



BSI Standards Publication

**Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of fungicidal or yeasticidal activity in the medical area — Test method and requirements (phase 2, step 1)**

**National foreword**

This British Standard is the UK implementation of EN 13624:2013. It supersedes BS EN 13624:2003 which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee CH/216, Chemical disinfectants and antiseptics.

A list of organizations represented on this committee can be obtained on request to its secretary.

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

© The British Standards Institution 2013.  
Published by BSI Standards Limited 2013

ISBN 978 0 580 69791 3

ICS 11.080.20

**Compliance with a British Standard cannot confer immunity from legal obligations.**

This British Standard was published under the authority of the Standards Policy and Strategy Committee on 31 October 2013.

**Amendments issued since publication**

Date	Text affected
------	---------------

---

EUROPEAN STANDARD

**EN 13624**

NORME EUROPÉENNE

EUROPÄISCHE NORM

September 2013

ICS 11.080.20

Supersedes EN 13624:2003

English Version

## Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of fungicidal or yeasticidal activity in the medical area - Test method and requirements (phase 2, step 1)

Désinfectants chimiques et antiseptiques - Essai quantitatif de suspension pour l'évaluation de l'activité fongicide ou levuricide en médecine - Méthode d'essai et prescriptions (phase 2, étape 1)

Chemische Desinfektionsmittel und Antiseptika - Quantitativer Suspensionsversuch zur Bestimmung der fungiziden oder levuroziden Wirkung im humanmedizinischen Bereich - Prüfverfahren und Anforderungen (Phase 2, Stufe 1)

This European Standard was approved by CEN on 3 August 2013.

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. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the CEN-CENELEC Management Centre or to any CEN member.

This European Standard exists 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, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and United Kingdom.



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

**Management Centre: Avenue Marnix 17, B-1000 Brussels**

## Contents

Page

Foreword.....	3
Introduction .....	4
1 Scope .....	5
2 Normative references .....	5
3 Terms and definitions .....	5
4 Requirements .....	5
5 Test method.....	7
5.1 Principle.....	7
5.2 Materials and reagents.....	7
5.3 Apparatus and glassware .....	10
5.4 Preparation of test organism suspensions and product test solutions .....	12
5.5 Procedure for assessing the fungicidal and yeasticidal activity of the product .....	17
5.6 Experimental data and calculation.....	24
5.7 Verification of methodology .....	30
5.8 Expression of results and precision.....	30
5.9 Interpretation of results – conclusion .....	31
5.10 Test report .....	32
Annex A (informative) Referenced strains in national collections.....	35
Annex B (informative) Neutralizers and rinsing liquids .....	36
Annex C (informative) Graphical representation of test procedures.....	38
C.1 Dilution-neutralization method.....	38
C.2 Membrane filtration method .....	40
C.3 Dilution-neutralization method (modified method for ready-to-use products) .....	42
C.4 Membrane filtration method (modified method for ready-to-use products).....	44
Annex D (informative) Example of a typical test report.....	46
Annex E (informative) Precision of the test result .....	50
Annex ZA (informative) Relationship between this European Standard and the Essential Requirements of EU Directive 93/42/EEC.....	53
Bibliography .....	54

## Foreword

This document (EN 13624:2013) has been prepared by Technical Committee CEN/TC 216 “Chemical disinfectants and antiseptics”, the secretariat of which is held by AFNOR.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by March 2014, and conflicting national standards shall be withdrawn at the latest by March 2014.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

This document supersedes EN 13624:2003.

The document was revised to adapt it to the latest state of science, to correct errors and ambiguities, to harmonise the structure and wording with other tests of CEN/TC 216 existing or in preparation and to improve the readability of the standard and thereby make it more understandable. The following is a list of significant technical changes since the last edition:

- The Scope was expanded for the following fields of application within the medical area, i.e. products for surgical and/or hygienic handrub and/or handwash and disinfectants for other surfaces than instrument surfaces.
- “Obligatory test conditions” were replaced by “minimum test conditions” (test temperatures and contact times can be chosen within limits) that have to be performed to pass the test.
- An additional modified method is described to test ready-to-use products in a higher concentration than 80 %, i.e. 97 %.
- The quality of the cultured conidiospores of *Aspergillus brasiliensis* is described in greater detail (media, limits and the control methods) resulting from work done in WG 3 of CEN/TC 216.
- The neutralization time was shortened to 10 s for products with contact times of 10 min or less.
- The Annex ZA was reformulated to more accurately describe the relationship with the Medical Device Directive.

Data obtained using the former version of EN 13624 may still be used, if the quality of the conidiospores of *Aspergillus brasiliensis* had been controlled and had met the requirements in this standard (5.4.1.4.2).

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association, and supports essential requirements of EU Directive(s).

For relationship with EU Directive(s), see informative Annex ZA, which is an integral part of this document.

According to the CEN-CENELEC Internal Regulations, the national standards organisations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

## Introduction

This European Standard specifies a suspension test for establishing whether a chemical disinfectant or an antiseptic has a fungicidal or yeasticidal activity in the area and fields described in the scope.

This laboratory test takes into account practical conditions of application of the product including contact time, temperature, test organisms and interfering substances, i.e. conditions which may influence its action in practical situations. Each utilisation concentration of the chemical disinfectant or antiseptic found by this test corresponds to the chosen experimental conditions.

## 1 Scope

This European Standard specifies a test method and the minimum requirements for fungicidal or yeasticidal activity of chemical disinfectant and antiseptic products that form a homogeneous, physically stable preparation when diluted with hard water, or - in the case of ready-to-use products - with water. Products can only be tested at a concentration of 80 % or less (97 % with a modified method for special cases) as some dilution is always produced by adding the test organisms and interfering substance.

This European Standard applies to products that are used in the medical area in the fields of hygienic handrub, hygienic handwash, surgical handrub, surgical handwash, instrument disinfection by immersion, and surface disinfection by wiping, spraying, flooding or other means.

This European Standard applies to areas and situations where disinfection or antiseptics is medically indicated. Such indications occur in patient care, for example:

- in hospitals, in community medical facilities and in dental institutions;
- in clinics of schools, of kindergartens and of nursing homes;

and may occur in the workplace and in the home. It may also include services such as laundries and kitchens supplying products directly for the patients.

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

NOTE 2 This method corresponds to a phase 2 step 1 test.

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

## 2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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 4793:1980, *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 hygienic handwash at least a 2 lg reduction), when tested in accordance with Table 1 and Clause 5.

Table 1 — Minimum and additional test conditions

Test conditions	Hygienic handrub and handwash	Surgical handrub and handwash	Instrument disinfection	Surface disinfection
<b>Minimum spectrum of test organisms</b>	<i>Candida albicans</i> (vegetative cells)	<i>Candida albicans</i> (vegetative cells)	a) <b>fungicidal activity:</b> <i>Aspergillus brasiliensis</i> (conidiospores) <i>Candida albicans</i> (veg. cells) b) <b>yeastocidal activity:</b> <i>Candida albicans</i> (veg. cells)	a) <b>fungicidal activity:</b> <i>Aspergillus brasiliensis</i> (conidiospores) <i>Candida albicans</i> (veg. cells) b) <b>yeastocidal activity:</b> <i>Candida albicans</i> (veg. cells)
additional	Any relevant test organism			
Test temperature	according to the manufacturer's recommendation, but at/ between			
	20 °C	20 °C	20 °C and 70 °C	4 °C and 30 °C
Contact time	according to the manufacturer's recommendation, but between			
	30 s and 60 s	1 min and 5 min	60 min	5 min or 60 min <sup>a</sup>
<b>Interfering substance</b> clean conditions	0,3 g/l bovine albumin solution (hygienic handrub) <sup>b</sup>	0,3 g/l bovine albumin solution (surgical handrub) <sup>b</sup>	0,3 g/l bovine albumin solution	0,3 g/l bovine albumin solution
dirty conditions	3,0 g/l bovine albumin solution plus 3,0 ml/l erythrocytes (hygienic handwash) <sup>c</sup>	3,0 g/l bovine albumin solution plus 3,0 ml/l erythrocytes (surgical and handwash) <sup>c</sup>	and/or 3,0 g/l bovine albumin solution plus 3,0 ml/l erythrocytes	and/or 3,0 g/l bovine albumin solution plus 3,0 ml/l erythrocytes
b) additional	—	—	any relevant substance	any relevant substance
NOTE For the additional conditions, the concentration defined as a result can be lower than the one obtained under the minimum test conditions.				
<sup>a</sup> The contact times for surface disinfectants stated in this table are chosen on the basis of the practical conditions of the product. The recommended contact time for the use of the product is within the responsibility of the manufacturer. Products intended to disinfect surfaces that are likely to come into contact with the patient and/or the medical staff and surfaces, which are frequently touched by different people, leading to the transmission of microorganisms to the patient, shall be tested with a contact time of maximum 5 min. The same applies where the contact time of the product shall be limited for practical reasons. Products for other surfaces than stated above may be tested with a contact time of maximum 60 min.				
<sup>b</sup> Hygienic and surgical handrub shall be tested as a minimum under clean conditions.				
<sup>c</sup> Hygienic and surgical handwash shall be tested as a minimum under dirty conditions.				



## 5 Test method

### 5.1 Principle

**5.1.1** A sample of the product as delivered and/or diluted with hard water (or water for ready to use products) is added to a test suspension of fungi (yeast cells or mould spores) in a solution of an interfering substance. The mixture is maintained at the temperature and the contact time specified in Clause 4 and 5.5.1.1. At the end of this contact time, an aliquot is taken; the fungicidal and/or the fungistatic action in this portion is immediately neutralized or suppressed by a validated method. The method of choice is dilution-neutralization. If a suitable neutralizer cannot be found, membrane filtration is used. The numbers of surviving fungi in each sample are determined and the reduction is calculated.

NOTE Handwash products are always prediluted with hard water (5.2.2.7). The resulting solution is regarded as a ready-to-use product (5.4.2).

**5.1.2** The test is performed using the vegetative cells of *Candida albicans* and the conidiospores of *Aspergillus brasiliensis* (fungicidal activity) or only the vegetative cells of *Candida albicans* (yeastocidal activity) as test-organisms (Clause 4, Table 1).

**5.1.3** Additional contact times and temperatures are specified (Clause 4, Table 1). Additional interfering substances and test organisms may be used.

### 5.2 Materials and reagents

#### 5.2.1 Test organisms

The fungicidal activity shall be evaluated using the following strains as test organisms selected according to Clause 4 (Table 1)<sup>1)</sup>:

- *Candida albicans* ATCC 10231;
- *Aspergillus brasiliensis* (former "A.niger") ATCC 16404.

The yeastocidal activity shall be evaluated using only *Candida albicans*.

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

The required incubation temperature for these test organisms is  $(30 \pm 1) ^\circ\text{C}$  (5.3.2.3).

If additional test organisms are used, they shall be incubated under optimum growth conditions (temperature, time, atmosphere, media) noted in the test report. If the additional test organisms selected do not correspond to the specified strains, their suitability for supplying the required inocula shall be verified. If these additional test organisms 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 collection under a reference for five years.

---

1) The ATCC numbers are the collection numbers of strains supplied by these culture collections. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named.

## 5.2.2 Culture media and reagents

### 5.2.2.1 General

All weights of chemical substances given in this European Standard refer to the anhydrous salts. Hydrated forms may be used as an alternative, but the weights required shall be adjusted to allow for consequent molecular weight differences.

The reagents shall be of analytical grade and/or appropriate for microbiological purposes. They shall be free from substances that are toxic or inhibitory to the test organisms.

To improve reproducibility, it is recommended that commercially available dehydrated material is used for the preparation of culture media. The manufacturer's instructions relating to the preparation of these products should be rigorously followed.

For each culture medium and reagent, a time limitation for use should be fixed.

All specified pH values are measured at  $(20 \pm 1)$  °C.

### 5.2.2.2 Water

The water shall be freshly glass-distilled water and not demineralised water. If distilled water of adequate quality is not available, water for injections (see bibliographic reference [1]) can be used.

Sterilise in the autoclave [5.3.2.1 a)]. Sterilisation is not necessary if the water is used, e.g. for preparation of culture media and subsequently sterilised.

See 5.2.2.7 for the procedure to prepare hard water.

### 5.2.2.3 Malt extract agar (MEA)

Malt extract agar, consisting of:

Malt extract (food grade (e.g. Christomalt powder from Difal) or an equivalent extract that is not highly purified and not only based on maltose (e.g. Malt extract from OXOID)) <sup>2)</sup>	30,0 g
Agar	15,0 g
Water (5.2.2.2)	to 1 000,0 ml

Sterilise in the autoclave (5.3.1). After sterilisation, the pH (5.3.2.4) of the medium shall be equivalent to  $5,6 \pm 0,2$ .

In case of an encountering (problems with neutralization (5.5.1.2 and 5.5.1.3), it may be necessary to add neutralizer to MEA. Annex B gives guidance on the neutralizers that may be used. It is recommended not to use neutralizer that causes opalescence in the agar.

If there are problems with producing at least 75 % spiny conidiospores, see 5.4.1.4.2.

### 5.2.2.4 Diluent

Tryptone sodium chloride solution, consisting of:

---

2) This Malt extract from OXOID is an example of a suitable product available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of this product.

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

Sterilise in the autoclave [5.3.2.1 a)]. After sterilisation, the pH (5.3.2.4) of the diluent shall be equivalent to  $7,0 \pm 0,2$ .

#### 5.2.2.5 Neutralizer

The neutralizer shall be validated for the product being tested in accordance with 5.5.1.2, 5.5.1.3 and 5.5.2. It shall be sterile.

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

#### 5.2.2.6 Rinsing liquid (for membrane filtration)

The rinsing liquid shall be validated for the product being tested in accordance with 5.5.1.2, 5.5.1.3 and 5.5.3. It shall be sterile, compatible with the filter membrane and capable of filtration through the filter membrane under the test conditions described in 5.5.3.

NOTE Information on rinsing liquids 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 products

For the preparation of 1 l of hard water, the procedure is as follows:

- prepare solution A: dissolve 19,84 g magnesium chloride ( $MgCl_2$ ) and 46,24 g calcium chloride ( $CaCl_2$ ) in water (5.2.2.2) and dilute to 1 000 ml. Sterilise by membrane filtration (5.3.2.7) 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 (5.3.2.8) for no longer than one month;
- prepare solution B: dissolve 35,02 g sodium bicarbonate ( $NaHCO_3$ ) in water (5.2.2.2) and dilute to 1 000 ml. Sterilise by membrane filtration (5.3.2.7). Store the solution in the refrigerator (5.3.2.8) for no longer than one week;
- place 600 ml to 700 ml of water (5.2.2.2) in a 1 000 ml volumetric flask (5.3.2.12) and add 6,0 ml (5.3.2.9) of solution A, then 8,0 ml of solution B. Mix and dilute to 1 000 ml with water (5.2.2.2). The pH (5.3.2.4) of the hard water shall be  $7,0 \pm 0,2$ . 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 product test solutions (5.4.2), the addition of the product to the hard water produces different final water hardness in each test tube. In any case the final hardness expressed as calcium carbonate ( $CaCO_3$ ) is in the test tube lower than 375 mg/l.

#### 5.2.2.8 Interfering substance

##### 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 10 times its final concentration in the test (50 times in the case of the modified method, see 5.2.2.8.4).

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

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

#### **5.2.2.8.2 Clean conditions (bovine albumin solution – low concentration)**

Dissolve 0,30 g of bovine albumin fraction V (suitable for microbiological purposes) in 100 ml of diluent (5.2.2.4).

Sterilise by membrane filtration (5.3.2.7), keep in a refrigerator (5.3.2.8) and use within one month.

The final concentration of the bovine albumin in the test procedure (5.5) shall be 0,3 g/l.

#### **5.2.2.8.3 Dirty conditions (mixture of bovine albumin solutions – high concentration with sheep erythrocytes)**

Dissolve 3,00 g of bovine albumin fraction V (suitable for microbiological purposes) in 97 ml of diluent (5.2.2.4).

Sterilise by membrane filtration (5.3.2.7).

Prepare at least 8,0 ml fresh defibrinated sheep blood (5.2.2.9). Centrifuge the erythrocytes at 800  $g_N$  for 10 min (5.3.2.13). After discarding the supernatant, resuspend erythrocytes in diluent (5.2.2.4). Repeat this procedure at least 3 times, until the supernatant is colourless.

Resuspend 3 ml of the packed sheep erythrocytes in the 97 ml of sterilised bovine albumin solution (see above). To avoid later contamination this mixture should be split in portions probably needed per day and kept in separate containers for a maximum of 7 d in a refrigerator (5.3.2.8).

The final concentration of bovine albumin and sheep erythrocytes in the test procedure (5.5) shall be 3 g/l and 3 ml/l respectively.

#### **5.2.2.8.4 Clean and dirty conditions for the modified method for ready-to-use products (5.5.4)**

Follow the procedures for preparation according to 5.2.2.8.2 and 5.2.2.8.3, but prepare the interfering substance in fivefold higher concentrations.

- a) Clean conditions (5.2.2.8.2) – dissolve 1,50 g bovine albumin (instead of 0,3 g) in 100 ml of diluent;
- b) Dirty conditions (5.2.2.8.3) – dissolve 15,0 g bovine albumin (instead of 3,0 g) in 85 ml of diluent (instead of 97 ml).

Prepare at least 40 ml (instead of 8,0 ml) sheep blood. Resuspend 15 ml (instead of 3,0 ml) of the packed sheep erythrocytes in 85 ml of sterilised bovine albumin solution (see above).

#### **5.2.2.9 Defibrinated sheep blood**

The defibrinated sheep blood should be sterile (aseptic blood-letting and preparation), pooled from more than one sheep and can be acquired from a commercial supplier.

### **5.3 Apparatus and glassware**

#### **5.3.1 General**

Sterilise all glassware and parts of the 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) by moist heat, in the autoclave [5.3.2.1 a)];
- b) by dry heat, in the hot air oven [5.3.2.1 b)].

### 5.3.2 Usual microbiological laboratory equipment<sup>3)</sup>

and, in particular, the following:

#### 5.3.2.1 Apparatus for moist and dry heat sterilisation:

- a) for moist heat sterilisation, an autoclave capable of being maintained at  $(121_{0}^{+3})$  °C for a minimum holding time of 15 min;
- b) for dry heat sterilisation, a hot air oven capable of being maintained at  $(180_{0}^{+5})$  °C for a minimum holding time of 30 min, at  $(170_{0}^{+5})$  °C for a minimum holding time of 1 h or at  $(160_{0}^{+5})$  °C for a minimum holding time of 2 h.

**5.3.2.2 Water baths**, capable of being controlled at  $(20 \pm 1)$  °C, at  $(45 \pm 1)$  °C (to maintain melted MEA in case of pour plate technique) and at additional test temperatures  $\pm 1$  °C (5.5.1).

**5.3.2.3 Incubator**, capable of being controlled at  $(30 \pm 1)$  °C.

**5.3.2.4 pH-meter**, having an inaccuracy of calibration of no more than  $\pm 0,1$  pH units at  $(20 \pm 1)$  °C. A puncture electrode or a flat membrane electrode should be used for measuring the pH of the agar media (5.2.2.3).

**5.3.2.5 Stopwatch.**

**5.3.2.6 Shakers.**

- a) Electromechanical agitator, e.g. Vortex<sup>®</sup> mixer<sup>4)</sup>;
- b) Mechanical shaker.

**5.3.2.7 Membrane filtration apparatus**, constructed of a material compatible with the substances to be filtered, with a filter holder of at least 50 ml volume, and suitable for use of filters of diameter 47 mm to 50 mm and 0,45 µm pore size for sterilisation of hard water (5.2.2.7), bovine albumin (5.2.2.8.2, 5.2.2.8.3 and 5.2.2.8.4), and if the membrane filtration method is used (5.5.3).

The vacuum source used shall give an even filtration flow rate. In order to obtain a uniform distribution of the micro-organisms over the membrane and to prevent overlong filtration, the device shall be set so as to obtain the filtration of 100 ml of rinsing liquid in 20 s to 40 s.

**5.3.2.8 Refrigerator**, capable of being controlled at 2 °C to 8 °C.

**5.3.2.9 Graduated pipettes**, of nominal capacities 10 ml and 1 ml and 0,1 ml, or calibrated automatic pipettes.

**5.3.2.10 Petri dishes**, (plates) of size 90 mm to 100 mm.

---

3) Disposable sterile equipment is an acceptable alternative to reusable glassware.

4) Vortex<sup>®</sup> is an example of a suitable product available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of this product.

**5.3.2.11 Glass beads** (diameter 3 mm to 4 mm).

**5.3.2.12 Volumetric flasks.**

**5.3.2.13 Centrifuge** (800 g<sub>N</sub>).

**5.3.2.14 Flasks with ventilated caps: Roux bottles or similar flasks.**

**5.3.2.15 Fritted filters:** Porosity of 40 µm to 100 µm according to ISO 4793:1980, Clause 2.

## **5.4 Preparation of test organism suspensions and product test solutions**

### **5.4.1 Test organism suspensions (test and validation suspension)**

#### **5.4.1.1 General**

For each test organism, two different suspensions have to be prepared: the “test suspension” to perform the test and the “validation suspension” to perform the controls and method validation.

#### **5.4.1.2 Preservation and stock cultures of test organisms**

The test organisms and their stock cultures shall be prepared and kept in accordance with EN 12353.

#### **5.4.1.3 Working culture of test organisms**

##### **5.4.1.3.1 *Candida albicans* (yeast)**

In order to prepare the working culture of *Candida albicans* (5.2.1), subculture from the stock culture (5.4.1.2) by streaking onto MEA (5.2.2.3) slopes or plates and incubate (5.3.2.3). 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. The second and (if produced) third subculture are the working cultures.

If it is not possible to prepare the second subculture on a particular day, a 72 h subculture may be used for subsequent sub-culturing, provided that the subculture has been kept in the incubator (5.3.2.3) during the 72 h period.

Never produce and use a fourth subculture.

##### **5.4.1.3.2 *Aspergillus brasiliensis* (previously *A. niger*) (mould)**

For *Aspergillus brasiliensis* (5.2.1) use only the first subculture grown on MEA (5.2.2.3) in Petri dishes (5.3.2.10) or flasks with ventilated caps (5.3.2.14) and incubate for 7 d to 9 d. No further subculturing is needed. Stacking the Petri dishes during the incubation could result in inhomogeneous temperature.

At the end of incubation, all the cultures have to show a dark brown or black surface with only a few small white or grey spots.

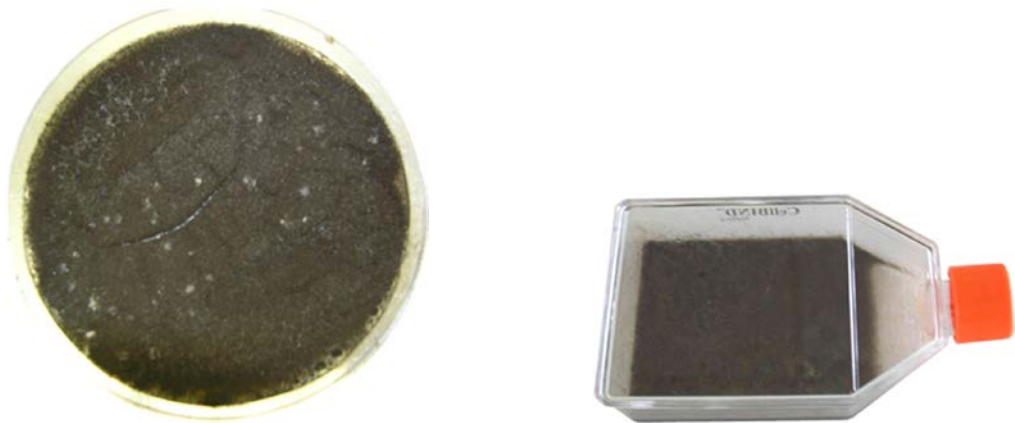


Figure 1 — Photos N°1: Examples of appropriate cultures of *A.brasiliensis* ATCC 16404 after 7 d of incubation at 30 °C

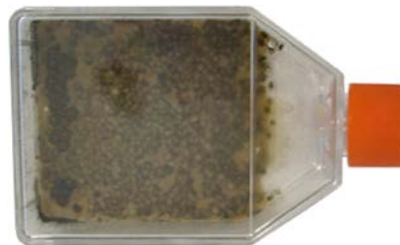


Figure 2 — Photo N°2: Example of inappropriate (not usable) culture of *A.brasiliensis* ATCC 16404 after 7 d of incubation at 30 °C

#### 5.4.1.3.3 Other test organisms (yeasts or moulds)

For additional test organisms, any departure from this method of culturing the yeast or the mould or of preparing the suspensions shall be noted, giving the reasons in the test report.

#### 5.4.1.4 Test suspension (N)

##### 5.4.1.4.1 *Candida albicans*

- Take 10 ml of diluent (5.2.2.4) and place in a 100 ml flask with 5 g of glass beads (5.3.2.11). Take the working culture (5.4.1.3.1) and transfer loopfuls of the cells into the diluent (5.2.2.4). The cells should be suspended in the diluent by rubbing the loop against the wet wall of the flask to dislodge the cells before immersing in the diluent. Shake the flask for 3 min using a mechanical shaker [5.3.2.6 b)]. Aspirate the suspension from the glass beads and transfer to a tube.
- Adjust the number of cells in the suspension to  $1,5 \times 10^7$  cfu/ml<sup>5)</sup> to  $5,0 \times 10^7$  cfu/ml using diluent (5.2.2.4) ( $1,5 \times 10^8$  cfu/ml to  $5,0 \times 10^8$  cfu/ml in the case of the modified method, 5.5.4), estimating the number of cfu by any suitable means. Maintain this test suspension in the water bath at 20 °C and use within 2 h. Adjust the temperature according to 5.5.1.1 a) and 5.5.1.4 only immediately before the start of the test.

The use of a spectrophotometer for adjusting the number of cells is highly recommended (about 620 nm wavelength – cuvette 10 mm path length). Each laboratory should therefore produce calibration data knowing that suitable values of optical density are generally found between 0,200 and 0,350. To achieve reproducible results of this measurement it may be necessary to dilute the test suspension, e.g. 1+9.

---

5) cfu/ml = colony forming unit(s) per millilitre.

NOTE A colorimeter is a suitable alternative.

- c) For counting, prepare  $10^{-5}$  and  $10^{-6}$  dilutions ( $10^{-6}$  and  $10^{-7}$  dilutions in the case of the modified method – 5.5.4) of the test suspension using diluent (5.2.2.4). Mix [(5.3.2.6a)].

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

- 1) When using the pour plate technique, transfer each 1,0 ml sample into separate Petri dishes and add 15 ml to 20 ml melted MEA (5.2.2.3), cooled to  $(45 \pm 1) ^\circ\text{C}$ .
- 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 MEA (5.2.2.3).

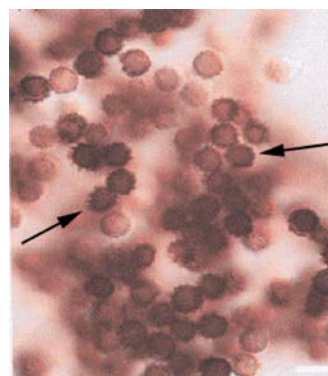
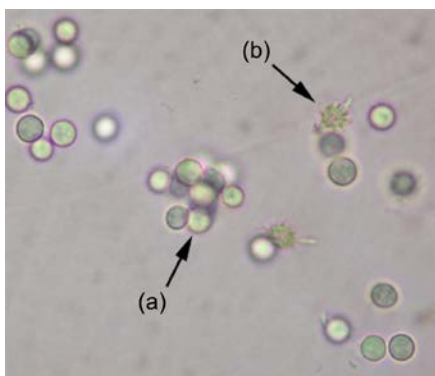
The technique used for counting of the test suspension has to be used for all other countings, 5.4.1.5 d), 5.5.2.2 c) and d), 5.5.2.3 b), 5.5.2.4 b) and 5.5.2.5 b).

For incubation and counting, see 5.4.1.6.

#### 5.4.1.4.2 *Aspergillus brasiliensis*

The procedure for preparing the *Aspergillus brasiliensis* test suspension is as follows:

- a) Take the working culture (5.4.1.3.2) and suspend the conidiospores in 10 ml of sterile 0,05 % (w/v) polysorbate 80 solution in water (5.2.2.2). Using a 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 5 g of glass beads (5.3.2.11). Filter the suspension through a fritted filter (5.3.2.15);
- b) Carry out a microscopic examination under x 400 magnification immediately after the preparation to show:
  - 1) the presence of a high concentration of characteristic mature conidiospores, i.e. spiny conidiospores (versus smooth spores). See Figure 3 and Figure 4.



**Figure 3 — Photo N°3 Observation of conidiospores under light microscope: Presence of smooth (a) and spiny (b) spores [inappropriate (not usable) suspension]**

**Figure 4 — Photo N°4 Observation of conidiospores under light microscope: High concentration of characteristic mature spores with spiny aspect (appropriate suspension)**

If there are less than 75 % spiny conidiospores it may be due to the *Aspergillus* strain used or to the media. Therefore use another strain, preferably from another supplier, and/or try out another media;



- 2) the absence of conidiospore germination (check at least ten fields of view);
- 3) if germinated conidiospores are present, discard the suspension;
- 4) the absence of mycelia fragments (check at least ten fields of view).

If mycelia are present, proceed to a second fritted filtration. If mycelia are still present, discard the suspension.

- d) Adjust the number of conidiospores in the suspension to  $1,5 \times 10^7$  cfu/ml to  $5,0 \times 10^7$  cfu/ml ( $1,5 \times 10^8$  cfu/ml to  $5,0 \times 10^8$  cfu/ml in the case of the modified method – 5.5.4) using the diluent (5.2.2.4), estimating the number of cfu by any suitable means. Use the suspension within 4 h in a water bath controlled at  $(20 \pm 1)$  °C (5.3.2.2). In any case, adjust the temperature according to 5.5.1.4 only immediately before the start of the test (5.5.2, 5.5.3 or 5.5.4).

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.

Each laboratory should therefore produce calibration data to establish the relationship between the counts obtained using the counting device and the counts (5.4.1.6) obtained by the pour plate or the spread plate technique [d)]. Experienced laboratories found a better fit to the required number of conidiospores when the conidiospore suspension count in the device was 10 % to 50 % higher than the number aimed at.

- e) For counting, prepare  $10^{-5}$  and  $10^{-6}$  dilutions ( $10^{-6}$  and  $10^{-7}$  dilutions in the case of the modified method 5.5.4) of the test suspension using diluent (5.2.2.4). Mix [5.3.2.6 a)].

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

- 1) 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 (5.2.2.3), cooled to  $(45 \pm 1)$  °C;
- 2) 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 (5.2.2.3) (i.e. in duplicate – at least eight plates).

The technique used for counting of the test suspension has to be used for all other countings, 5.4.1.5 d), 5.5.2.2 c) and d), 5.5.2.3 b), 5.5.2.4 b) and 5.5.2.5 b).

For incubation and counting, see 5.4.1.6.

#### 5.4.1.5 Validation suspension ( $N_V$ and $N_{VB}$ )

- a) To prepare the validation suspension, dilute the test suspension  $N$  (see 5.4.1.4.1 and 5.4.1.4.2) with the diluent (5.2.2.4) to obtain  $3,0 \times 10^2$  cfu/ml to  $1,6 \times 10^3$  cfu/ml (about one fourth (1 + 3) of the  $10^{-4}$  dilution).

NOTE In the case of the modified method (5.5.4) the procedure is the same, but one fourth (1+3) of the  $10^{-4}$  dilution resulting in  $3,0 \times 10^3$  cfu/ml to  $1,6 \times 10^4$  cfu/ml.

- b) For the neutralizer control  $N_{VB}$  (5.5.2.4) dilute the test suspension (5.4.1.4) with the diluent to obtain  $3,0 \times 10^4$  cfu/ml to  $1,6 \times 10^5$  cfu/ml [about one fourth (1+3) of the  $10^{-2}$  dilution].
- c) Maintain and use these validation suspensions ( $N_V$  and  $N_{VB}$ ) the same way as the test suspension [5.4.1.4.1 b) and 5.4.1.4.2 c)].
- d) For counting prepare with diluent (5.2.2.4) a  $10^{-1}$  dilution, in the case of the modified method (5.5.4) a

- e)  $10^{-2}$  dilution and in the case of the neutralizer control  $N_{VB}$  [see b)] a  $10^{-3}$  dilution. Mix [5.3.2.6 a)]. Take a sample of 1,0 ml in duplicate and inoculate using the pour plate or the spread plate technique [with *Candida albicans*, see 5.4.1.4.1 c); with *Aspergillus brasiliensis*, see 5.4.1.4.2 d)].

For incubation and counting, see 5.4.1.6.

#### 5.4.1.6 Incubation and counting of the test and the validation suspensions

- a) Incubate (5.3.2.3) the plates for 42 h to 48 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of cfu. Only for *Aspergillus brasiliensis*: incubate the plates for a further 20 h to 24 h and – if the number of colonies has increased – for a third additional period of 20 h to 24 h. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.
- b) Note for each plate the exact number of colonies but record > 165 (for moulds) or > 330 (for yeasts) for any counts higher than 165 and 330 respectively and determine the  $V_C$ -values according to 5.6.2.2.
- c) Calculate the numbers of cfu/ml in the test suspension  $N$  and in the validation suspensions  $N_V$  and  $N_{VB}$  (neutralizer control 5.5.2.4) using the method given in 5.6.2.3 and 5.6.2.5. Verify according to 5.7.

#### 5.4.2 Product test solutions

The concentration of a product test solution shall be 1,25 times the desired test concentration because it is diluted to 80 % during the test and the method validation (5.5.2 or 5.5.3).

Product test solutions shall be prepared in hard water (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 (5.8.2). The product as received may be used as one of the product test solutions; in this case the highest tested concentration is 80 %. Ready to use products may be tested at 97 % [modified method (5.5.4.)] In this case, the “real test concentration” is 97 %.

Dilutions of ready-to-use products shall be prepared in water (5.2.2.2) instead of hard water. Handwash products are always prediluted with hard water (5.2.2.7) to achieve a 62,5 % solution. The solution simulates the addition of tap water in practice (1:1). Such a product is nevertheless regarded as a “ready-to-use product”. The modified method (5.5.4) cannot be used since 62,5 % represents the highest accepted concentration (50 %), multiplied by 1,25.

For solid products, dissolve the product as received by weighing at least  $1,0 \text{ g} \pm 10 \text{ mg}$  of the product in a volumetric flask and filling up with hard water (5.2.2.7). Subsequent dilutions (= lower concentrations) shall be prepared in volumetric flasks (5.3.2.12) on a volume/volume basis in hard water (5.2.2.7).

For liquid products, dilutions of the product shall be prepared with hard water in volumetric flasks (5.3.2.12) on a volume/volume basis.

The product test solutions shall be prepared freshly and used in the test within 2 h. They shall give a physically homogenous preparation, stable during the whole procedure. If during the procedure a visible inhomogeneity appears due to the formation of a precipitate or flocculate (for example, through the addition of the interfering substance), it shall be recorded in the test report.

NOTE Counting micro-organisms embedded in a precipitate or flocculate is difficult and unreliable.

The concentration of the product stated in the test report shall be the desired test concentration. Record the test concentration in terms of mass per volume or volume per volume and details of the product sample as received.

## 5.5 Procedure for assessing the fungicidal and yeasticidal activity of the product

### 5.5.1 General

#### 5.5.1.1 Experimental conditions

The experimental conditions may be selected according to the practical use considered for the product (Clause 4):

- a) temperature  $\theta$  (in °C):  
The temperatures to be tested are specified in Clause 4, Table 1.  
The allowed deviation for each chosen temperature is  $\pm 1$  °C.
- b) contact time  $t$  (in min):  
The contact times to be tested are specified in Clause 4, Table 1.  
The allowed deviation for each chosen contact time is  $\pm 10$  s, except for 1 min or less where it is 5 s.
- c) interfering substance:  
The interfering substance to be tested is 0,30 g/l bovine albumin (5.2.2.8.2) representing clean conditions or a mixture of 3 ml/l sheep erythrocytes and 3,0 g/l bovine albumin (5.2.2.8.3) representing dirty conditions – according to Clause 4, Table 1 and practical applications. Additional interfering substances may be tested according to specific fields of application.
- d) test organisms:
  - 1) the test organisms for testing fungicidal activity are: *Candida albicans* and *Aspergillus brasiliensis* as specified in Clause 4, Table 1 and 5.2.1;
  - 2) the test organism for testing yeasticidal activity is: *Candida albicans* as specified in Clause 4, Table 1 and 5.2.1.

Additional test organisms may be tested.

#### 5.5.1.2 Choice of test method

The method of choice is the dilution-neutralization method. To determine a suitable neutralizer carry out the validation of the dilution neutralization method (5.5.2.2, 5.5.2.3 and 5.5.2.4 in connection with 5.5.2.5) using a neutralizer, chosen according to laboratory experience and published data.

If this neutralizer is not valid, repeat the validation test using an alternative neutralizer taking into account the information given in Annex B.

If both neutralizers are found to be invalid, the membrane filtration method (5.5.3) may be used.

In special circumstances it may be necessary to add neutralizer to MEA (5.2.2.3).

#### 5.5.1.3 General instructions for validation and control procedures

The neutralization and/or removal of the fungicidal and/or fungistatic activity of the product shall be controlled and validated - only for the highest product test concentration - for each of the used test organisms and for each experimental condition (interfering substance, temperature, contact time). These procedures (experimental condition control, neutralizer or filtration control and method validation) shall be performed at the same time with the test and with the same neutralizer – or rinsing liquid – used in the test.

In the case of ready-to-use-products use water (5.2.2.2) instead of hard water, but observe the exception with handwash products (5.1.1, NOTE).

If because of problems with neutralization a neutralizer has been added to MEA (5.5.1.2) used for the validation and control procedures the MEA used for the test shall contain the same amount of this neutralizer as well.

#### 5.5.1.4 Equilibration of temperature

Prior to testing, equilibrate all reagents (product test solutions (5.4.2), test suspension (5.4.1.4), validation suspension (5.4.1.5), diluent (5.2.2.4), hard water (5.2.2.7) and interfering substance (5.2.2.8) to the test temperature of  $\theta$  [5.5.1.1a)] using the water bath (5.3.2.2) controlled at  $\theta$ . Observe the provisions laid down in 5.4.1.4.1 b) and 5.4.1.4.2 c). Check that the temperature of the reagents is stabilised at  $\theta$ .

The neutralizer (5.2.2.5) or the rinsing liquid (5.2.2.6) and water (5.2.2.2) shall be equilibrated at a temperature of  $(20 \pm 1)$  °C.

In the case of ready-to-use-products, water (5.2.2.2) shall be additionally equilibrated to  $\theta$ .

#### 5.5.1.5 Precautions for manipulation of test organisms

Do not touch the upper part of the test tube sides when adding the test- or the validation suspensions (5.4.1).

### 5.5.2 Dilution-neutralization method<sup>6)</sup>

#### 5.5.2.1 General

The test and the control and validation procedures (5.5.2.2 through 5.5.2.5) shall be carried out at the same time.

#### 5.5.2.2 Test $N_a$ – determination of fungicidal or yeasticidal concentrations

The procedure for determining fungicidal or yeasticidal concentrations is as follows:

- a) Pipette 1,0 ml of the interfering substance (5.2.2.8) into a tube. Add 1,0 ml of the test suspension (5.4.1.4). Start the stopwatch (5.3.2.5) immediately, mix [5.3.2.6 a)] and place the tube in a water bath controlled at the chosen test temperature  $\theta$  [5.5.1.1 a)] for  $2 \text{ min} \pm 10 \text{ s}$ .
- b) At the end of this time, add 8,0 ml of one of the product test solutions (5.4.2). Restart the stopwatch at the beginning of the addition. Mix [5.3.2.6 a)] and place the tube in a water bath controlled at  $\theta$  for the chosen contact time  $t$  [5.5.1.1 b)]. Just before the end of  $t$ , mix [5.3.2.6 a)] again.
- c) At the end of  $t$ , take a 1,0 ml sample of the test mixture  $N_a$  and transfer into a tube containing 8,0 ml neutralizer (5.2.2.5) and 1,0 ml water (5.2.2.2). Mix [5.3.2.6 a)] and place in a water bath controlled at  $(20 \pm 1)$  °C. After a neutralization time of  $5 \text{ min} \pm 10 \text{ s}$  (for handrub and handwash products only  $10 \text{ s} \pm 1 \text{ s}$ ), mix [5.3.2.6 a)] and immediately take a sample of 1,0 ml of the neutralized test mixture  $N_a$  (containing neutralizer, product test solution, interfering substance and test suspension) in duplicate and inoculate using the 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 MEA (5.2.2.3), cooled to  $(45 \pm 1)$  °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 MEA (5.2.2.3).

---

6) For a graphical representation of this method, see Figure C.1 and Figure C.2.

- d) Additionally transfer 0,5 ml of this mixture into a tube containing 4,5 ml of neutralizer to obtain  $10^{-1}$  dilution of  $N_a$ , with hygienic handwash products mix and dilute additionally with neutralizer to obtain a  $10^{-2}$  dilution of  $N_a$ . Take samples of 1,0 ml from each dilution tube in duplicate and inoculate using the pour plate or spread plate technique.

For incubation and counting, see 5.5.2.6.

- e) Perform the procedure a) to d) using the other product test solutions at the same time.
- f) Perform the procedure a) to e) applying the other minimum and – if appropriate – other additional experimental conditions (5.5.1.1).

### 5.5.2.3 Experimental conditions control A – validation of the selected experimental conditions and/or verification of the absence of any lethal effect in the test conditions

To validate the selected experimental conditions and/or verify the absence of any lethal effect in the test conditions, the procedure is as follows:

- a) Pipette 1,0 ml of the interfering substance used in the test (5.5.2.2) into a tube. Add 1,0 ml of the validation suspension  $N_v$  (5.4.1.5). Start the stopwatch immediately, mix [5.3.2.6 a)] and place the tube in a water bath controlled at  $\theta$  for 2 min  $\pm$  10 s.
- b) At the end of this time, add 8,0 ml of hard water (5.2.2.7). [In the case of ready-to-use products (except handwash products (5.4.2)!): water (5.2.2.2) instead of hard water.] Restart the stopwatch at the beginning of the addition. Mix [5.3.2.6 a)] and place the tube in a water bath controlled at  $\theta$  for  $t$ . Just before the end of  $t$ , mix [5.3.2.6 a)] again.
- c) At the end of  $t$ , take a sample of 1,0 ml of this mixture A in duplicate and inoculate using the pour plate or the spread plate technique [5.5.2.2 c)].

For incubation and counting, see 5.5.2.6.

### 5.5.2.4 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) Pipette 9,0 ml of the neutralizer used in the test (5.5.2.2) into a tube. Add 1,0 ml of the validation suspension ( $N_{vB}$ ) [5.4.1.5 b)] containing  $3,0 \times 10^4$  cfu/ml to  $1,6 \times 10^5$  cfu/ml. Start the stopwatch at the beginning of the addition, mix [5.3.2.6 a)]. Transfer 0,5 ml of this mixture into a tube containing 4,5 ml of neutralizer to obtain  $10^{-1}$  dilution of  $N_{vB}$ , repeat this procedure to obtain  $10^{-2}$  dilution of  $N_{vB}$ . Place the tubes of the  $10^{-2}$  dilution of  $N_{vB}$  in a water bath controlled at  $(20 \pm 1)$  °C for 5 min  $\pm$  10 s (for contact times of 10 min or shorter only 10 s  $\pm$  1s). Just before the end of this time, mix [5.3.2.6 a)].

The high amount of neutralizer in relation to the test organisms reflects the additional dilutions with neutralizer – in the case of Na [5.5.2.2 c) and d)]  $10^{-1}$  and for handwash products  $10^{-1}$  and  $10^{-2}$ .

- b) At the end of this time, take a sample of 1,0 ml of this mixture B ( $10^{-2}$  dilution of  $N_{vB}$ ) in duplicate and inoculate using the pour plate or the spread plate technique [5.5.2.2 c)].

For incubation and counting, see 5.5.2.6.

### 5.5.2.5 Method validation C – dilution-neutralization validation

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

- a) Pipette 1,0 ml of the interfering substance used in the test (5.5.2.2) into a tube. Add 1,0 ml of the diluent (5.2.2.4) and then, starting a stopwatch, add 8,0 ml of the product test solution only of the highest

concentration used in the test (5.5.2.2). Mix [5.3.2.6 a)] and place the tube in a water bath controlled at  $\theta$  for  $t$ . Just before the end of  $t$ , mix [5.3.2.6 a)] again.

- b) At the end of  $t$  transfer 1,0 ml of the mixture into a tube containing 8,0 ml of neutralizer (used in 5.5.2.2). Restart the stopwatch at the beginning of the addition. Mix [5.3.2.6 a)] and place the tube in a water bath controlled at  $(20 \pm 1)^\circ\text{C}$  for  $5 \text{ min} \pm 10 \text{ s}$  (for handrub and handwash products only  $10 \text{ s} \pm 1 \text{ s}$ ). Add 1,0 ml of the validation suspension  $N_V$  (5.4.1.5). Start a stopwatch at the beginning of the addition and mix [5.3.2.6 a)]. Place the tube in a water bath controlled at  $(20 \pm 1)^\circ\text{C}$  for  $(30 \pm 1) \text{ min}$ . Just before the end of this time, mix [5.3.2.6 a)] again. At the end of this time, take a sample of 1,0 ml of the mixture  $C$  in duplicate and inoculate using the pour plate or the spread plate technique [5.5.2.2 c)].

For incubation and counting, see 5.5.2.6.

### 5.5.2.6 Incubation and counting of the test mixture and the control and validation mixtures

For incubation and counting of the test mixture and the control and validation mixtures, the procedure is as follows:

- a) Incubate (5.3.2.3) the plates for 42 h to 48 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of cfu. Only for *Aspergillus brasiliensis*: incubate the plates for a further 20 h to 24 h and – if the number of colonies has increased – for a third additional period of 20 h to 24 h. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.
- b) Note for each plate the exact number of colonies but record  $> 165$  (for moulds) or  $> 330$  (for yeasts) for any counts higher than 165 and 330 respectively and determine the  $V_C$ -values according to 5.6.2.2.
- c) Calculate the numbers of cfu/ml in the test mixture  $N_a$  and in the validation mixtures  $A$ ,  $B$  and  $C$  using the method given in 5.6.2.4 and 5.6.2.6. Verify according to 5.7.

### 5.5.3 Membrane filtration method<sup>7)</sup>

#### 5.5.3.1 General

The test and the control and validation procedures (5.5.3.2 through 5.5.3.5) shall be carried out at the same time.

Each membrane filtration apparatus (5.3.2.7) shall be filled with 50 ml of the rinsing liquid (5.2.2.6). The time required for filtering — if longer than one minute in exceptional cases — shall be recorded in the test report. When transferring the membranes to the surface of an agar plate, care should be taken to ensure that the test organisms are on the upper side of the membrane when placed on the plate, and to avoid trapping air between the membrane and agar surface.

#### 5.5.3.2 Test $N_a$ – determination of the fungicidal or yeasticidal concentrations

The procedure for determining the fungicidal or yeasticidal concentrations is as follows:

- a) See 5.5.2.2 a) and b).
- b) At the end of  $t$ , take a sample of 0,1 ml of the test mixture  $N_a$  (for hygienic handwash products  $1 \mu\text{l}$ ) in duplicate and transfer each 0,1 ml ( $1 \mu\text{l}$ ) sample into a separate membrane filtration apparatus (5.5.3.1). Filter immediately. Filter through at least 150 ml but no more than 500 ml of rinsing liquid (5.2.2.6). If the rinsing liquid is not water, complete the procedure by filtering 50 ml of water (5.2.2.2). Then transfer each of the membranes to the surface of separate MEA plates.

---

7) For a graphical representation of this method, see Figure C.3 and Figure C.4.

The amount of 1  $\mu\text{l}$  takes into account the 100 fold dilution of  $N_a$  [ $10^{-2}$  dilution in 5.5.2.2 d)] which enables the measurement of a 2 lg reduction. Since it is not recommended to pipette 1  $\mu\text{l}$  of microbial suspensions you may dilute for example 1 ml in 1 000 ml rinsing liquid (or 100  $\mu\text{l}$  in 100 ml) and pour 1 ml of this mixture into the membrane filtration apparatus.

- c) For incubation and counting, see 5.5.3.6.
- d) See 5.5.2.2 e).
- e) See 5.5.2.2 f).

### 5.5.3.3 Experimental conditions control A – validation of the selected experimental conditions and/or verification of the absence of any lethal effect in the test conditions

To validate the selected experimental conditions and/or verify the absence of any lethal effect in the test conditions, the procedure is as follows.

- a) See 5.5.2.3 a) and b).
- b) At the end of  $t$ , take a sample of 1,0 ml of this mixture A in duplicate and transfer each 1,0 ml sample into a separate membrane filtration apparatus (5.5.3.1). Filter immediately and additionally with 50 ml of water (5.2.2.2). Then transfer each of the membranes to the surface of separate MEA plates (5.2.2.3).

In the case of *Aspergillus brasiliensis* divide the sample in two, three or four portions of approximately equal size and transfer each portion into a separate membrane filtration apparatus (5.5.3.1), i.e. for duplicate four, six or eight membranes shall be inoculated.

NOTE The reason for dividing the samples is the upper limit for counting [5.6.2.2 a)].

For incubation and counting, see 5.5.3.6.

### 5.5.3.4 Filtration control B – validation of the filtration procedure

To validate the filtration procedure proceeds as follows:

Take 0,1 ml of the validation suspension  $N_v$  [5.4.1.5 a)] (Attention: not  $N_{vB}$  as described in 5.5.2.4!) in duplicate (suspension for control B) and transfer each 0,1 ml sample into a separate membrane filtration apparatus (5.5.3.1).

Filter immediately. Filter through the rinsing liquid (5.2.2.6) the same way as in the test [5.5.3.2 b)]. If the rinsing liquid is not water, complete the procedure by filtering 50 ml of water (5.2.2.2). Then transfer each of the membranes to the surface of separate MEA plates (5.2.2.3).

In the case of *Aspergillus brasiliensis* divide the sample in two, three or four portions of approximately equal size and transfer each portion into a separate membrane filtration apparatus (5.5.3.1), i.e. for duplicate four, six or eight membranes shall be inoculated.

NOTE The reason for dividing the samples is the upper limit for counting [5.6.2.2 a)].

For incubation and counting, see 5.5.3.6.

### 5.5.3.5 Method validation C – validation of the membrane filtration method or counting of the fungi on the membranes which have previously been in contact with the mixture of product and interfering substance

For validation of the membrane filtration method or counting of the fungi on the membranes which have previously been in contact with the mixture of product and interfering substance, the procedure is as follows:

- a) See 5.5.2.5 a);
- b) At the end of  $t$ , take 0,1 ml of the validation mixture  $C$  in duplicate and transfer each 0,1 ml sample into a separate membrane filtration apparatus (5.5.3.1). Filter immediately. Filter through the rinsing liquid (5.2.2.6) the same way as in the test [5.5.3.2 b)], then cover the membranes with 50 ml of the rinsing liquid (5.2.2.6) and add 0,1 ml of the validation suspension  $N_V$  [(5.4.1.5 a)]. Filter immediately again and additionally with 50 ml of water (5.2.2.2), then transfer each of the membranes to the surface of separate MEA plates (5.2.2.3).

In the case of *Aspergillus brasiliensis* divide the sample in two, three or four portions of approximately equal size and transfer each portion into a separate membrane filtration apparatus (5.5.3.1), i.e. for duplicate four, six or eight membranes shall be inoculated.

NOTE The reason for dividing the samples is the upper limit for counting [5.6.2.2 a)].

For incubation and counting, see 5.5.3.6.

### 5.5.3.6 Incubation and counting of test mixture and the validation mixtures

For incubation and counting of the test mixture and the control and validation mixtures, the procedure is as follows:

- a) Incubate (5.3.2.3) the plates for 42 h to 48 h. Discard any plates which are not countable for any reason. Count the colonies on the membranes.

Only for *Aspergillus brasiliensis*: Incubate the plates for a further 20 h to 24 h and – if the number of colonies has increased – for an additional third period of 20 h to 24 h. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. If the number has increased use only the higher number for further evaluation.

- b) Note for each plate the exact number of colonies but record “>55” (for moulds) or “>165” (for yeasts) for any counts higher than 55 and 165 respectively and determine the  $V_C$ -values according to 5.6.2.2.
- c) Calculate the numbers of cfu/ml in the test mixture  $N_a$  and in the validation mixtures  $A$ ,  $B$  and  $C$  using the method given in 5.6.2.4 and 5.6.2.6. Verify according to 5.7.

## 5.5.4 Modified method for ready-to-use products

### 5.5.4.1 General

Ready-to-use products may be tested according to the following modified test procedure, if the product under test does not pass the procedure in 5.5.2 or 5.5.3. The test suspension  $N$  and the validation suspension  $N_V$  have to be prepared in tenfold higher numbers, as described in 5.4.1.4 and 5.4.1.5.

The interfering substance has to be prepared in fivefold higher concentrations as described in 5.2.2.8.4.

NOTE The concentration of the product in the modified method is 97 % only.

### 5.5.4.2 Modified dilution-neutralization method

In the following only the modifications to 5.5.2 are described. See also Figure C.5 and Figure C.6.

#### 5.5.4.2.1 Test $N_a$

Pipette 0,2 ml of the 5 fold concentrated interfering substance (5.2.2.8.4) into a tube. Add 0,1 ml of the 10 fold concentrated test suspension (5.4.1.4). Start the stopwatch (5.3.2.5) immediately, mix [5.3.2.6 a)] and place the tube in a water bath controlled at the chosen test temperature [5.5.1.1 a)] for 2 min  $\pm$  10 s.



At the end of this time, add 9,7 ml of the undiluted product test solution (5.4.2). Restart the stopwatch at the beginning of the addition. Mix [5.3.2.6 a)] and place in a water bath controlled at  $\theta$  for the chosen contact time  $t$  [5.5.1.1 b)]. Just before the end of  $t$ , mix [5.3.2.6 a)] again. Follow the instructions in 5.5.2.2 c) and d), eventually f).

#### **5.5.4.2.2 Experimental conditions control A – validation of the selected experimental conditions and/or verification of the absence of any lethal effect in the test conditions**

Pipette 0,2 ml of the interfering substance used in the test (5.5.4.2.1) into a tube. Add 0,1 ml of the 10 fold concentrated validation suspension [5.4.1.5 a)] as described for this modified method. Start the stopwatch immediately, mix [5.3.2.6 a)] and place the tube in a water bath controlled at  $\theta$  for 2 min  $\pm$  10 s.

At the end of this time, add 9,7 ml of water (5.2.2.2). Restart the stopwatch at the beginning of the addition. Follow the instructions in 5.5.2.3 b) (last two sentences) and c).

#### **5.5.4.2.3 Neutralizer control B – verification of the absence of toxicity of the neutralizer**

Follow the procedure as described in 5.5.2.4.

#### **5.5.4.2.4 Method validation C – dilution-neutralization validation**

- a) Pipette 0,2 ml of the interfering substance used in the test (5.5.4.2.1) into a tube. Add 0,1 ml of the diluent (5.2.2.4) and then, starting a stopwatch, add 9,7 ml of the product test solution. Mix [5.3.2.6 a)] and place the tube in a water bath controlled at  $\theta$  for  $t$ . Just before the end of  $t$ , mix [5.3.2.6 a)] again.
- b) At the end of  $t$  transfer 1,1 ml of the mixture into a tube containing 8,8 ml of neutralizer (used in 5.5.4.2.1). Restart the stopwatch at the beginning of the addition. Mix [5.3.2.6 a)] and place the tube in a water bath controlled at  $(20 \pm 1) ^\circ\text{C}$  for 5 min  $\pm$  10 s (for handrub and handwash products only 10 s  $\pm$  1 s). Add 0,1 ml of the 10 fold concentrated validation suspension [5.4.1.5 a)] as described for this modified method. Start a stopwatch at the beginning of the addition and mix [5.3.2.6 a)]. Place the tube in a water bath controlled at  $(20 \pm 1) ^\circ\text{C}$  for  $(30 \pm 1)$  min. Just before the end of this time, mix [5.3.2.6 a)] again. At the end of this time, take a sample of 1,0 ml of the mixture "C" in duplicate and inoculate using the pour plate or the spread plate technique [5.5.2.2 c)].

For incubation and counting, see 5.5.2.6.

#### **5.5.4.3 Modified membrane filtration method**

In the following only the modifications to 5.5.3 are described, see also Figure C.7 and Figure C.8.

##### **5.5.4.3.1 Test $N_a$**

See 5.5.4.2.1, but follow after the mixing at the end of  $t$  5.5.3.2 b), c) and e).

##### **5.5.4.3.2 Experimental conditions control A – validation of the selected experimental conditions and/or verification of the absence of any lethal effect in the test conditions**

See 5.5.4.2.2 but follow only till the end of 5.5.2.3 a). Follow then 5.5.3.3 b).

##### **5.5.4.3.3 Filtration control "B" – validation of the filtration procedure**

Take 0,01 ml of the validation suspension  $N_v$  [5.4.1.5 a)] (Attention: not  $N_{VB}$  as described in 5.5.2.4!) in duplicate (suspension for control B) and transfer each 0,01 ml sample into a separate membrane filtration apparatus (5.5.3.1). Follow 5.5.3.4.

#### 5.5.4.3.4 Method validation C – validation of the membrane filtration method or counting of the fungi on the membranes which have previously been in contact with the mixture of product and interfering substance

- a) Follow 5.5.4.2.4 a).
- b) At the end of  $t$ , mix again [5.3.2.6 a)] and take 0,01 ml of the validation mixture  $C$  in duplicate and transfer each 0,01 ml sample into a separate membrane filtration apparatus (5.5.3.1). Filter immediately. Filter through the rinsing liquid (5.2.2.6) the same way as in the test [5.5.3.2 b)], then cover the membranes with 50 ml of the rinsing liquid (5.2.2.6) and add 0,01 ml of the 10 fold concentrated validation suspension [5.4.1.4 b) and 5.4.1.5]. Filter immediately again and additionally with 50 ml of water (5.2.2.2), then transfer each of the membranes to the surface of separate MEA plates (5.2.2.3).

For incubation and counting, see 5.5.3.6.

## 5.6 Experimental data and calculation

### 5.6.1 Explanation of terms and abbreviations

#### 5.6.1.1 Overview of the different suspensions and test mixtures

$N$ ,  $N_V$  and  $N_{VB}$  represent the fungal suspensions,  $N_a$  represents the fungicidal test mixture,  $A$  (experimental conditions control),  $B$  (neutralizer or filtration control),  $C$  (method validation) represent the different control test mixtures.

$N_{VB}$ ,  $N_0$ ,  $N_{V0}$ ,  $N_a$  and  $A$ ,  $B$  and  $C$  represent the number of cells counted per ml in the different test mixtures in accordance with Table 2.

**Table 2 — Number of cells counted per ml in the different test mixtures**

	Number of cells per ml in the fungal suspensions	Number of cells per ml in the test mixtures at the beginning of the contact time (time 0)	Number of survivors per ml in the test mixtures at the end of the contact time $t$ ( $A$ ) or 5 min ( $B$ ) or 30 min ( $C$ )
Test	$N$ Test suspension	$N_0^a$ (= $N/10$ )	$N_a$ (before neutralization or filtration)
Controls	$N_V$ Validation suspension $N_{VB}$ Validation suspension for control $B$	$N_{V0}^a$ (= $N_V/10$ = $N_{VB}/1\ 000$ )	$A$ , $B$ , $C$
<sup>a</sup> In the case of the modified method (5.5.4) $N_0$ is $N/100$ and $N_{V0}$ is $N_V/100$ .			

#### 5.6.1.2 $V_C$ -values

All experimental data are reported as  $V_C$ -values:

- a) in the dilution-neutralization method (test and controls), a  $V_C$ -value is the number of cfu counted per 1,0 ml sample;
- b) in the membrane filtration method, a  $V_C$ -value is the number of cfu counted per 0,1 ml sample of test mixture  $N_a$ , (1,0  $\mu$ l in the case of hygienic handwash) of filtration control ( $B$ ) and method validation ( $C$ ) and per 1,0 ml sample in the experimental condition control  $A$  (for the modified method 5.5.4.3 it is 0,1 ml of  $N_a$ , 1,0 ml of  $A$  and 0,01 ml of  $B$  and  $C$ ).

## 5.6.2 Calculation

### 5.6.2.1 General

The first step in the calculation is the determination of the  $V_C$ -values, the second the calculation of  $N$ ,  $N_0$ ,  $N_a$ ,  $N_V$ ,  $N_{V0}$ ,  $N_{VB}$ ,  $A$ ,  $B$  and  $C$ . The third step is the calculation of the reduction  $R$  (5.8).

### 5.6.2.2 Determination of $V_C$ -values

The  $V_C$ -values are determined as follows.

- a) The usual limits for counting fungi on agar plates are between 15 and 150 colonies for moulds and between 15 and 300 colonies for yeasts. In this European Standard, a deviation of 10 % is accepted, so the limits are 14 and 165 for moulds and 14 and 330 for yeasts. On membranes the usual upper limits are different: 50 for moulds and 150 for yeasts, therefore with the 10 % deviation, the limits are 55 for moulds and 165 for yeasts.

NOTE The lower limit (14) is based on the fact that the variability is increasing the smaller the number counted in the sample (1 ml or 0,1 ml) is, and therefore subsequent calculations may lead to wrong results. The lower limit refers only to the sample (and not necessarily to the counting on one plate), e.g. three plates per 1 ml sample with 3 cfu, 8 cfu and 5 colony-forming units give a  $V_C$  value of 16. The upper limits (55, 165 and 330) reflect the imprecision of counting confluent colonies and growth inhibition due to nutriment depletion. They refer only to the counting on one plate, and not necessarily to the sample.

- b) For counting the test suspension  $N$  (5.4.1.6), the validation suspensions  $N_V$  and  $N_{VB}$  (5.4.1.6) and for all countings of the dilution-neutralization method (5.5.2.6), determine and record the  $V_C$ -values according to the number of plates used per 1 ml (or other volumes for membrane filtration and/or handwash products) sample (5.6.1.2).

If more than one plate per sample has been used to determine the  $V_C$ -value, the countings per plate should be noted.

If the count on one plate is higher than 165 / 330, report the number as ">165" / ">330". If more than one plate per sample has been used and at least one of them shows a number higher than 165 (or 330), report this  $V_C$ -values as "more than sum of the counts," e. g. for "> 330, 310, 302", report "> 942".

If a  $V_C$ -value is lower than 14, report the number (but substitute by "< 14" for further calculations in the case of  $N_a$ ).

For the membrane-filtration method (5.5.3), the countings on the membranes are the  $V_C$ -values (5.6.1.2). Report the  $V_C$ -values below the lower limit (14) or above the upper limit (55/165) as described above.

- c) Only  $V_C$ -values within the counting limits are taken into account for further calculation, except in the case of  $N_a$  (5.6.2.4).

### 5.6.2.3 Calculation of $N$ and $N_0$

$N$  is the number of cells per ml in the test suspension (5.4.1.4; 5.6.1.1).

Since two dilutions of the test suspension (5.4.1.4 in connection with 5.4.1.6) are evaluated, calculate the number of cfu/ml as the weighted mean count using the following formula:

$$N = \frac{c}{(n_1 + 0,1 n_2) 10^{-5}} \quad (1)$$

where

- $c$  is the sum of  $V_C$ -values taken into account;
- $n_1$  is the number of  $V_C$ -values taken into account in the lower dilution, i.e.  $10^{-5}$ ;
- $n_2$  is the number of  $V_C$ -values taken into account in the higher dilution, i.e.  $10^{-6}$ ;
- $10^{-5}$  is the dilution factor corresponding to the lower dilution.

NOTE 1 For the modified method (5.5.4), the lower dilution is  $10^{-6}$  and the higher dilution is  $10^{-7}$ .

NOTE 2 For the modified method (5.5.4),  $N$  is tenfold higher and, therefore, the dilutions to be evaluated are tenfold higher ( $10^{-6}$  instead of  $10^{-5}$ , and  $10^{-7}$  instead of  $10^{-6}$ ).

The formula given above should be changed accordingly.

Round off the results calculated to two significant figures. For this, if the last figure is below 5, the preceding figure is not modified; if the last figure is more than 5, the preceding figure is increased by one unit; if the last figure is equal to 5, round off the preceding figure to the next nearest even figure. Proceed stepwise until two significant figures are obtained. As a result, the number of cfu/ml is expressed by a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

EXAMPLE

$$N = \frac{168 + 213 + 20 + 25}{(2 + 0,1 \times 2) 10^{-5}} = \frac{426}{2,2 \times 10^{-5}} = 1,9363 \times 10^7 = 1,9 \times 10^7 \text{ (cfu / ml)}$$

$N_0$  is the number of cells per ml in the test mixture [5.5.2.2 a)] at the beginning of the contact time (time “zero” = 0). It is one-tenth of the weighted mean of  $N$  due to the tenfold dilution by the addition of the product and interfering substance. It is one hundredth in the case of the modified method (5.5.4) as only 0,1 ml of  $N$  are used in the test.

#### 5.6.2.4 Calculation of $N_a$

$N_a$  is the number of survivors per ml in the test mixture [5.5.2.2 a) or 5.5.3.2 a)] at the end of the contact time and before neutralization or membrane filtration. It is tenfold higher than the  $V_C$ -values due to the addition of neutralizer and water [5.5.2.2 b)] or the sample volume of 0,1 ml [5.5.3.2 b)] in the membrane filtration method.

- a) Calculate the mean for each dilution step  $N_a^0$ ,  $N_a^{-1}$  and for handwash products additionally  $N_a^{-2}$  using the following formula:

$$Na^0, Na^{-1}, Na^{-2} = 10 c / n \tag{2}$$

where

- $c$  is the sum of  $V_C$ -values taken into account;
- $n$  is the number of  $V_C$ -values taken into account.

If one or both of the duplicate  $V_C$ -values are either below the lower or above the upper limit, express the results as “less than” or “more than”.

**a1** duplicate  $V_C$ -values  $N_a^{-1}$ : 2, 16

EXAMPLE 1

$$N_a^{-1} = \frac{(<14 + 16) \times 10}{2} \times 10 = <150 \times 10^1 = <1500 = <1,5 \times 10^3$$

**a2** duplicate  $V_C$ -values  $N_a^{-2}$  (membrane filtration): > 165, > 165

EXAMPLE 2

$$N_a^{-2} = \frac{(>165 + >165) \times 100}{2} \times 10 = >1650 \times 10^2 = >165000 = >1,6 \times 10^5$$

**a3** duplicate  $V_C$ -values (two spread plates per 1,0 ml sample): > 660, 600

EXAMPLE 3

$$N_a^0 = \frac{(>660 + 600) \times 10}{2} = >6300 = >6,3 \times 10^3$$

b) For calculation of  $N_a$  use only  $N_a^0$ ,  $N_a^{-1}$ ,  $N_a^{-2}$  results, where one or both  $V_C$ -values are within the counting limits. Exceptions and rules for special cases:

NOTE Although  $10^{-2}$  dilutions are only prepared when handwash products are tested, the following examples include this dilution step.

**b1** If all subsequent dilutions of  $N_a$  show mean values of „more than”, take only the highest dilution ( $10^{-1}$ , with handwash products  $10^{-2}$ ) as result for  $N_a$ .

EXAMPLE 4

	$V_{C1}$	$V_{C2}$	mean x 10	
$N_a^0$	> 660	> 660	> 6 600	$N_a = > 6\,600 \times 10^2 = > 6,6 \times 10^5$
$N_a^{-1}$	> 660	> 660	> 6 600	
$N_a^{-2}$	> 660	> 660	> 6 600	

**b2** If all subsequent dilutions of  $N_a$  show mean values of „less than”, take only the lowest dilution ( $10^0$ ) as result for  $N_a$ .

EXAMPLE 5

	$V_{C1}$	$V_{C2}$	mean x 10	
$N_a^0$	< 14	18	< 160	$N_a = < 160 \times 10^0 = < 1,6 \times 10^2$
$N_a^{-1}$	< 14	< 14	< 140	
$N_a^{-2}$	< 14	< 14	< 140	

**b3** If one or both duplicate  $V_C$ -values in only one dilution of  $N_a$  are within the counting limits, use this result as  $N_a$ .

EXAMPLE 6 (membrane filtration, *Aspergillus brasiliensis*):

	$V_{C1}$	$V_{C2}$	mean x 10	
$N_a^0$	> 55	> 55	> 550	$N_a = 730 \times 10^1 = 7,3 \times 10^3$
$N_a^{-1}$	46	27	730	
$N_a^{-2}$	< 14	< 14	< 140	

**b4** If the higher dilution in two subsequent dilutions of  $N_a$  shows a mean value of „less than” and the lower dilution shows a mean value of „more than”, take only the lower dilution as  $N_a$ -value.

EXAMPLE 7

	$V_{C1}$	$V_{C2}$	mean x 10	
$N_a^0$	> 660	> 660	> 6 600	$N_a = > 6 600 \times 10^0 = > 6,6 \times 10^3$
$N_a^{-1}$	< 14	29	< 215	
$N_a^{-2}$	< 14	< 14	< 140	

c) Use maximum 2 subsequent dilutions for calculating  $N_a$  as a weighted mean. Exceptions and rules for special cases:

**c1** If one or both duplicate  $V_C$ -values in three or more subsequent dilutions of  $N_a$  (including  $N_a^0$ ) are within the counting limits (e.g.  $N_a^{-2}$ : 17, 23;  $N_a^{-1}$ : 120, 135;  $N_a^0$ : 308, > 330) the whole test is invalid (5.7.1).

**c2** If two subsequent dilutions of  $N_a$  show duplicate  $V_C$  values within the counting limits calculate  $N_a$  as the weighted mean using Formula (3):

$$N_a = \frac{c \times 10}{2,2 \times 10^Z} \quad (3)$$

where

$c$  is the sum of  $V_C$ -values taken into account;

$Z$  is the dilution factor corresponding to the lower dilution, e.g.  $N_a^0$  is the lower dilution in comparison with  $N_a^{-1}$

**c3** If in two subsequent dilutions of  $N_a$  both  $V_C$  values of the higher dilution are within the counting limits and one  $V_C$  value of the lower dilution is „more than”, calculate  $N_a$  as the weighted mean, using Formula (3), see **c2**.

EXAMPLE 8

	$V_{C1}$	$V_{C2}$	mean x 10	
$N_a^0$	> 538	320	> 4 290	$N_a = \frac{(> 538 + 320 + 46 + 57) \times 10}{2,2 \times 10^0} = > 4 368,18 = > 4,4 \times 10^3$
$N_a^{-1}$	46	57	515	
$N_a^{-2}$	< 14	< 14	< 140	

**c4** If in two subsequent dilutions of  $N_a$  one of the higher dilution duplicate values shows „< 14”, take only the lower dilution as result for  $N_a$ .

EXAMPLE 9

	$Vc_1$	$Vc_2$	mean x 10	
$N_a^0$	> 660	> 660	> 6 600	$N_a = > 6\,145 \times 10^1 = > 6,1 \times 10^4$
$N_a^{-1}$	569	> 660	> 6 145	
$N_a^{-2}$	< 14	26	< 200	

**5.6.2.5 Calculation of  $N_V$ ,  $N_{V0}$  and  $N_{VB}$**

$N_V$  is the number of cells per ml in the validation suspension [5.4.1.5 a)]. It is tenfold higher than the counts in terms of  $V_C$ -values due to the dilution step of  $10^{-1}$  [5.4.1.5 b)].

$N_{V0}$  is the number of cells per ml in the mixtures *A*, *B* and *C* at the beginning of the contact time (time 0) (5.6.1.1). In the case of neutralizer control *B* – dilution-neutralization method (5.2.2.4) it is the number of cells per ml after 100 fold dilution.  $N_{V0}$  is one-tenth of the mean of the  $V_C$ -values of  $N_V$  [5.4.1.6 c)] taken into account, in case of  $N_{VB}$  it is one thousandth.

Calculate  $N_V$ ,  $N_{VB}$  and  $N_{V0}$  using the following formulae:

$$N_V = 10 c / n \quad (4)$$

$$N_{VB} = 1\,000 c / n \quad (5)$$

$$N_{V0} = c / n \quad (6)$$

where

*c* is the sum of  $V_C$ -values taken into account;

*n* is the number of  $V_C$ -values taken into account.

**5.6.2.6 Calculation of *A*, *B* and *C***

*A*, *B* and *C* are the numbers of survivors in the experimental conditions control *A* (5.5.2.3 or 5.5.3.3), neutralizer control *B* (5.5.2.4) or filtration control (5.5.3.4) and method validation *C* (5.5.2.5 or 5.5.3.5) at the end of the contact time *t* (*A*) or the defined times 5 min (*B*) and 30 min (*C*). They correspond to the mean of the  $V_C$ -values of the mixtures *A*, *B* and *C* taken into account.

Calculate *A*, *B* and *C* using the following formula:

$$A, B, C = c / n \quad (7)$$

where

*c* is the sum of  $V_C$ -values taken into account;

*n* is the number of  $V_C$ -values taken into account.

## 5.7 Verification of methodology

### 5.7.1 General

A test is valid if:

- all results meet the criteria of 5.7.3; and
- the requirements of 5.8.2 are fulfilled; and
- it is not invalidated by a result described under 5.6.2.4 c) first special case (c1).

### 5.7.2 Control of weighted mean counts

For results calculated by weighted mean of two subsequent dilutions (e.g.  $N$ ), the quotient of the means of the two results shall be not higher than 15 and not lower than 5. Results below the lower limit are taken as the lower limit number (14). Results above the respective upper limit [5.6.2.2 b)] are taken as the upper limit number.

EXAMPLE For  $N$ :  $10^{-5}$  dilution: (168 + 215) cfu/ml,  $10^{-6}$  dilution: (20 + < 14) cfu/ml; (168 + 215) / (20 + 14) = 383/34 = 11,26 = between 5 and 15.

When the counts obtained on plates are out of limits fixed for the determination of  $V_C$ -values [5.6.2.2 b)], check for the weighted mean as mentioned above but use only the  $V_C$ -values within the counting limits for the calculation of  $N$ .

### 5.7.3 Basic limits

For each test organism check that:

- |    |   |   |  |
|----|---|---|--|
| a) | $N$   | is between $1,5 \times 10^7$ and $5,0 \times 10^7$                          | $(7,17 \leq \lg N \leq 7,70)$                    |
|    | $N$ (modified method 5.5.4)   | is between $1,5 \times 10^8$ and $5,0 \times 10^8$                          | $(8,17 \leq \lg N \leq 8,70)$                    |
|    | $N_0$   | is between $1,5 \times 10^6$ and $5,0 \times 10^6$                          | $(6,17 \leq \lg N_0 \leq 6,70)$                  |
| b) | $N_{V0}$  | is between 30 and 160   | $(3,0 \times 10^1 \text{ and } 1,6 \times 10^2)$ |
|    | $N_V$   | is between $3,0 \times 10^2$ and $1,6 \times 10^3$                          |  |
|    | $N_V$ (modified method 5.5.4)   | is between $3,0 \times 10^3$ and $1,6 \times 10^4$                          |  |
|    | $N_{VB}$ (5.5.2.4)  | is between $3,0 \times 10^4$ and $1,6 \times 10^5$                          |  |
| c) | $A, B, C$   | are equal to or greater than $0,5 \times N_{V0}$                            |  |
|    | $B$ (dil.-neutr.)   | is equal to or greater than $0,0005 \times N_{VB}$ (half of one thousandth) |  |
| d) | Control of weighted mean counts (5.7.2): quotient is not lower than 5 and not higher than 15. |   |  |

NOTE In case of using the modified method for ready-to-use products (5.5.4) the limits for  $N$  and  $N_V$  are 10 fold higher, but  $N_0$ ,  $N_{V0}$  and  $N_{VB}$  are unchanged.

### 5.7.4 Additional limits for *Aspergillus brasiliensis*

Check the presence of a high concentration of spiny conidiospores in the *Aspergillus brasiliensis* conidiospore suspension of at least 75 % [5.4.1.4.2 b) 1)].

## 5.8 Expression of results and precision

### 5.8.1 Reduction

The reduction ( $R = N_0 / N_a$ ) is expressed in logarithm.



For each test organism record the number of cfu/ml in the test suspension  $N$  (5.6.2.3) and in the test  $N_a$  (5.6.2.4). Calculate  $N_0$  (5.6.2.3).

For each product concentration and each experimental condition, calculate and record the decimal log reduction (lg) separately using the formula:

$$\lg R = \lg N_0 - \lg N_a \quad (8)$$

For the controls and validation of the dilution-neutralization method or membrane filtration method, record  $N_{V0}$  (5.6.2.5), the results of  $A$ ,  $B$  and  $C$  (5.6.2.6) and their comparison with  $N_{V0}$  [(5.7.3 c)].

### **5.8.2 Control of active and non-active product test solution (5.4.2)**

At least one concentration per test [5.5.2.2 a) - c) or 5.5.3.2 a) - c) or 5.5.4] shall demonstrate a 4 lg or more reduction (for hygienic handwash products 2 lg or more) and at least one concentration shall demonstrate a lg reduction of less than 4 (for hygienic handwash products less than 2).

### **5.8.3 Limiting test organism and fungicidal and yeasticidal concentration**

#### **5.8.3.1 Fungicidal concentration**

For each test organism, record the lowest concentration of the product which passes the test. Record as the limiting test organism the test organism requiring the highest of these concentrations (it is the least susceptible to the product in the chosen experimental conditions).

The lowest concentration of the product active on the limiting test organism is the fungicidal concentration determined according to this European Standard.

#### **5.8.3.2 Yeasticidal concentration**

Record the lowest concentration of the product which passes the test with *Candida albicans* ( $\lg R \geq 4$ , but  $\lg R \geq 2$  for handwash products). The lowest concentration of the product active on *Candida albicans* is the yeasticidal concentration determined according to this European Standard.

### **5.8.4 Precision, repetitions**

Taking into account the precision of the methodology determined by a statistical analysis based on data provided by a collaborative study, repetition of the test [for a precision of  $\pm 1$  lg in reduction: 4 repetitions in the best case, 5 repetitions in the worst case (Annex E)] is recommended. The number of repetitions shall be decided according to the required level of precision, taking into account the intended use of the test results.

Repetition means the complete test procedure with separately prepared test - and validation suspensions. The repetitions of the test may be restricted to the limiting test organism. The mean of the results of the repetitions - not each single result - shall demonstrate at least a 4 lg reduction (in the case of handwash products 2 lg) and shall also be calculated and recorded.

## **5.9 Interpretation of results – conclusion**

### **5.9.1 General**

According to the chosen experimental conditions the fungicidal concentrations determined according to this standard may differ (Clause 4). A product can only pass the test if the requirements of 5.8.2 are fulfilled.

### 5.9.2 Fungicidal activity for instrument disinfection products

The product shall be deemed to have passed the EN 13624 Standard if it demonstrates in a valid test for instrument disinfection products at least a 4 lg reduction within 60 min at the lowest temperature recommended by the manufacturer, min. 20 °C and max. 70 °C, with the chosen interfering substance (clean or dirty conditions) under the conditions defined by this standard when the test organisms are *Aspergillus brasiliensis* and *Candida albicans*.

### 5.9.3 Fungicidal activity for surface disinfection products

The product shall be deemed to have passed the EN 13624 Standard if it demonstrates in a valid test for surface disinfection products at least a 4 lg reduction within max. 5 min (or between 6 min and 60 min for products used on surfaces which do not require an action within 5 min or shorter) at min. 4 °C and max. 30 °C with the chosen interfering substance (clean or dirty conditions) under the conditions defined by this standard when the test organisms are *Aspergillus brasiliensis* and *Candida albicans*.

### 5.9.4 Yeasticidal activity for handrub and handwash products

The product shall be deemed to have passed the EN 13624 Standard (yeasticidal activity) if it demonstrates in a valid test for handrub and handwash products at 20 °C under the conditions defined by this standard when the test organism is *Candida albicans* at least a

- 4 lg reduction within 1 min under clean conditions (hygienic handrub);
- 4 lg reduction within 5 min under clean conditions (surgical handrub);
- 2 lg reduction within 1 min under dirty conditions (hygienic handwash);
- 4 lg reduction within 5 min under dirty conditions (surgical handwash).

### 5.9.5 Yeasticidal activity for instrument disinfection products

The product shall be deemed to have passed the EN 13624 Standard (yeasticidal activity) if it demonstrates in a valid test for instrument disinfection products at least a 4 lg reduction within 60 min at the lowest temperature recommended by the manufacturer, min. 20 °C and max. 60 °C, with the chosen interfering substance (clean or dirty conditions) under the conditions defined by this standard when the test organism is *Candida albicans*.

### 5.9.6 Yeasticidal activity for surface disinfection products

The product shall be deemed to have passed the EN 13624 Standard (yeasticidal activity) if it demonstrates in a valid test for surface disinfection products at least a 4 lg reduction within max. 5 min (or between 6 min and 60 min for products used on surfaces which do not require an action within 5 min or shorter) at min. 4 °C and max. 30 °C with the chosen interfering substance (clean or dirty conditions) under the conditions defined by this standard when the test organism is *Candida albicans*.

### 5.9.7 Qualification for certain fields of application

See EN 14885.

## 5.10 Test report

The test report shall refer to this European Standard (EN 13624).

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

- a) identification of the testing laboratory;

- b) identification of the client;
- c) identification of the sample:
  - 1) name of the product;
  - 2) batch number and — if available — expiry date;
  - 3) manufacturer – if not known: supplier;
  - 4) date of delivery;
  - 5) storage conditions;
  - 6) product diluent recommended by the manufacturer for use;
  - 7) active substance(s) and its/their concentration(s) (optional);
  - 8) appearance of the product;
- d) test method and its validation:
  - 1) if the dilution-neutralization method is used, full details of the test for validation of the neutralizer shall be given;
  - 2) if the membrane filtration method is used, full details of the procedure which was carried out in order to justify the use of the membrane filtration method shall be given;
- e) experimental conditions:
  - 1) date(s) of test (period of analysis);
  - 2) diluent used for product test solution (hard water or distilled water);
  - 3) product test concentrations (= *desired* test concentrations according to 5.4.2);
  - 4) appearance of the product dilutions;
  - 5) contact time(s);
  - 6) test temperature(s);
  - 7) interfering substance;
  - 8) stability and appearance of the mixture during the procedure (note the formation of any precipitate or flocculant);
  - 9) temperature of incubation;
  - 10) neutralizer or rinsing liquid;
  - 11) identification of the fungal strains used;
- f) test results:
  - 1) controls and validation;
  - 2) evaluation of fungicidal or yeasticidal activity;
  - 3) number of repetitions per test organism;

- g) special remarks;
- h) conclusion;
- i) locality, date and identified signature.

NOTE An example of a typical test report is given in Annex D.

**Annex A**  
(informative)

**Referenced strains in national collections**

<i>Candida albicans</i>	ATCC	10231
	CIP	4872
	DSM	1386
	CBS	6431
	NCTC	3179
<i>Aspergillus brasiliensis</i>	ATCC	16404
	DSM	1988
	CBS	733.88
	CIP	1431.83
	NCTC	2275
	IMI	149007

## Annex B (informative)

### Neutralizers and rinsing liquids

Examples of neutralizers of the residual antimicrobial activity of chemical disinfectants and antiseptics and of rinsing liquids

**Important! Neutralizers of the residual antimicrobial activity of chemical disinfectants and antiseptics and rinsing liquids shall be validated according to the prescriptions of the standard.**

**Table B.1 — Neutralizers and rinsing liquids (1 of 2)**

Antimicrobial agent	Chemical compounds able to neutralize residual antimicrobial activity	Examples of suitable neutralizers and of rinsing liquids (for membrane filtration methods) <sup>a</sup>
Quaternary ammonium compounds and fatty amines Amphoteric compounds	Lecithin, Saponin, Polysorbate 80, Sodium dodecyl sulphate, Ethylene oxide condensate of fatty alcohol (non-ionic surfactants) <sup>b</sup>	<ul style="list-style-type: none"> <li>— Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l.</li> <li>— Polysorbate 80, 30 g/l + sodium dodecyl sulphate, 4 g/l + lecithin, 3 g/l.</li> <li>— Ethylene oxide condensate of fatty alcohol, 3 g/l + lecithin, 20 g/l + polysorbate 80, 5 g/l.</li> <li>— Rinsing liquid : tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.</li> </ul>
Biguanides and similar compounds	Lecithin <sup>c</sup> , Saponin, Polysorbate 80	<ul style="list-style-type: none"> <li>— Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l.</li> </ul> <p><i>Rinsing liquid : tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.</i></p>
Oxidizing compounds (Chlorine, iodine, hydrogen peroxide, peracetic acid, hypochlorites, etc...)	Sodium thiosulphate <sup>d</sup> Catalase [for hydrogen peroxide or products releasing hydrogen peroxide]	<ul style="list-style-type: none"> <li>— Sodium thiosulphate, 3 g/l to 20 g/l + polysorbate 80, 30 g/l + lecithin, 3 g/l.</li> <li>— Polysorbate 80, 50 g/l + catalase 0,25 g/l + lecithin 10 g/l.</li> </ul> <p><i>Rinsing liquid : sodium thiosulphate, 3 g/l.</i></p>
Aldehydes	L – histidine Glycine	<ul style="list-style-type: none"> <li>— Polysorbate 80, 30 g/l + lecithin, 3 g/l + L-histidine, 1 g/l (or + glycine, 1 g/l).</li> <li>— Polysorbate 80, 30 g/l + saponin, 30 g/l + L-histidine, 1 g/l (or + glycine, 1 g/l).</li> </ul> <p><i>Rinsing liquid : polysorbate 80, 5 g/l + L-histidine, 0,5 g/l (or + glycine, 1 g/l).</i></p>
Phenolic and related compounds: orthophenylphenol, phenoxyethanol, triclosan, phenylethanol, etc. Anilides	Lecithin Polysorbate 80 Ethylene oxide condensate of fatty alcohol <sup>b</sup>	<ul style="list-style-type: none"> <li>— Polysorbate 80, 30 g/l + lecithin, 3 g/l.</li> <li>— Ethylene oxide condensate of fatty alcohol, 7 g/l + lecithin, 20 g/l, + polysorbate 80, 4 g/l.</li> </ul> <p><i>Rinsing liquid : tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.</i></p>

Table B.1 (2 of 2)

Antimicrobial agent	Chemical compounds able to neutralize residual antimicrobial activity	Examples of suitable neutralizers and of rinsing liquids (for membrane filtration methods) <sup>a</sup>
Alcohols	Lecithin, Saponin, Polysorbate 80 <sup>e</sup>	<p>— Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l.</p> <p><i>Rinsing liquid : tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.</i></p>
<p><sup>a</sup> According to the pH of the tested product, the pH of the neutralizer or the rinsing liquid may be adjusted at a suitable value or prepared in phosphate buffer [ex: phosphate buffer 0,25 mol/l: potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) 34 g; distilled water (500 ml); adjusted to pH 7,2 ± 0,2 with sodium hydroxide (NaOH) 1 mol/l; distilled water up to 1 000 ml].</p> <p><sup>b</sup> The carbon chain-length varies from C<sub>12</sub> to C<sub>18</sub> carbon atoms.</p> <p><sup>c</sup> Egg and soya; egg is preferable.</p> <p><sup>d</sup> The toxic effect of sodium thiosulphate differs from one test organism to another.</p> <p><sup>e</sup> For the neutralization of short chain alcohols (less than C<sub>5</sub>), simple dilution may be appropriate. Care should be taken if the alcohol-based products contain additional antimicrobial agents.</p>		

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

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

## Annex C (informative)

### Graphical representation of test procedures

#### C.1 Dilution-neutralization method

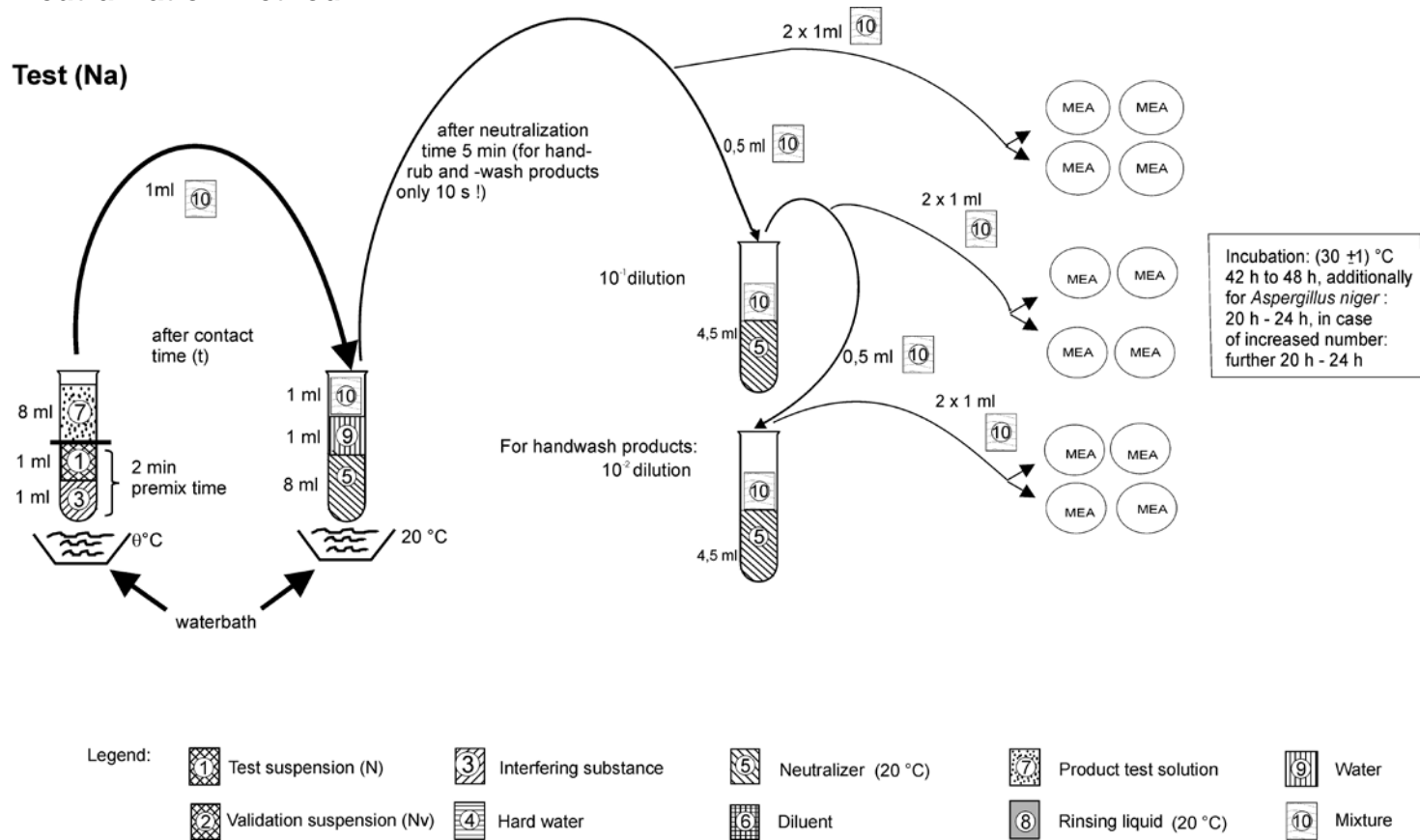


Figure C.1 — Dilution-neutralization method – Test procedure



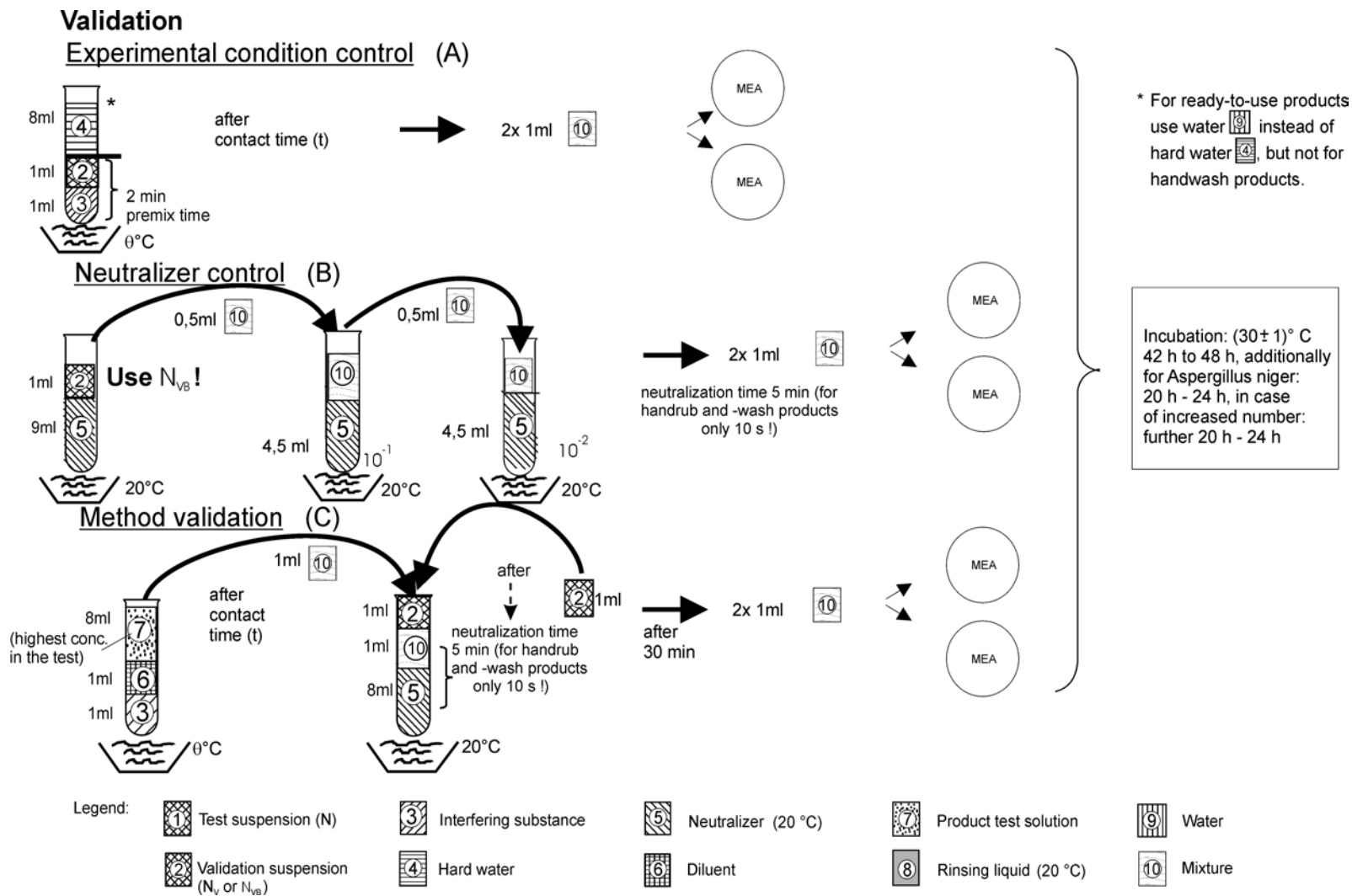


Figure C.2 — Dilution-neutralization method - Validation

## C.2 Membrane filtration method

### Test (Na)

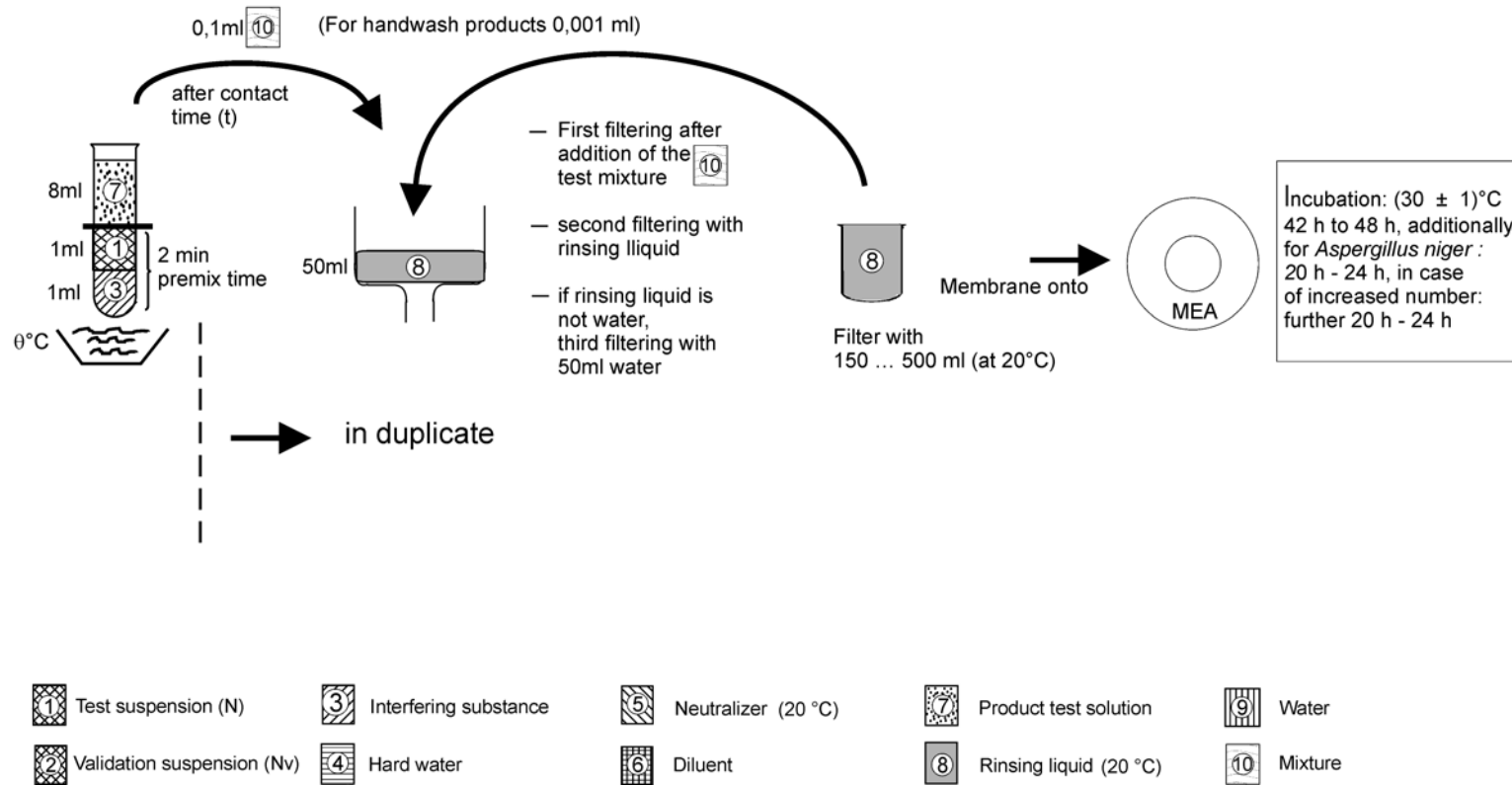


Figure C.3 — Membrane filtration method - Test procedure

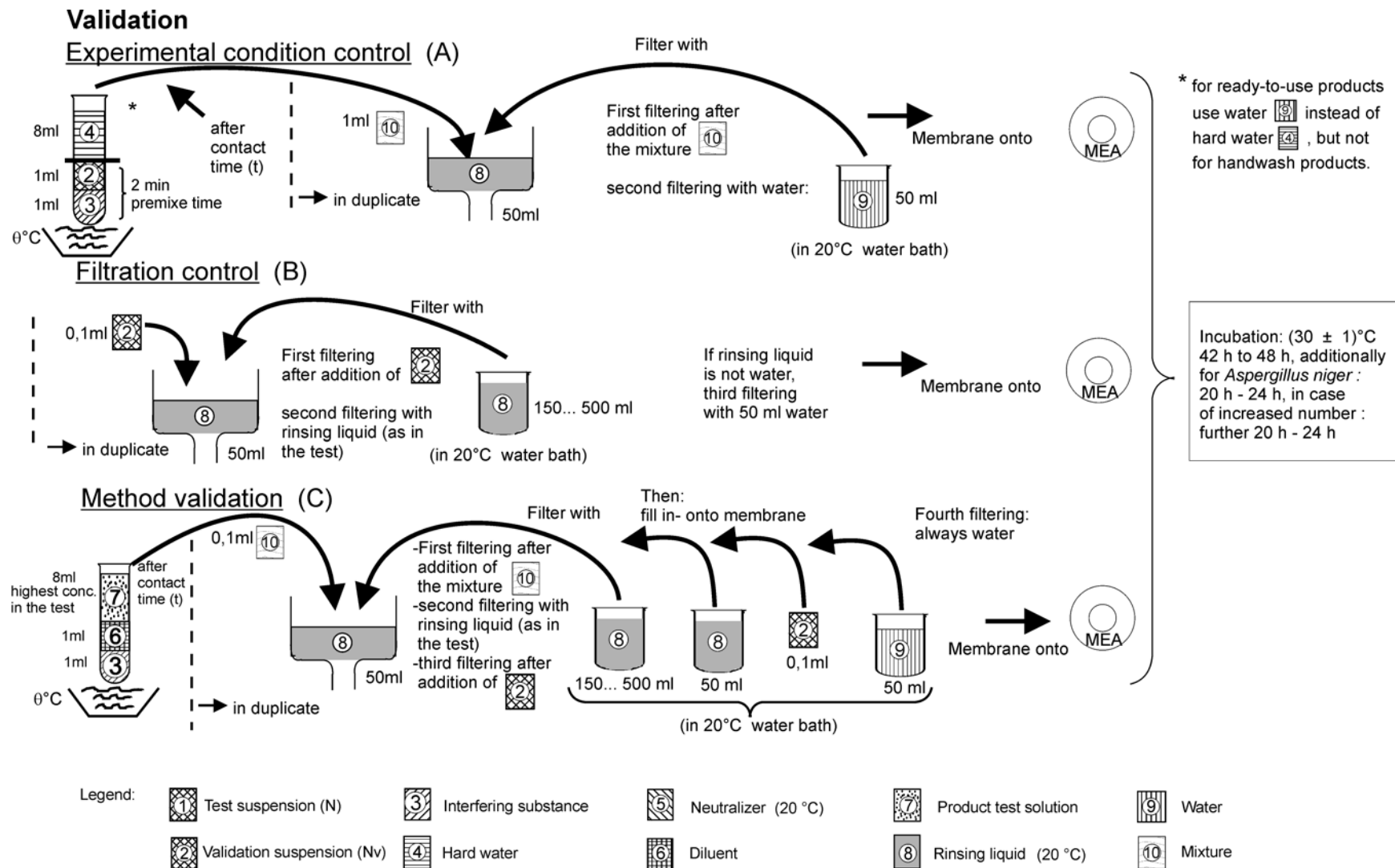


Figure C.4 — Membrane filtration method – Validation

### C.3 Dilution-neutralization method (modified method for ready-to-use products)

#### Test (Na)

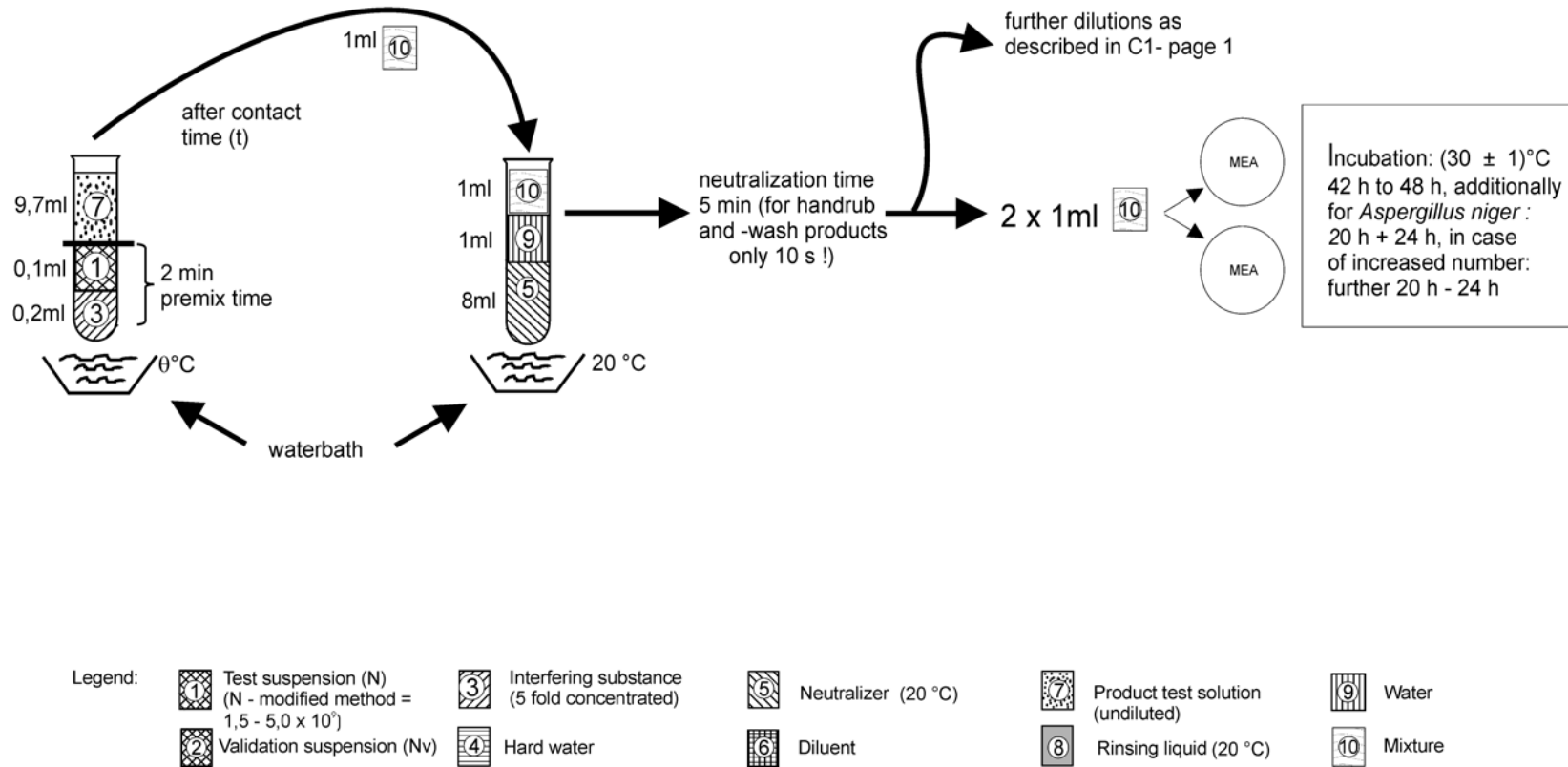
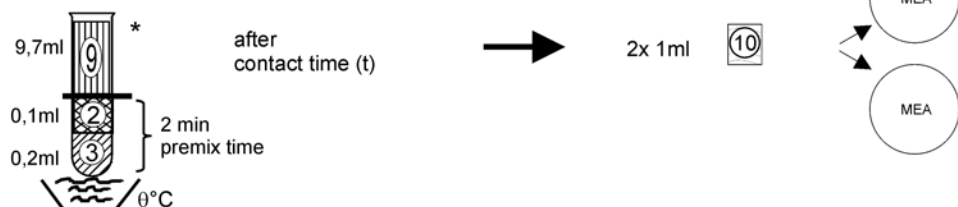


Figure C.5 — Dilution-neutralization method (modified method for ready-to-use products) - Test procedure

**Validation**

Experimental condition control (A)



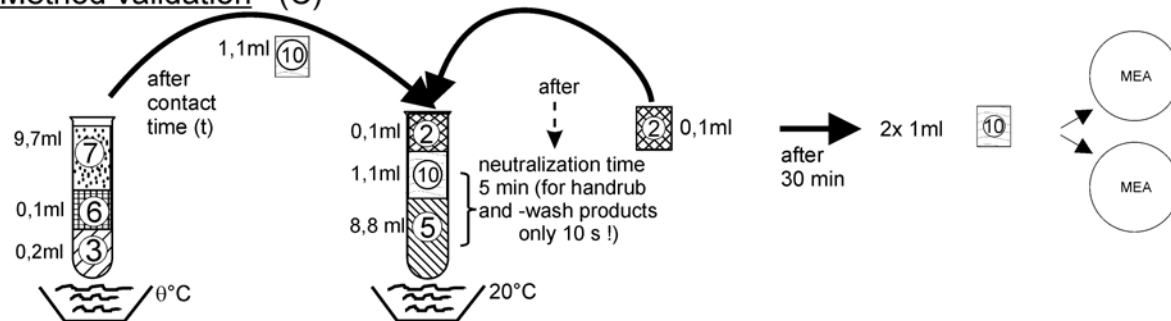
Neutralizer control (B)

**See figure C.2**

\* for handwash products  
use hard water (4)  
instead of water (9)

Incubation:  $(30 \pm 1)^{\circ}\text{C}$   
42 h to 48 h, additionally  
for *Aspergillus niger* :  
20 h - 24 h, in case  
of increased number:  
further 20 h - 24 h

Method validation (C)



- Legend:
- |   |   |                     |                                   |         |
|---|---|---------------------|-----------------------------------|---------|
| Test suspension (N)   | Interfering substance (5 fold concentrated) | Neutralizer (20 °C) | Product test solution (undiluted) | Water   |
| Validation suspension (Nv - modified method = $3,0 \times 10^3 - 1,6 \times 10^4$ ) | Hard water                                  | Diluent             | Rinsing liquid (20 °C)            | Mixture |

**Figure C.6 — Dilution-neutralization method (modified method for ready-to-use products) – Validation**

### C.4 Membrane filtration method (modified method for ready-to-use products)

#### Test (Na)

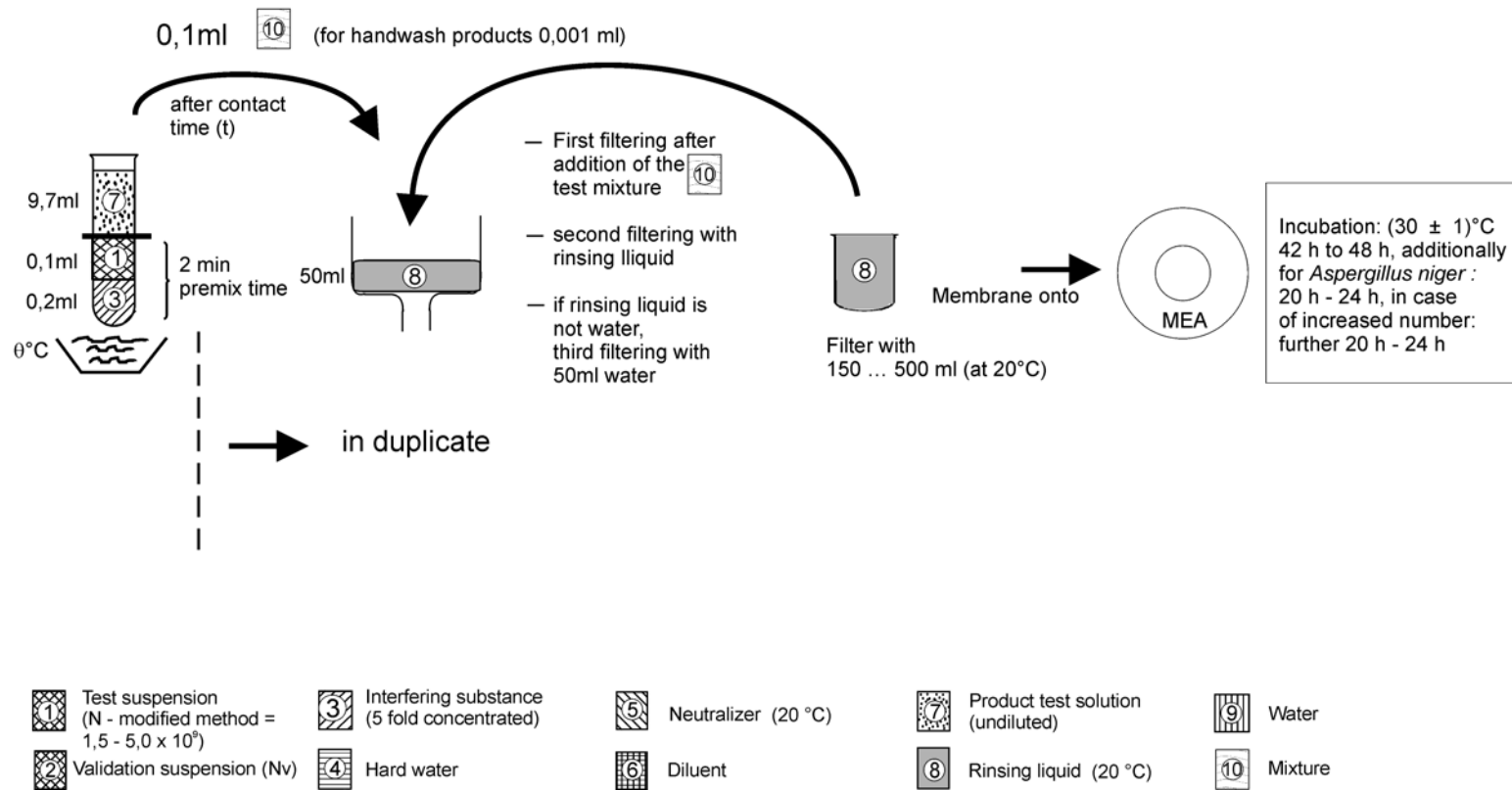


Figure C.7 — Membrane filtration method (modified method for ready-to-use products) - Test procedure

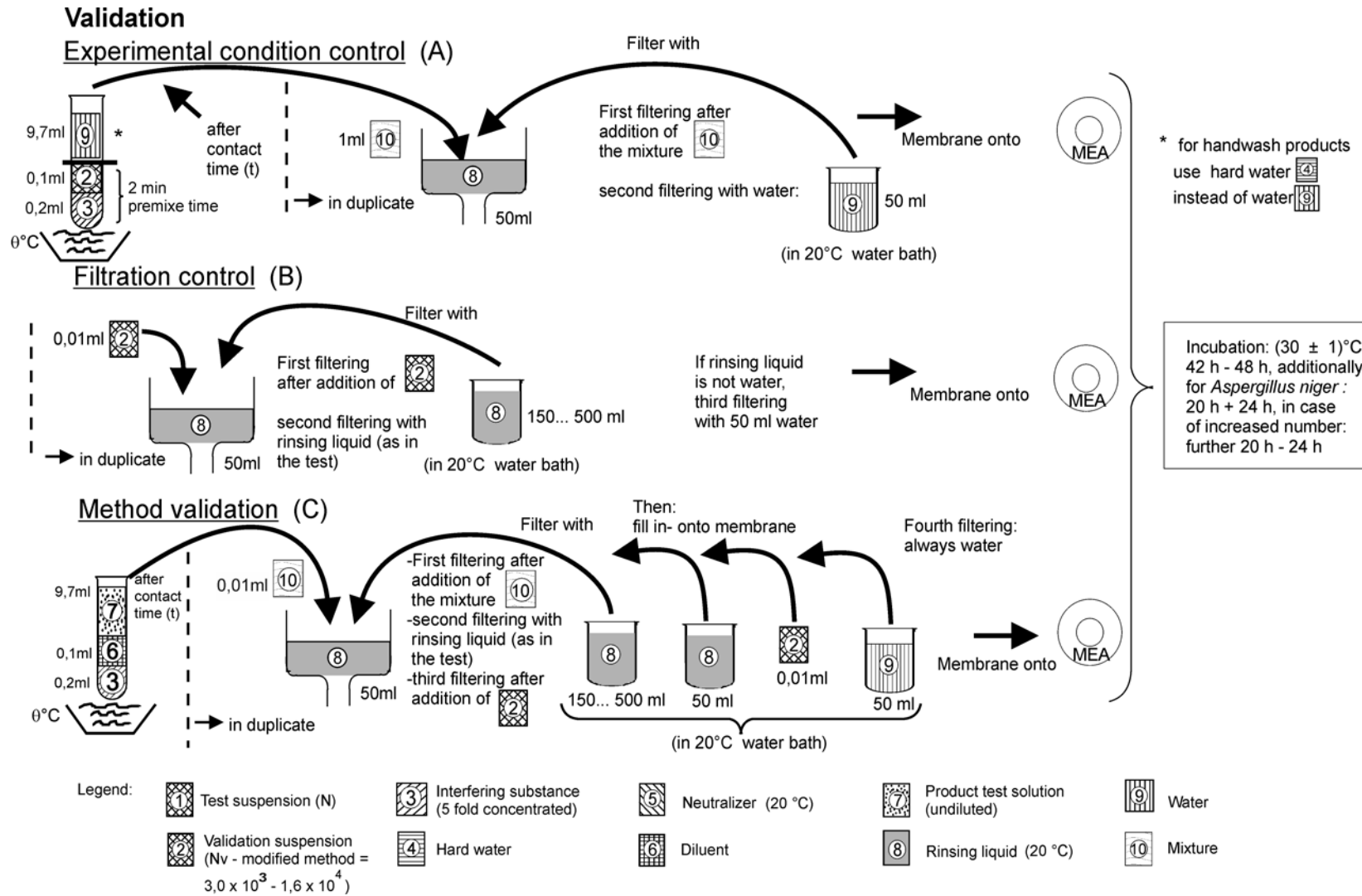


Figure C.8 — Membrane filtration method (modified method for ready-to-use products) — Validation

## Annex D (informative)

### Example of a typical test report

NOTE 1 All names and examples in Annex D are fictitious apart from those used in this European Standard.

NOTE 2 Only the test results of one replicate for *Aspergillus brasiliensis* is given as an example.

NOTE 3 The results of a hygienic handrub product tested according to the modified method 5.5.4 (with *Candida albicans*) is given without a complete test report as an additional example.

Test reports for yeasticidal activity should be entitled "EN 13624, YEASTICIDAL ACTIVITY" and be presented in the same format.

---

HHQ Laboratories  
Antiseptville/Euroland  
Tel. ++011.57 83 62-0  
Fax ++011-57 83 62-19  
e-mail: [h.h.Q.lab@net.com](mailto:h.h.Q.lab@net.com)

## TEST REPORT

### EN 13624, FUNGICIDAL ACTIVITY

**Client:** Cult Formulations Inc., Mannheim/Euroland

#### Disinfectant-sample

**Name of the product:** JP (instrument disinfectant)

**Batch number:** 11-11-78

**Manufacturer** or – if not known – **supplier:** MDI Formulations Inc. (manufacturer)

**Storage conditions (temp. and other):** Room temperature, darkness

**Appearance of the product:** Liquid, clear, yellowish

**Active substance(s) and their concentration(s):** Not indicated

**Product diluent recommended by the manufacturer for use:** Potable water

#### Period of testing

**Date of delivery of the product:** 2008-05-01

**Dates of tests:** see "Test results" (attached)

#### Experimental conditions

**Product diluent: hard water; concentrations of the product tested:** see "Test results" (attached)



**Minimum conditions:**

test-organisms: *Aspergillus brasiliensis* ATCC 16404, *Candida albicans* ATCC 10231

Test temperature: 20 °C; contact time: 60 min;  
interfering substance: 0,3 g/l bovine albumin = clean conditions;  
incubation temperature: 30 °C

**Additional conditions:**

Test organism: *Penicillium* xxx ATCC .....;  
test temperature: 40 °C; contact time: 60 min;  
interfering substance: 3,0 g/l bovine albumin plus 3,0 ml/l erythrocytes = dirty conditions  
[test results: see attached sheets]

**Special remarks regarding the results:**

1. All controls and validation were within the basic limits.
2. At least one concentration of the product demonstrated a 4 lg reduction of less than 4 lg.
3. No precipitate during the test procedure (test mixtures were homogeneous).

**Conclusion:**

For the product JP (batch 11-11-78), the fungicidal concentration for instrument or surface disinfection determined according to the EN 13624 Standard (minimum conditions) under clean conditions is:

1,0 % (v/v)

(the mean reduction of seven repetitions with the limiting test organism *Aspergillus brasiliensis* was  $1,2 \times 10^4$  and *Candida albicans* were tested once and showed a 4 lg reduction or more at a lower concentration than *Aspergillus brasiliensis*).

For the product JP (batch 11-11-78), the fungicidal concentration for specific instrument disinfection determined according to the EN 13624 Standard at 40 °C, with 60 min contact time under dirty conditions, using *Penicillium* xxxx ATCC ..... as test organism is:

0,50 % (v/v)

Antiseptville, 2008-11-11

Alexander May, MD, PhD, Scientific Director

### Test results (fungicidal suspension test)

EN.... 13624....(Phase 2, step 1) Product name: *JP.....(instrument disinfectant)* Batch No.: ... 11-11-78

Remarks:

Dilution neutralization method  Pour plate  Spread plate  Number of plates .....2 / ml  
 Neutralizer:.....*Lecithin 3,0 g/l* in diluent  
 Membrane filtration method  Rinsing liquid: .....  
 Test temperature: 20 °C interfering substance:.....bovine albumin: 0,3 g/l .....  
 Test organism: ...*Aspergillus brasiliensis* ATCC 16404 .....Incubation temperature: 30 °C  
 Internal lab. No.:... *QS 70/00....* Date of test: 2007-06-06 Responsible person: Fang.... Signature: *Fang*

Diluent used for product test solutions: .. *hard water...*  
 Appearance of the product test solutions: ..... *clear.....*

#### Validation and controls

Concentration of spiny conidiospores of *Aspergillus brasiliensis* 75 % or more in the conidiospore suspension:  
 yes  no

Validation suspension ( $N_{V0}$ )			Experimental conditions control (A)			Neutralizer or filtration control (B)			Method validation (C) Product conc.: 10 ml/l		
$V_{C1}$	86 (40 + 46)	$\bar{x} = 89$	$V_{C1}$	79 (43 + 36)	$\bar{x} = 81,5$	$V_{C1}$	86 (42 + 44)	$\bar{x} = 88,5$	$V_{C1}$	75 (35 + 40)	$\bar{x} = 81$
$V_{C2}$	92 (47 + 45)		$V_{C2}$	84 (39 + 45)		$V_{C2}$	91 (43 + 48)		$V_{C2}$	87 (41 + 46)	
30 ≤ $\bar{x}$ of $N_{V0}$ ≤ 160?			$\bar{x}$ of A is ≥ 0,5 x $\bar{x}$ of $N_{V0}$ ?			$\bar{x}$ of B is ≥ 0,5 x $\bar{x}$ of $N_{V0}$ or $N_{VB}/1000$ ?			$\bar{x}$ of C is ≥ 0,5 x $\bar{x}$ of $N_{V0}$ ?		
Yes <input checked="" type="checkbox"/> NO <input type="checkbox"/>			Yes <input checked="" type="checkbox"/> NO <input type="checkbox"/>			Yes <input checked="" type="checkbox"/> NO <input type="checkbox"/>			Yes <input checked="" type="checkbox"/> NO <input type="checkbox"/>		
Validation suspension ( $N_{VB}$ )			$V_{C1} = 99$ (49 + 50)	$V_{C2} = 71$ (39 + 32)	$\bar{x} = 85$	30 ≤ $\bar{x}$ of $N_{VB} / 1\ 000$ ≤ 160? Yes <input checked="" type="checkbox"/> NO <input type="checkbox"/>					

#### Test suspension and test

Test-suspension ( $N$ and $N_0$ ):	$N$	$V_{C1}$	$V_{C2}$	$\bar{x}_{wm} = 193,64 \times 10^5$ ; $\lg N = 7,29$ $N_0 = N/10$ ; $\lg N_0 = 6,29$ $6,17 \leq \lg N_0 \leq 6,70$ ?
	$10^{-5}$	168	213	
	$10^{-6}$	20	25	Yes <input checked="" type="checkbox"/> NO <input type="checkbox"/>

Concentration of the product	Dilution step	$V_{C1}$	$V_{C2}$	$N_a = (\bar{x} \text{ or } \bar{x}_{wm} \times 10)$	$\lg N_a$	$\lg R$ ( $N_0 = 6,29$ )	Contact time (min)
0,5	$10^0$	> 330	> 330	8 100	3,91	2,38	60
	$10^{-1}$	77	85				
0,75	$10^0$	122	154	1 395,5	3,15	3,14	60
	$10^{-1}$	14	17				
1,00	$10^0$	7	0	< 140	< 2,15	> 4,14	60
	$10^{-1}$	0	0				

Countings per plate for

$N = 10^{-6}$ : 80+88; 105+108;  $10^{-7}$ : 9+11; 15+10;  
 $N_a = 0,50\%$   $V_{C1}$ : 42+35  $V_{C2}$ : 44+41;  $0,75\%$   $V_{C1}$ : 66+56  $V_{C2}$ : 71+83;  $1,00\%$   $V_{C1}$ : 1+6  $V_{C2}$ : 0;

Explanations:

$V_C$  = count per ml (one plate or more)

$\bar{x}$  = average of  $V_{C1}$  and  $V_{C2}$  (1. + 2. duplicate)

$\bar{x}_{wm}$  = weighted mean of  $\bar{x}$

$R$  = reduction ( $\lg R = \lg N_0 - \lg N_a$ )

**Test results (yeastcidal suspension test)**

EN....13624....(Phase 2, step 1) Product name: BU....(hygienic handrub) Batch No.: ...26-01-48

Remarks: Ready-to-use product see 5.5.4.

Dilution neutralization method  Pour plate  Spread plate  Number of plates .....2 / ml  
 Neutralizer:.....Lecithin 3,0 g/l in diluent .....  
 Membrane filtration method  Rinsing liquid: .....  
 Test temperature: 20 °C interfering substance:....bovine albumin: 0,3 g/l .....  
 Test organism: ...Candida albicans ATCC 10231 .....Incubation temperature: 36 °C  
 Internal lab. No.:...QS 68/12.... Date of test: 2007-05-27 Responsible person: ... Fang.... Signature: Fang

Diluent used for product test solutions: .. water...  
 Appearance of the product test solutions: ..... clear.....

**Validation and controls**

Validation suspension ( $N_{V0}$ )			Experimental conditions control (A)			Neutralizer or filtration control (B)			Method validation (C) Product conc.: 100 ml/l		
$V_{C1}$	86 (40 + 46)	$\bar{x} = 89$	$V_{C1}$ 30 s	79	$\bar{x} = 81,5$	$V_{C1}$	86 (42 + 44)	$\bar{x} = 88,5$	$V_{C1}$ 30 s	75	$\bar{x} =$
			$V_{C2}$ 30 s	84					$V_{C2}$ 30 s	87	81
$V_{C2}$	92 (47 + 45)		$V_{C1}$ 1 min	75	$\bar{x} = 78,5$	$V_{C2}$	91 (43 + 48)		$V_{C1}$ 1 min	82	$\bar{x} =$
			$V_{C2}$ 1 min	82					$V_{C2}$ 1 min	79	80,5
$30 \leq \bar{x} \text{ of } N_{V0} \leq 160?$			$\bar{x} \text{ of A is } \geq 0,5 \times \bar{x} \text{ of } N_{V0}?$			$\bar{x} \text{ of B is } \geq 0,5 \times \bar{x} \text{ of } N_{V0} \text{ or } N_{VB}/1\ 000?$			$\bar{x} \text{ of C is } \geq 0,5 \times \bar{x} \text{ of } N_{V0}?$		
Yes <input checked="" type="checkbox"/> NO <input type="checkbox"/>			Yes <input checked="" type="checkbox"/> NO <input type="checkbox"/>			Yes <input checked="" type="checkbox"/> NO <input type="checkbox"/>			Yes <input checked="" type="checkbox"/> NO <input type="checkbox"/>		
Validation suspension ( $N_{VB}$ )			$V_{C1} = 99$ (49 + 50)	$V_{C2} = 71$ (39 + 32)	$\bar{x} = 85$	$30 \leq \bar{x} \text{ of } N_{VB}/1\ 000 \leq 160?$ Yes <input checked="" type="checkbox"/> NO <input type="checkbox"/>					

**Test suspension and test**

Test-suspension ( $N$ and $N_0$ ):	$N$	$V_{C1}$	$V_{C2}$	$\bar{x}_{wm} = 193,64 \times 10^7$ ; $\lg N = 8,29$ $N_0 = N/100$ ; $\lg N_0 = 6,29$ $6,17 \leq \lg N_0 \leq 6,70?$ Yes <input checked="" type="checkbox"/> NO <input type="checkbox"/>
	$10^{-7}$	168	213	
	$10^{-8}$	20	25	

Concentration of the product	Dilution step	$V_{C1}$	$V_{C2}$	$N_a = (\bar{x} \text{ or } \bar{x}_{wm} \times 10)$	$\lg N_a$	$\lg R$ ( $N_0 = 6,29$ )	Contact time (min)
97	$10^0$	> 660	> 660	8 100	3,91	2,38	0,5 (30 s)
	$10^{-1}$	77	85				
97	$10^0$	7	0	< 140	< 2,15	> 4,14	1
	$10^{-1}$	0	0				

Countings per plate for

$N = 10^{-7}$ : 80+88; 105+108;  $10^{-8}$ : 9+11; 15+10  
 $N_a = 0,5 \text{ min}$   $V_{C1}$ : 42+35;  $V_{C2}$ : 44+41 **1 min**:  $V_{C1}$ : 1+6;  $V_{C2}$ : 0;  
**A 30 s**: 43+36; 39+45; **1 min**: 45+30; 43+39;  
**C 30 s**: 35+40; 41+46; **1 min**: 37+45; 35+44;

Explanations:

$V_C$  = count per ml (one plate or more)  
 $\bar{x}$  = average of  $V_{C1}$  and  $V_{C2}$  (1. + 2. duplicate)

$\bar{x}_{wm}$  = weighted mean of  $\bar{x}$   
 $R$  = reduction ( $\lg R = \lg N_0 - \lg N_a$ )

## Annex E (informative)

### Precision of the test result

The scope of the study is to determine the precision of the test method within and between a random sample of laboratories. The sample size necessary to achieve a precision of the reduction of  $\pm 1$  lg is determined.

In order to determine the required sample size it is necessary to quantify the residual, unexplained variance in different experiments. This variance is estimated by the help of a statistical mixed model including the random effect of laboratories and the fixed effect of the combination of a specified test organism, a product and a contact time. Reduction is determined with different concentrations of the products labeled  $R_1$ ,  $R_2$ ,  $R_3$  (and in that case  $R_4$ ). Separate statistical analyses are performed for each reduction.

The statistical analyses are done using different data sets. All data available are used in the “unrestricted” case in contrast to the “restricted” case, where a measured reduction is excluded from the statistical analysis if it is below 0 or above 5.

The study involved eight laboratories from different European countries and was carried out in the years 2001-2003. Each laboratory repeated the test at least once.

Beside the random effect of laboratories, a possible influence of the test-organism, the product and the contact time on the independent variable reduction is analysed.

The experimental design is not balanced, i.e. not all possible combinations have been tested. Therefore, a new variable called *Influence* was created which combines each of the three variables (test organism, product and contact time) with their different possible appearances, e.g. *A. brasiliensis* – Glutaraldehyde – 15 min into the one influence “1” (see Table E.1).

**Table E.1 — Number of repetitions (unrestricted data set) for a given test organism, product, contact time and laboratory and independent variable  $R_1$ ,  $R_2$  and  $R_3$**

Each cell shows the number of the repetitions for the given reduction  $R_1$ ,  $R_2$  and  $R_3$  separated by a semicolon.

Test organism	Product tested	Contact time	Influence	Laboratory							
				1	2	3	4	5	6	7	8
A. <i>brasiliensis</i>	Glutaraldehyde	15	1	5;5;5	4;4;4;	-;-;	6;6;6	3;5;5	3;3;3	5;5;5	-;-;
		60	2	-;-;	3;3;2	-;-;	-;-;	-;-;	-;-;	-;-;	-;-;
	Peracetic acid	5	3	5;5;5	8;8;4	-;-;	6;6;6	5;5;5	1;1;1	6;6;6	-;-;
		15	4	-;-;	4;4;1	-;-;	-;-;	-;-;	-;-;	-;-;	-;-;
C. <i>albicans</i>	Glutaraldehyde	15	5	6;1;0	6;6;6	6;6;0	6;6;0	8;9;3	5;5;0	-;-;	5;5;0
		60	6	3;1;0	6;6;4	6;6;0	6;6;0	7;6;0	3;3;3	-;-;	7;7;0
	Peracetic acid	5	7	6;0;0	6;5;3	6;6;0	6;6;0	7;7;0	4;4;4	-;-;	4;4;0
		15	8	-;-;	4;3;2	-;-;	-;-;	-;-;	-;-;	-;-;	-;-;
		60	9	-;-;	-;-;	-;-;	-;-;	-;-;	-;-;	-;-;	2;2;0

Table E.1 shows for example that the test organism *Aspergillus brasiliensis* was not tested with all combinations of the influential variables “Product tested (Glutaraldehyde)” and “contact time (15 minutes)” in two of the eight laboratories. Furthermore, one laboratory repeated the determination of the reduction  $R_1$ ,  $R_2$  and  $R_3$  only 3 times at maximum which is not enough to do statistical analysis. Nevertheless, the new design is the best way to estimate the residual variance needed for the calculation of the precision of the test results and its dependency on the number of repetitions.

## Results

Unrestricted and restricted mixed effects models using  $R_1$  to  $R_3$  as dependent variable and influence as fixed and laboratory as random effect are used to estimate the variance components.

**Table E.2 — Results of statistical analyses**

(“Est.Var.”: estimated variance; “SE”: standard error; “Z”: test value; “Pr Z”: significance; “N” = number of results included)

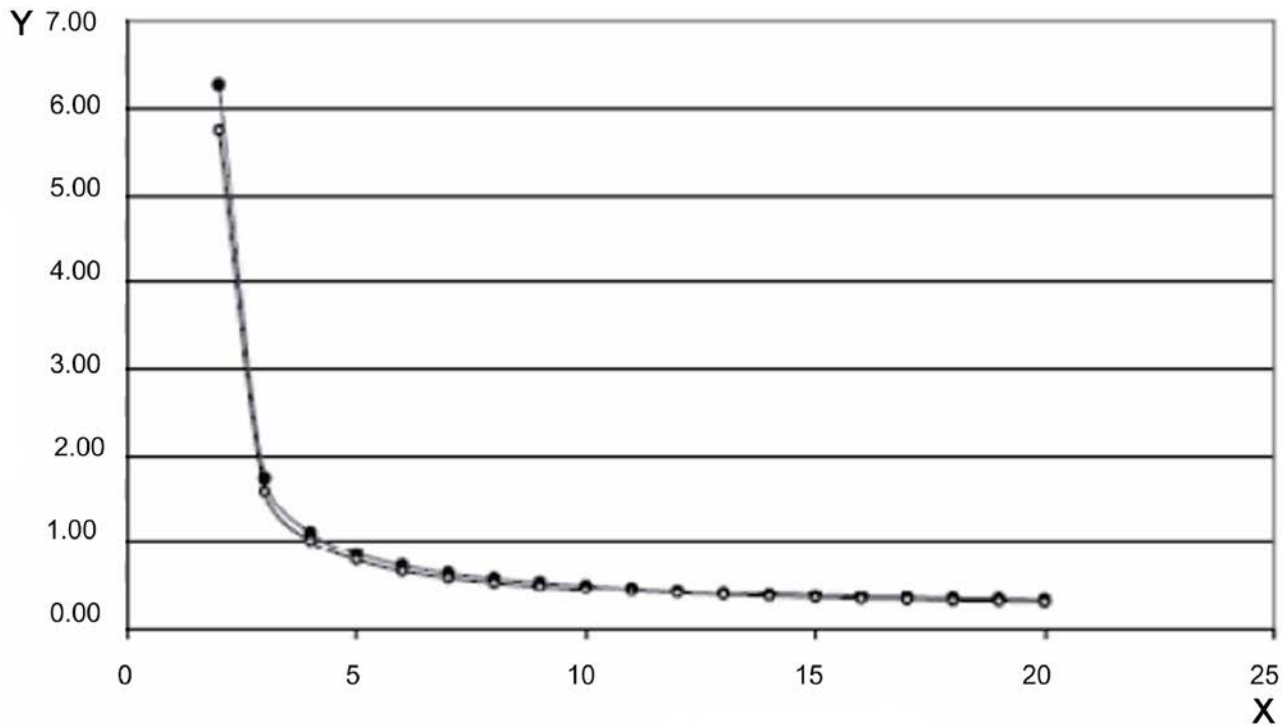
	Unstricted					Restricted				
	Variance component	Est. Var.	SE	Z	Pr Z	Variance component	Est. Var.	SE	Z	Pr Z
$R_1$	Laboratory	0,325 0	0,239 2	1,36	0,087 1	Laboratory	0,292 2	0,220 9	1,32	0,092 9
	Residual	0,484 5	0,055 4	8,74	< 0,000 1	Residual	0,407 0	0,051 4	8,62	< 0,000 1
	N=189					N=185				
$R_2$	Laboratory	0,049 3	0,081 6	0,60	0,272 7	Laboratory	0,049 3	0,081 6	0,60	0,272 7
	Residual	0,424 9	0,050 9	8,34	< 0,000 1	Residual	0,424 9	0,050 9	8,34	< 0,000 1
	N=176					N=176				
$R_3$	Laboratory	0,284 4	0,206 1	1,38	0,083 8	Laboratory	0,284 4	0,206 1	1,38	0,083 8
	Residual	0,430 6	0,076 6	5,62	< 0,000 1	Residual	0,430 6	0,076 6	5,62	< 0,000 1
	N=76					N=76				

“Laboratory” in Table E.2 means the interlaboratory variance and “Residual” means the unexplained residual intralaboratory variance.

The fixed effect *Influence* is significant for all reductions  $R_1$  to  $R_3$  (restricted or unrestricted).

The estimated residual variances (worst case  $s^2 = 0,484 5$  and best case  $s^2 = 0,407 0$ ) given in Table E.2 are used to determine the precision of the test results. The formula used for the calculation of the precision of the test results can be found in EN 1040:2005, Annex E.

As seen from Figure E.1 below, the number of repetitions of four in the best case or five in the worst case or more gives a precision of the reduction of  $\pm 1$  lg or less.



**Key**

- X number of repetitions
- Y precision [in lg terms]
- worst case
- best case

**Figure E.1 — Dependency of the precision of the reduction (y-axis) on the number of repetitions (x-axis) for worst and best case**

**Annex ZA**  
(informative)

**Relationship between this European Standard and the Essential Requirements of EU Directive 93/42/EEC**

This European Standard has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association to provide a means of conforming to Essential Requirements of the New Approach Directive 93/42/EEC.

Once this standard is cited in the Official Journal of the European Union under that Directive and has been implemented as a national standard in at least one Member State, compliance with the clauses of this standard given in Table ZA.1 confers, within the limits of the scope of this standard, a presumption of conformity with the corresponding Essential Requirements of that Directive and associated EFTA regulations.

**Table ZA.1 — Correspondence between this European Standard and Directive 93/42/EEC**

Clauses of this EN	Essential Requirements (ERs) of Directive 93/42/EEC	Qualifying remarks/Notes
1, 2, 3 and 4	6a (in connection with Annex X, 1.1d)	This standard is intended for products whose main efficacy claim is of a microbicidal nature, e.g. chemical disinfectants and antiseptics. Complying with the requirements of this standard demonstrates the fungicidal or yeasticidal activity of the product. Generally, at least one additional European Standard (phase 2, step 2) has to be complied with to demonstrate a sufficient performance evaluation of such products.

**WARNING** — Other requirements and other EU Directives may be applicable to the product(s) falling within the scope of this European Standard.

## Bibliography

- [1] European Pharmacopoeia (EP), Edition 1997 supplement 2000, *Water for injections*.
- [2] 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)*





# British Standards Institution (BSI)

BSI is the national body responsible for preparing British Standards and other standards-related publications, information and services.

BSI is incorporated by Royal Charter. British Standards and other standardization products are published by BSI Standards Limited.

## About us

We bring together business, industry, government, consumers, innovators and others to shape their combined experience and expertise into standards-based solutions.

The knowledge embodied in our standards has been carefully assembled in a dependable format and refined through our open consultation process. Organizations of all sizes and across all sectors choose standards to help them achieve their goals.

## Information on standards

We can provide you with the knowledge that your organization needs to succeed. Find out more about British Standards by visiting our website at [bsigroup.com/standards](http://bsigroup.com/standards) or contacting our Customer Services team or Knowledge Centre.

## Buying standards

You can buy and download PDF versions of BSI publications, including British and adopted European and international standards, through our website at [bsigroup.com/shop](http://bsigroup.com/shop), where hard copies can also be purchased.

If you need international and foreign standards from other Standards Development Organizations, hard copies can be ordered from our Customer Services team.

## Subscriptions

Our range of subscription services are designed to make using standards easier for you. For further information on our subscription products go to [bsigroup.com/subscriptions](http://bsigroup.com/subscriptions).

With **British Standards Online (BSOL)** you'll have instant access to over 55,000 British and adopted European and international standards from your desktop. It's available 24/7 and is refreshed daily so you'll always be up to date.

You can keep in touch with standards developments and receive substantial discounts on the purchase price of standards, both in single copy and subscription format, by becoming a **BSI Subscribing Member**.

**PLUS** is an updating service exclusive to BSI Subscribing Members. You will automatically receive the latest hard copy of your standards when they're revised or replaced.

To find out more about becoming a BSI Subscribing Member and the benefits of membership, please visit [bsigroup.com/shop](http://bsigroup.com/shop).

With a **Multi-User Network Licence (MUNL)** you are able to host standards publications on your intranet. Licences can cover as few or as many users as you wish. With updates supplied as soon as they're available, you can be sure your documentation is current. For further information, email [bsmusales@bsigroup.com](mailto:bsmusales@bsigroup.com).

## BSI Group Headquarters

389 Chiswick High Road London W4 4AL UK

## Revisions

Our British Standards and other publications are updated by amendment or revision.

We continually improve the quality of our products and services to benefit your business. If you find an inaccuracy or ambiguity within a British Standard or other BSI publication please inform the Knowledge Centre.

## Copyright

All the data, software and documentation set out in all British Standards and other BSI publications are the property of and copyrighted by BSI, or some person or entity that owns copyright in the information used (such as the international standardization bodies) and has formally licensed such information to BSI for commercial publication and use. Except as permitted under the Copyright, Designs and Patents Act 1988 no extract may be reproduced, stored in a retrieval system or transmitted in any form or by any means – electronic, photocopying, recording or otherwise – without prior written permission from BSI. Details and advice can be obtained from the Copyright & Licensing Department.

## Useful Contacts:

### Customer Services

**Tel:** +44 845 086 9001

**Email (orders):** [orders@bsigroup.com](mailto:orders@bsigroup.com)

**Email (enquiries):** [cservices@bsigroup.com](mailto:cservices@bsigroup.com)

### Subscriptions

**Tel:** +44 845 086 9001

**Email:** [subscriptions@bsigroup.com](mailto:subscriptions@bsigroup.com)

### Knowledge Centre

**Tel:** +44 20 8996 7004

**Email:** [knowledgecentre@bsigroup.com](mailto:knowledgecentre@bsigroup.com)

### Copyright & Licensing

**Tel:** +44 20 8996 7070

**Email:** [copyright@bsigroup.com](mailto:copyright@bsigroup.com)



...making excellence a habit.™