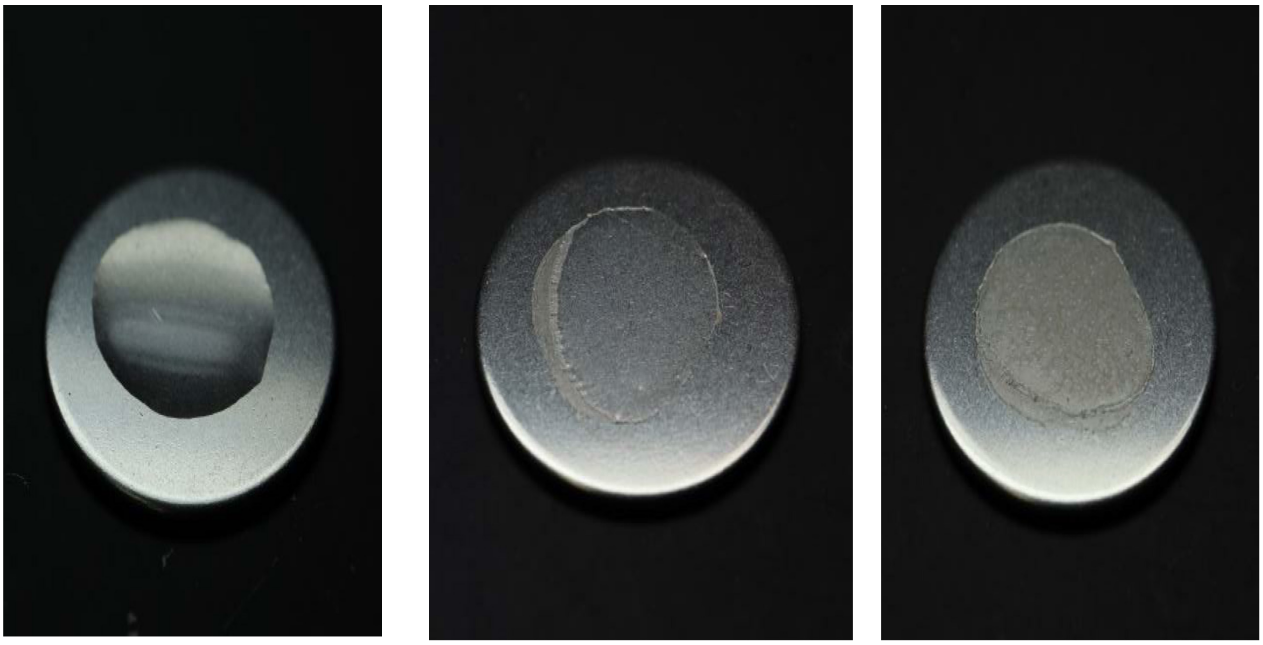


Key

R	recovery of *P. aeruginosa* [$\log_{10}$ cfy/mL
t	drying time [min]
w	weight of the test suspension [µg]
N_C min	minimum value of N_C
P	chosen drying endpoint after which the recovery of *P. aeruginosa* is sufficiently high

Figure F.1 — Example of a drying curve of *P. aeruginosa* ATCC 15442, dried according to the EN 13697:2019. The red line shows the minimum value of A for bacterial test organisms

The respective drying time after which 2-8 µL of liquid remains on the carrier [e.g. 15 min as shown in Figure F.2 b)] is then to be used as the fixed drying end point.



a) b) c)

Figure F.2 — Carriers inoculated with 0,05 µL test suspension after a drying time of a) 0 min, b) 15 min (2-8 µL remaining test suspension), c) 25 min drying time (= visibly dry)

Bibliography

[1] EN 1040:2005, *Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics — Test method and requirements (phase 1)*

[2] EN 10088-1, *Stainless steels — Part 1: List of stainless steels*

[3] EN 10088-2, *Stainless steels — Part 2: Technical delivery conditions for sheet/plate and strip of corrosion resisting steels for general purposes*