

English Version

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

Antiseptiques et désinfectants chimiques - Essai quantitatif de suspension pour l'évaluation de l'activité fongicide ou levuricide des antiseptiques et des désinfectants chimiques utilisés dans le domaine vétérinaire - Méthode d'essai et prescriptions (phase 2, étape 1)

Chemische Desinfektionsmittel und Antiseptika - Quantitativer Suspensionsversuch zur Bestimmung der fungiziden oder levuroziden Wirkung chemischer Desinfektionsmittel und Antiseptika für den Veterinärbereich - Prüfverfahren und Anforderungen (Phase 2, Stufe 1)

This European Standard was approved by CEN on 28 July 2005.

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## Foreword

This European Standard (EN 1657:2005) 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of an identical text or by endorsement, at the latest by June 2006, and conflicting national standards shall be withdrawn at the latest by June 2006.

This European Standard supersedes EN 1657:2000.

It was revised to include the results of a collaborative trial (ANDISTAND), to correct obvious errors and ambiguities, to harmonize the structure and wording with other quantitative suspension tests of CEN/TC 216 existing or in preparation, and to improve the readability of the standard and thereby make it more understandable.

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

## **Introduction**

This European Standard specifies a suspension test for establishing whether a chemical disinfectant or antiseptic has a fungicidal or yeasticidal activity in the 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.

The conditions are intended to cover general purposes and to allow reference between laboratories and product types. Each utilization concentration of the chemical disinfectant or antiseptic found by this test corresponds to defined experimental conditions. However, for some applications the recommendations of use of a product may differ and therefore additional test conditions need to be used.

## 1 Scope

This 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, 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 veterinary area – i.e. in the breeding, husbandry, production, transport and disposal of all animals except when in the food chain following death and entry to the processing industry.

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 (Annex F).

## 2 Normative references

The following referenced documents are indispensable for the application of this European Standard. 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 microbial strains used for the determination of bactericidal and fungicidal activity*

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

## 3 Terms and definitions

For the purposes of this European Standard, the following terms and definitions apply.

### 3.1

#### **product**

chemical agent or formulation used as chemical disinfectant or antiseptic

### 3.2

#### **fungicide**

product that kills fungi (moulds and yeasts) and their spores under defined conditions

NOTE The adjective derived from "fungicide" is "fungicidal".

### 3.3

#### **fungicidal activity**

capability of a product to produce a reduction in the number of viable vegetative yeast cells and mould spores of relevant test organisms under defined conditions

### 3.4

#### **fungistatic activity**

capability of a product to inhibit the growth of fungi (moulds and/or yeasts ) under defined conditions

### 3.5

#### **yeasticide**

product that kills yeasts under defined conditions

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

### 3.6

#### yeastocidal activity

capability of a product to produce a reduction in the number of viable yeast cells of relevant test organisms under defined conditions

## 4 Requirements

The product shall demonstrate at least a 4 decimal log (lg) reduction when diluted with hard water (**5.2.2.7**) or – in the case of ready-to-use products – with water (**5.2.2.2**) and tested in accordance with **Clause 5** under simulated low-level soiling (3 g/l bovine albumin solution – **5.2.2.8.2**) or simulated high-level soiling (10 g/l bovine albumin solution plus 10 g/l yeast extract – **5.2.2.8.3**) according to its practical applications and under the other obligatory test conditions (one or two selected test organisms, 10 °C, 30 min).

The fungicidal activity shall be evaluated using the following two test organisms:

- *Candida albicans* (vegetative cells);
- *Aspergillus niger* (spores).

The yeastocidal activity shall be evaluated using the following test organism:

- *Candida albicans* (vegetative cells).

Where indicated, additional specific fungicidal or yeastocidal activity shall be determined applying other contact times, temperatures and test organisms in accordance with **5.2.1** and **5.5.1.1** in order to take into account intended specific use conditions.

NOTE For these additional conditions, the concentration defined as a result can be lower than the one obtained under the obligatory test 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  $(10 \pm 1) ^\circ\text{C}$  for  $30 \text{ min} \pm 10 \text{ s}$  (obligatory test conditions). At the end of this contact time, an aliquot is taken, and the fungicidal and/or the fungistatic activity 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.

**5.1.2** The test is performed using the vegetative cells of *Candida albicans* and the spores of *Aspergillus niger* (fungicidal activity) or only the vegetative cells of *Candida albicans* (yeastocidal activity) as test organisms (obligatory test conditions).

**5.1.3** Additional and optional contact times and temperatures are specified. Additional test organisms can 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:<sup>1)</sup>

— *Candida albicans* ATCC 10231;

— *Aspergillus niger* ATCC 16404.

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

NOTE See Annex A for strain references 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.

### 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.

NOTE 1 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.

NOTE 2 For each culture medium and reagent, a limitation for use should be fixed.

#### 5.2.2.2 Water

The water shall be freshly glass-distilled water and not demineralized water.

Sterilize in the autoclave [5.3.2.1 a)].

NOTE 1 Sterilization is not necessary if the water is used e.g. for preparation of culture media and subsequently sterilized.

NOTE 2 If distilled water of adequate quality is not available, water for injections (see bibliographic reference [1]) can be used.

NOTE 3 See 5.2.2.7 for the procedure to prepare hard water.

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1) The ATCC numbers are the collection numbers of strains supplied by the American Type Culture Collection (ATCC). 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.

### 5.2.2.3 Malt extract agar (MEA)

Malt extract agar, consisting of:

Malt extract	30,0 g
Soya peptone, papaic digest of soybean meal	3,0 g
Agar	15,0 g
Water (5.2.2.2)	to 1 000,0 ml

Sterilize in the autoclave [5.3.2.1 a)]. After sterilization, the pH of the medium shall be equivalent to  $5,6 \pm 0,2$  when measured at  $(20 \pm 1) ^\circ\text{C}$ .

NOTE In case of encountering problems with neutralization (5.5.1.2 and 5.5.1.3), it may be necessary to add neutralizer to the MEA. Annex B gives guidance on the neutralizers that may be used.

### 5.2.2.4 Diluent

Tryptone sodium chloride solution, consisting of:

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

Sterilize in the autoclave [5.3.2.1 a)]. After sterilization, the pH of the diluent shall be equivalent to  $7,0 \pm 0,2$  when measured at  $(20 \pm 1) ^\circ\text{C}$ .

### 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 ( $\text{MgCl}_2$ ) and 46,24 g calcium chloride ( $\text{CaCl}_2$ ) in water (5.2.2.2) and dilute to 1 000 ml. Sterilize 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 ( $\text{NaHCO}_3$ ) in water (5.2.2.2) and dilute to 1 000 ml. Sterilize 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 of the hard water shall be  $7,0 \pm 0,2$ , when measured at  $(20 \pm 1) ^\circ\text{C}$  (5.3.2.4). 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 a different final water hardness in each test tube. In any case the final hardness is lower than 300 mg/l of calcium carbonate ( $\text{CaCO}_3$ ) in the test tube.

### 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.

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 Low-level soiling (bovine albumin solution)

Dissolve 3,0 g of bovine albumin fraction V (suitable for microbiological purposes) in 100 ml of water (5.2.2.2).

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

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

#### 5.2.2.8.3 High-level soiling (mixture of bovine albumin solution with yeast extract)

Dissolve 50,0 g yeast extract powder in 150 ml of water (5.2.2.2) in a 250 ml volumetric flask (5.3.2.12) and allow foam to collapse. Make up to the mark with water (5.2.2.2). Transfer to a clean dry bottle and sterilize in an autoclave [5.3.2.1 a)]. Allow to cool to  $(20 \pm 1) ^\circ\text{C}$ .

Pipette 25 ml of this solution into a 50 ml volumetric flask (5.3.2.12) and add 10 ml of water (5.2.2.2). Dissolve 5,0 g of bovine albumin fraction V (suitable for microbiological purposes) in the solution with shaking and allow foam to collapse. Make up to the mark with water (5.2.2.2), sterilize by membrane filtration (5.3.2.7), keep in the refrigerator (5.3.2.8) and use within one month.

The final concentration in the test procedure (5.5) is 10,0 g/l yeast extract and 10,0 g/l bovine albumin.

### 5.3 Apparatus and glassware

#### 5.3.1 General

Sterilize 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>2)</sup> and, in particular, the following:

##### 5.3.2.1 Apparatus for sterilization:

- a) for moist heat sterilization, an autoclave capable of being maintained at  $(121^{+3}_0)$  °C for a minimum holding time of 15 min;
- b) for dry heat sterilization, a hot air oven capable of being maintained at  $(180^{+5}_0)$  °C for a minimum holding time of 30 min, at  $(170^{+5}_0)$  °C for a minimum holding time of 1 h or at  $(160^{+5}_0)$  °C for a minimum holding time of 2 h.

**5.3.2.2 Water baths**, capable of being controlled at  $(10 \pm 1)$  °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  $(\pm 1)$  °C.

NOTE 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>3)</sup>
- b) Mechanical shaker

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

The apparatus shall have a filter holder of at least 50 ml volume. It shall be suitable for use with filters of diameter 47 mm to 50 mm and 0,45 µm pore size for sterilization of hard water (5.2.2.7) and bovine albumin (5.2.2.8), 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.

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

3) 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.

- 5.3.2.8 Refrigerator**, capable of being controlled at 2 °C to 8 °C.
- 5.3.2.9 Graduated pipettes**, of nominal capacities 10 ml, 1 ml and 0,1 ml, or calibrated automatic pipettes.
- 5.3.2.10 Petri dishes (plates)**, of size 90 mm to 100 mm.
- 5.3.2.11 Glass beads**, 3 mm to 4 mm in diameter.
- 5.3.2.12 Volumetric flasks**.
- 5.3.2.13 Fritted filter**, with porosity of 40 µm to 100 µm according to ISO 4793.
- 5.3.2.14 Centrifuge** (2 000  $g_N$ ).
- 5.3.2.15 Roux bottles or similar flasks**.

## **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), prepare a subculture from the stock culture (5.4.1.2) by streaking onto MEA (5.2.2.3) slopes or plates (5.3.2.10) 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 subcultures 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 subculturing, 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 niger* (mould)**

For *Aspergillus niger* (5.2.1), use only the first subculture grown on MEA (5.2.2.3) in Roux bottles (5.3.2.15) and incubate for 9 d to 11 d. No further subculturing is needed.

##### **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*

The procedure for preparing the *Candida albicans* test suspension is as follows:

- a) 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 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 [5.3.2.6b)]. Aspirate the suspension from the glass beads and transfer to a tube;
- b) adjust the number of cells in the suspension to  $1,5 \times 10^7$  cfu/ml<sup>4)</sup> to  $5,0 \times 10^7$  cfu/ml using diluent (5.2.2.4), estimating the number of cfu by any suitable means. Maintain this test suspension in the water bath at the test temperature  $\theta$  [5.5.1.1 a)] and use within 2 h.

NOTE The use of a spectrophotometer for adjusting the number of cells is highly recommended (approximately 620 nm wavelength – cuvette 10 mm path length). Each laboratory should therefore produce calibration data for each test organism knowing that suitable values of optical density are generally found between 0,200 and 0,350. A colorimeter is a suitable alternative.

- c) For counting, prepare  $10^{-5}$  and  $10^{-6}$  dilutions 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).

For incubation and counting, see 5.4.1.6.

##### 5.4.1.4.2 *Aspergillus niger*

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

- a) take the working culture (5.4.1.3.2) and suspend the spores 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 (5.3.2.13);
- b) carry out a microscopic examination under x 400 magnification immediately after the preparation and just before the test, to show the absence of mycelia fragments and spore germination (check at least ten fields of view for absence of both).

If germinated spores are present, discard the suspension.

If mycelia are present, set up a washing process (centrifugation) as follows. Transfer the filtered suspension to centrifuge tubes. The filtered suspension is centrifuged (5.3.2.14) at  $2\,000\,g_N$  for 20 min. The conidiospores are washed at least twice by resuspension in diluent (5.2.2.4) and subsequent centrifugation. If mycelia are still present, repeat the washing process;

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

- c) Adjust the number of spores in the suspension to  $1,5 \times 10^7$  cfu/ml to  $5,0 \times 10^7$  cfu/ml using the diluent (5.2.2.4), estimating the number of cfu by any suitable means. Use the suspension within 4 h. It can be stored up to 2 d in the refrigerator and shall then be checked just before the test for absence of germinated spores [see b)]. In any case, adjust the temperature according to 5.5.1.4 only immediately before the start of the test (5.5.2 or 5.5.3).

NOTE 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 [5.4.1.4.2 e)]. Experienced laboratories found a better fit to the required number of spores when the spore suspension count in the device was 10 % to 50 % higher than the number aimed at;

- d) For counting, prepare  $10^{-5}$  and  $10^{-6}$  dilutions 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 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  $(45 \pm 1) ^\circ\text{C}$ ;
- 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).

For incubation and counting, see 5.4.1.6.

#### 5.4.1.5 Validation suspension (“Nv”)

- a) To prepare the validation suspension, dilute the test suspension (5.4.1.4.1 and 5.4.1.4.2) with the diluent (5.2.2.4) to obtain the fungal count of  $3,0 \times 10^2$  cfu/ml to  $1,6 \times 10^3$  cfu/ml [about one-fourth (1 + 3) of the  $10^{-4}$  dilution].
- b) For counting, prepare a  $10^{-1}$  dilution with diluent (5.2.2.4). Mix [5.3.2.6a)]. Take a sample of 1,0 ml in duplicate and inoculate using the pour plate or the spread plate technique [with *Candida albicans*, 5.4.1.4.1 c); with *Aspergillus niger*, 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

For incubation and counting of the test and the validation suspensions, the procedure is as follows:

- a) incubate (5.3.2.3) the plates for 42 h to 48 h. Discard any plates that are not countable for any reason. Count the plates and determine the number of cfu..

Only for *Aspergillus niger*: 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 that no longer show well-separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation;

- b) 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 Vc values according to 5.6.2.2;
- c) calculate the numbers of cfu/ml in the test suspension “N” and in the validation suspension “Nv” using the methods 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 %.

Dilutions of ready-to-use products, i.e. products that are not diluted when applied, shall be prepared in water (5.2.2.2).

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 (5.2.2.7) on a volume/volume basis using volumetric flasks (5.3.2.12).

The product test solutions shall be prepared freshly and used in the test within 2 h. They shall give a physically homogeneous preparation that is stable during the whole procedure. If during the procedure a visible inhomogeneity appears due to the formation of a precipitate or flocculant (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 flocculant 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 or yeasticidal activity of the product

#### 5.5.1 General

##### 5.5.1.1 Experimental conditions (obligatory and additional)

Besides the obligatory temperature, contact time, interfering substances and test organisms, additional experimental conditions (including test organisms) may be selected according to the practical use considered for the product (Clause 4), as follows:

a) temperature  $\theta$  (in °C):

- the obligatory temperature to be tested is  $\theta = 10 \text{ °C}$ ;
- additional temperature may be chosen from  $4 \text{ °C}$ ,  $20 \text{ °C}$  or  $40 \text{ °C}$ ;
- the allowed deviation for each chosen temperature is  $\pm 1 \text{ °C}$ ;

b) contact time  $t$  (in min):

- the obligatory contact time to be tested is  $t = 30$  min;
- additional contact times may be chosen from 1 min, 5 min, 15 min or 60 min;
- the allowed deviation for each chosen contact time is  $\pm 10$  s, except for 1 min, for which it is  $\pm 5$  s;

c) interfering substance:

- the obligatory interfering substance to be tested is 3,0 g/l bovine albumin (5.2.2.8.2) for low-level soiling or 10 g/l bovine albumin plus 10 g/l yeast extract (5.2.2.8.3) for high-level soiling, according to practical applications;

d) test organisms (5.2.1):

- the obligatory test organisms for testing fungicidal activity are *Candida albicans* and *Aspergillus niger*;
- the obligatory test organism for testing yeasticidal activity is *Candida albicans*.

Additional test organisms may be tested.

#### 5.5.1.2 Choice of test method (dilution-neutralization or membrane filtration)

The method of choice is the dilution-neutralization method (5.5.2). To determine a suitable neutralizer, carry out the validation of the dilution neutralization method (5.5.2.3, 5.5.2.4 and 5.5.2.5 in connection with 5.5.2.6) 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 containing a combination of polysorbate 80 (30 g/l), saponin (30 g/l), L-histidine (1 g/l), lecithin (3 g/l), sodium thiosulphate (5 g/l) in either diluent (5.2.2.4) or phosphate buffer 0,002 5 mol/l (Annex B).

If both neutralizers are found to be invalid, the membrane filtration method (5.5.3) may be used in place of the dilution-neutralization method.

NOTE In special circumstances, it may be necessary to add neutralizer to the 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.

If because of problems with neutralization a neutralizer has been added to the 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  $\theta$  [5.5.1.1 a)] using the water bath (5.3.2.2) controlled at  $\theta$ . Check that the temperature of the reagents is stabilized 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  $(20 \pm 1) ^\circ\text{C}$ .

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 validation suspensions (5.4.1).

### 5.5.2 Dilution-neutralization method<sup>5)</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 "Na" – 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 [5.3.2.6a)] and place the tube in a water bath controlled at the chosen test temperature  $\theta$  [5.5.1.1 a)] for  $2 \text{ min} \pm 10 \text{ s}$ .

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 [5.3.2.6a)] 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 [5.3.2.6a)] again;

- b) at the end of  $t$ , take a 1,0 ml sample of the test mixture "Na" and transfer into a tube containing 8,0 ml neutralizer (5.2.2.5) and 1,0 ml water (5.2.2.2). Mix [5.3.2.6a)] and place in a water bath controlled at  $(20 \pm 1)^\circ\text{C}$ . After a neutralization time of  $5 \text{ min} \pm 10 \text{ s}$ , mix [5.3.2.6a)] and immediately take a sample of 1,0 ml of the neutralized test mixture "Na" (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  $45 ^\circ\text{C} \pm 1 ^\circ\text{C}$ .
  - 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).

For incubation and counting, see 5.5.2.6.

- c) perform the procedure a) and b) using the other product test solutions at the same time;
- d) perform the procedure a) to c) applying the other obligatory 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:

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5) For a graphical representation of this method, see Annex C.



- 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 (5.4.1.5). Start the stopwatch immediately, mix [5.3.2.6a)] and place the tube in a water bath controlled at  $\theta$  for  $2 \text{ min} \pm 10 \text{ s}$ .

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: water (5.2.2.2) instead of hard water.] Restart the stopwatch at the beginning of the addition. Mix [5.3.2.6a)] and place the tube in a water bath controlled at  $\theta$  for  $t$ . Just before the end of  $t$ , mix [5.3.2.6a)] again.

- b) 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 b)].

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 8,0 ml of the neutralizer – used in the test (5.5.2.2) – and 1,0 ml of water (5.2.2.2) into a tube. Add 1,0 ml of the validation suspension (5.4.1.5). Start the stopwatch at the beginning of the addition, mix [5.3.2.6a)], and place the tube in a water bath controlled at  $(20 \pm 1)^\circ\text{C}$  for  $5 \text{ min} \pm 10 \text{ s}$ . Just before the end of this time, mix [5.3.2.6a)];
- b) at the end of this time, take a sample of 1,0 ml of this mixture "B" in duplicate and inoculate using the pour plate or the spread plate technique [5.5.2.2 b)].

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 [5.3.2.6a)] and place the tube in a water bath controlled at  $\theta$  for  $t$ . Just before the end of  $t$ , mix [5.3.2.6a)] 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 [5.3.2.6a)] and place the tube in a water bath controlled at  $(20 \pm 1)^\circ\text{C}$  for  $5 \text{ min} \pm 10 \text{ s}$ . Add 1,0 ml of the validation suspension (5.4.1.5). Start a stopwatch at the beginning of the addition and mix [5.3.2.6a)]. 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 [5.3.2.6a)] 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 b)].

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 that are not countable for any reason. Count the plates and determine the number of colony forming units.

Only for *Aspergillus niger*: 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 that no longer show well-separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation;

- b) 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 colony-forming units per millilitre in the test mixture "Na" 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>6)</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 in parallel and separately for each experimental condition (5.5.1.1).

Each membrane filtration apparatus shall be equipped with a membrane of 0,45 µm pore size and 47 mm to 50 mm diameter (5.3.2.7) and 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 "Na" – 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).
- b) at the end of  $t$ , take a sample of 0,1 ml of the test mixture "Na" in duplicate and transfer each 0,1 ml 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.

For incubation and counting see 5.5.3.6.

- c) see 5.5.2.2 c);
- d) see 5.5.2.2 d).

#### 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);
- 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 niger* 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 sample is the upper limit for counting [5.6.2.2 a)]

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6) For a graphical representation of this method, see Annex C.

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 proceed as follows.

Take 0,1 ml of the validation suspension (**5.4.1.5**) 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 niger* 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 sample 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 (**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**).

In the case of *Aspergillus niger* 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 sample 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 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 that are not countable for any reason. Count the colonies on the membranes.

Only for *Aspergillus niger*: 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 that no longer show well-separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation;

- b) 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 in accordance with 5.6.2.2;
- c) calculate the numbers of cfu/ml in the test mixture "Na" 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.

## 5.6 Experimental data and calculation

### 5.6.1 Explanation of terms and abbreviations

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

$N$  and  $N_v$  represent the fungal suspensions,  $Na$  represents the fungicidal test mixture,  $A$  (experimental conditions control),  $B$  (neutralizer or filtration control),  $C$  (method validation) represent the different control test mixtures.

$N$ ,  $N_v$ ,  $N_0$ ,  $N_{v0}$ ,  $Na$  and  $A$ ,  $B$  and  $C$  represent the number of cells counted per ml in the different test mixtures in accordance with Table 1.

**Table 1 — 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 $t$ or 5 min ( $B$ ) or 30 min ( $C$ )
Test	$N$ Test suspension	$N_0 (= N/10)$	$Na$ (before neutralization or filtration)
Controls	$N_v$ Validation suspension	$N_{v0} (= N_v/10)$	$A, B, C$

#### 5.6.1.2 $V_c$ values

All experimental data are reported as  $V_c$  values:

- in the dilution-neutralization method (test and controls), a  $V_c$  value is the number of colony-forming units counted per 1,0 ml sample;
- in the membrane filtration method, a  $V_c$  value is the number of colony-forming units counted per 0,1 ml sample of test mixture "Na" and per 1,0 ml sample in the controls.

## 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  $N$ ,  $N_0$ ,  $Na$ ,  $N_v$ ,  $N_{v0}$ ,  $A$ ,  $B$  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 nutrient 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 suspension  $N_v$  (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 sample (5.6.1.2).

NOTE If more than one plate per 1 ml 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 (or 330), report the number as "> 165" (or "> 330"). If more than one plate per 1 ml sample has been used and at least one of them shows a number higher than 165 (or 330), report this  $V_c$  value as "> sum of the counts" (e.g. for ">165, 132, 144", report "> 441").

If a  $V_c$  value is lower than 14, report the number (but substitute by "< 14" for further calculation 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 (165 or 55) as described above;

- c) Only  $V_c$  values within the respective 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 equation:

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

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.

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

$$N = \frac{139+154+14+17}{(2+0,1 \times 2)10^{-5}} = \frac{324}{2,2 \times 10^{-5}} = 1,4727 \times 10^7 = 1,5 \times 10^7 \text{ (cfu/ml)}$$

$N_0$  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 the interfering substance.

#### 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.

Calculate  $N_a$  using the following equation:

$$N_a = 10c / n$$

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”.

#### EXAMPLES

a) duplicate  $V_c$  values: 2, 16

$$N_a = \frac{(<14 + 16) \times 10}{2} = i.e. < 150$$

b) duplicate  $V_c$  values (pour plate, *Aspergillus niger*): > 165, > 165

$$N_a = \frac{(>165 + >165) \times 10}{2} = i.e. > 1\,650$$

c) duplicate  $V_c$  values (membrane-filtration, *Aspergillus niger*): 40, > 55

$$N_a = \frac{(40 + >55) \times 10}{2} = i.e. > 475$$

d) duplicate  $V_c$  values (two spread plates per 1,0 ml sample, *Candida albicans*): > 660, 600

$$N_a = \frac{(>660 + 600) \times 10}{2} = i.e. > 6\,300$$

#### 5.6.2.5 Calculation of $N_v$ and $N_{v0}$

$N_v$  is the number of cells per ml in the validation suspension (5.4.1.5). 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).

$N_{v0}$  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). It is one-tenth of the mean of the  $V_c$  values of  $N_v$  [5.4.1.6 c)] taken into account.

Calculate  $N_v$  and  $N_{v_0}$  using the following equations:

$$N_v = 10c / n$$

$$N_{v_0} = 10c / n$$

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 equation:

$$A, B, C = c/n$$

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.

#### 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 mean 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:  $> 165 + 150$  cfu;  $10^{-6}$  dilution:  $20 + 25$  cfu;  $(165 + 150) / (20 + 25) = 315/45 = 7,0 =$  between 5 and 15.

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

### 5.7.3 Basic limits

For each test organism check that:

- a)  $N$  is between  $1,5 \times 10^7$  and  $5,0 \times 10^7$  ( $7,17 \leq \lg N \leq 7,70$ );  
 $N_0$  is between  $1,5 \times 10^6$  and  $5,0 \times 10^6$  ( $6,17 \leq \lg N_0 \leq 6,70$ );
- b)  $Nv_0$  is between 30 and 160 ( $3,0 \times 10^1$  and  $1,6 \times 10^2$ );  
( $Nv$  is between  $3,0 \times 10^2$  and  $1,6 \times 10^3$ );
- c)  $A, B, C$  are equal to or greater than  $0,5 \times Nv_0$ ;
- d) control of weighted mean counts (5.7.2): quotient is not lower than 5 and not higher than 15.

## 5.8 Expression of results and precision

### 5.8.1 Reduction

The reduction ( $R = N_0/Na$ ) 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  $Na$  (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 equation:

$$\lg R = \lg N_0 - \lg Na$$

For the controls and validation of the dilution-neutralization method or membrane filtration method, record  $Nv_0$  (5.6.2.5), the results of  $A, B$  and  $C$  (5.6.2.6) and their comparison with  $Nv_0$  [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)] shall demonstrate a 4  $\lg$  or more reduction and at least one concentration shall demonstrate a  $\lg$  reduction of less than 4.

### 5.8.3 Limiting test organism and fungicidal/yeastcidal concentration

#### 5.8.3.1 Fungicidal concentration

For each test organism, record the lowest concentration of the product which passes the test ( $\lg R \geq 4$ ). 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 Yeastcidal concentration

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



#### 5.8.4 Precision, replicates

Taking into account the precision of the methodology determined by a statistical analysis based on data provided by a collaborative study, replication of the test (seven replicates for a precision of  $\pm 1$  lg in reduction) is recommended (**Annex E**). Replication means the complete test procedure with separately prepared test – and validation suspensions. The replicate of the test may be restricted to the limiting test organism. The mean of the results of the replicates – not each single result – shall demonstrate at least a 4 lg reduction and shall also be calculated and recorded.

### 5.9 Interpretation of results – conclusion

#### 5.9.1 General

According to the chosen experimental conditions (obligatory or obligatory and additional) the fungicidal concentrations determined according to this European Standard may differ (**Clause 4**).

#### 5.9.2 Fungicidal activity for general purposes

The product shall be deemed to have passed the EN 1657 standard if it demonstrates in a valid test at least a 4 lg reduction within 30 min or less at 10 °C with the chosen interfering substance simulating low-level or high-level soiling under the conditions defined by this European Standard when the test organisms are *Candida albicans* and *Aspergillus niger*.

The fungicidal concentration for general purposes is the concentration active on the limiting strain.

#### 5.9.3 Fungicidal activity for specific purposes

The fungicidal concentration for a specific purpose is the concentration of the tested product for which at least a 4 lg reduction is demonstrated in a valid test under the additional chosen test conditions when the test organisms are mould and yeast strains. The product shall have passed the EN 1657 standard (fungicidal activity) under the obligatory test conditions. The fungicidal concentration for specific purposes may be lower than the one determined for general purposes.

#### 5.9.4 Yeasticidal activity for general purposes

The product shall be deemed to have passed the EN 1657 standard (yeasticidal activity) if it demonstrates in a valid test at least a 4 lg reduction within 30 min or less at 10 °C with the chosen interfering substance simulating low-level or high-level soiling under the conditions defined by this European Standard when the test organism is *Candida albicans*.

The yeasticidal concentration for general purposes is the concentration active on *Candida albicans*.

#### 5.9.5 Yeasticidal activity for specific purposes

The yeasticidal concentration for a specific purpose is the concentration of the tested product for which at least a 4 lg reduction is demonstrated in a valid test under the additional chosen test conditions when the test organisms are only yeast strains. The product shall have passed the EN 1657 standard (yeasticidal activity) under the obligatory test conditions. The yeasticidal concentration for specific purposes may be lower than the one determined for general purposes.

### 5.10 Test report

The test report shall refer to this European Standard (EN 1657) mentioning if fungicidal activity or only yeasticidal activity has been tested.

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 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 product dilutions;
  - 5) contact time(s);
  - 6) test temperature(s);
  - 7) interfering substance(s);
  - 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 yeastocidal activity;
  - 3) number of replicates 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 niger</i> :	ATCC	16404
	DSM	1988
	CBS	733.88
	CIP	1431.83
	NCTC	2275
	IMI	149007

## Annex B (informative)

### Suitable neutralizers and rinsing liquids

#### B.1 General

The weights given in **B.2** to **B.4** refer to the anhydrous salts (**5.2.2.1**).

The lists in **B.2** to **B.4** are not exhaustive and other reagents may be used.

#### B.2 Neutralizers

Any of the following neutralizers may be used:

- lecithin 3 g/l; polysorbate 80<sup>7)</sup> 30 g/l; sodium thiosulphate ( $\text{Na}_2\text{S}_2\text{O}_3$ ) 5 g/l; L-histidine 1 g/l; saponin 30 g/l in diluent (**5.2.2.4**) or in phosphate buffer 0,002 5 mol/l);
- phosphate buffer 0,25 mol/l:
  - potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) 34 g;
  - water (**5.2.2.2**) 500 ml;
  - adjusted to pH  $7,2 \pm 0,2$  with sodium hydroxide ( $\text{NaOH}$ ) 1 mol/l;
  - water (**5.2.2.2**) up to 1 000 ml;
  - sterilized in an autoclave (**5.3.1**);
- fresh egg yolk diluted to 5 % or 0,5 % (v/v);
- 30 g/l polysorbate 80; 4 g/l sodium dodecyl sulphate ( $\text{C}_{12}\text{H}_{25}\text{NaO}_4\text{S}$ ); lecithin 3 g/l;
- fresh egg yolk diluted to 5 % (v/v); 40 g/l polysorbate 80;
- 7 % (v/v) ethylene oxide condensate of fatty alcohol; 20 g/l lecithin; 4 % (w/v); polysorbate 80;
- 4 % (v/v) ethylene oxide condensate of fatty alcohol; 4 g/l lecithin;
- 30 g/l polysorbate 80; lecithin 3 g/l; L-histidine 1 g/l;
- glycine as a function of the product concentration;
- 30 g/l polysorbate 80; lecithin 3 g/l;
- phospholipid emulsion (commercial) at 50 mg/ml (diluted 1 to 10);

---

7) Analytical quality, non-hydrolyzed in accordance with the European Pharmacopoeia. TWEEN 80 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.

- sodium thioglycollate at 0,5 g/l or 5 g/l;
- L-cysteine at 0,8 g/l or 1,5 g/l;
- thiomalic acid at 0,075 % (v/v) – adjusted to pH 7,0 with sodium hydroxide (NaOH);
- sodium thiosulphate at 5 g/l;
- catalase or peroxidase: one unit (U) of these enzymes catalyzes at  $(25 \pm 1)$  °C the decomposition of 1  $\mu$  mol of hydrogen peroxide per minute at pH 7,0;
- polysorbate 80 30 g/l ; saponin 30 g/l ; L-histidine 1 g/l ; L-cysteine 1 g/l.

### **B.3 Rinsing liquids**

Any of the following rinsing liquids may be used:

- water (5.2.2.2);
- diluent (5.2.2.4);
- aqueous solution of 0,1 % (w/v) polysorbate 80;
- aqueous solution of 0,5 % (w/v) polysorbate 80;
- aqueous solution of 0,5 % (w/v) polysorbate 80 and 0,7 g/l lecithin;
- neutralizer. Check first if the time required for filtration exceeds 1 min;
- buffer solutions.

### **B.4 Neutralizer added to the agar for counting**

Any of the following neutralizers may be used:

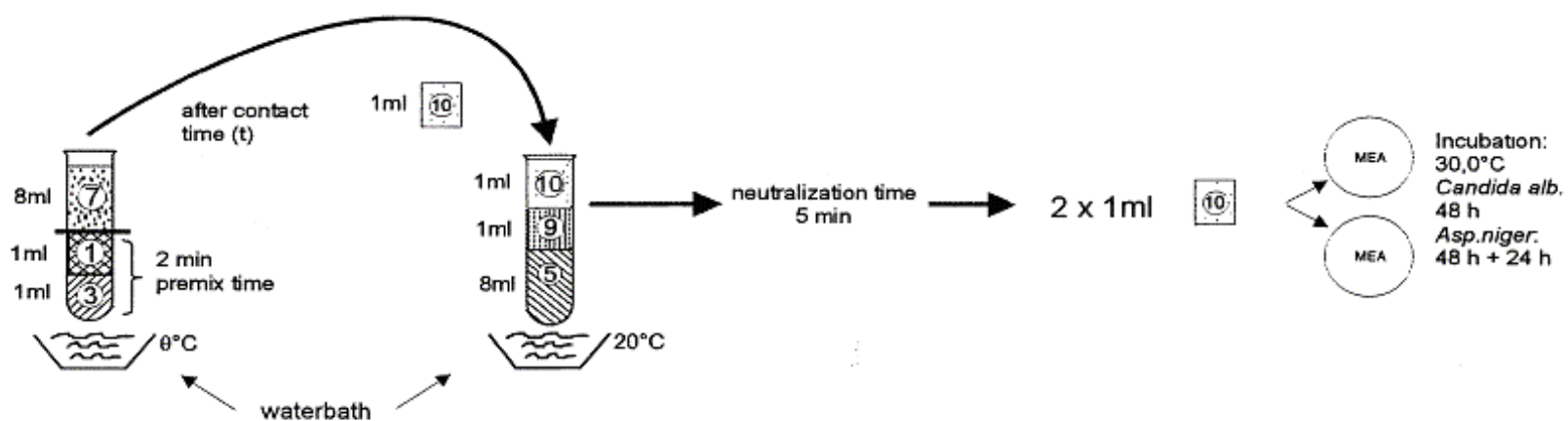
- 10 % (v/v) of a solution containing 0,7 g/l lecithin and 5 % (w/v) polysorbate 80;
- 10 % (v/v) of a solution containing 10 g/l lecithin and 5 % (w/v) polysorbate 80;
- 10 % (v/v) of a solution containing fresh egg yolk 1,5 % (v/v) and 5 % (w/v) polysorbate 80.

## Annex C (informative)

### Graphical representation of test procedures

#### C.1 — Dilution-neutralization method

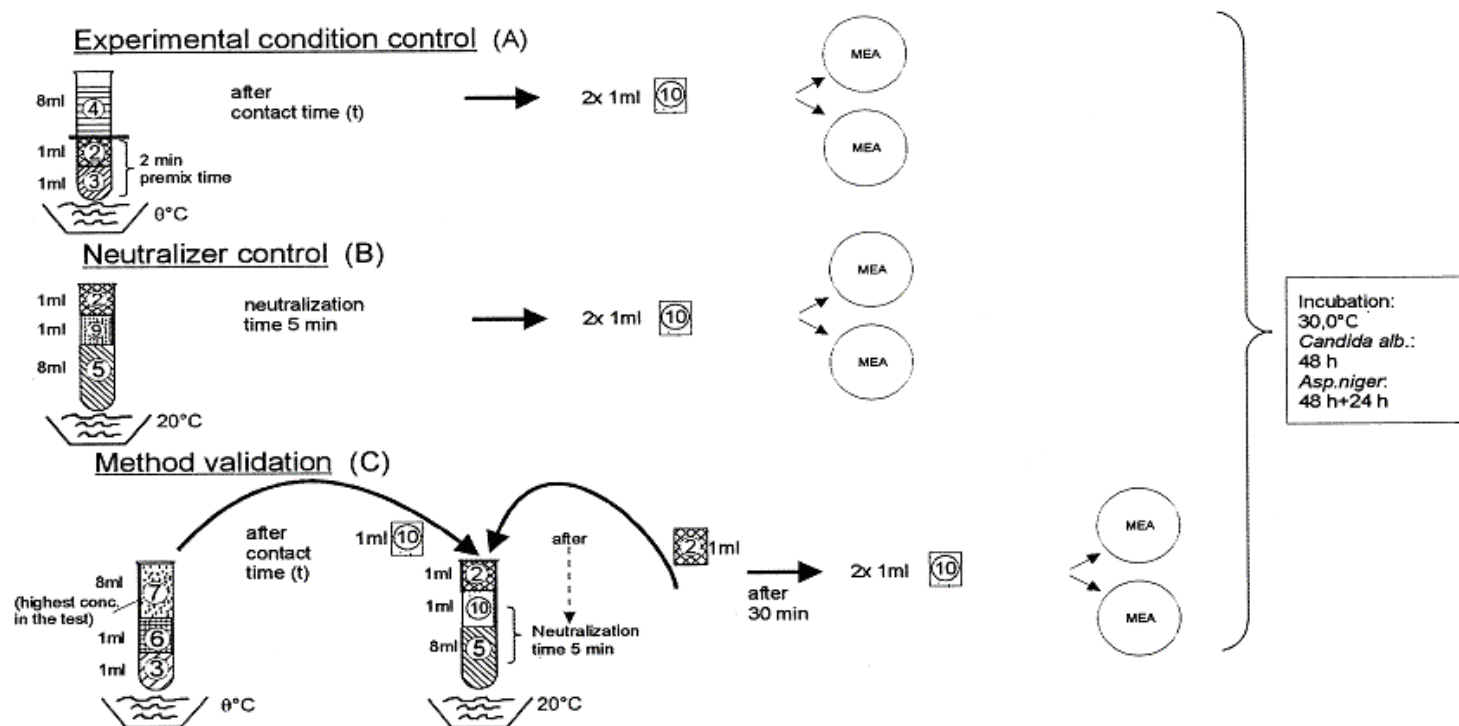
##### Test (Na)



#### Key

1	Test suspension (N)	3	Interfering substance	5	Neutralizer (20 °C)	7	Product test solution	9	Water
2	Validation suspension (Nv)	4	Hard water	6	Diluent	8	Rinsing liquid (20 °C)	10	Mixture

# Validation



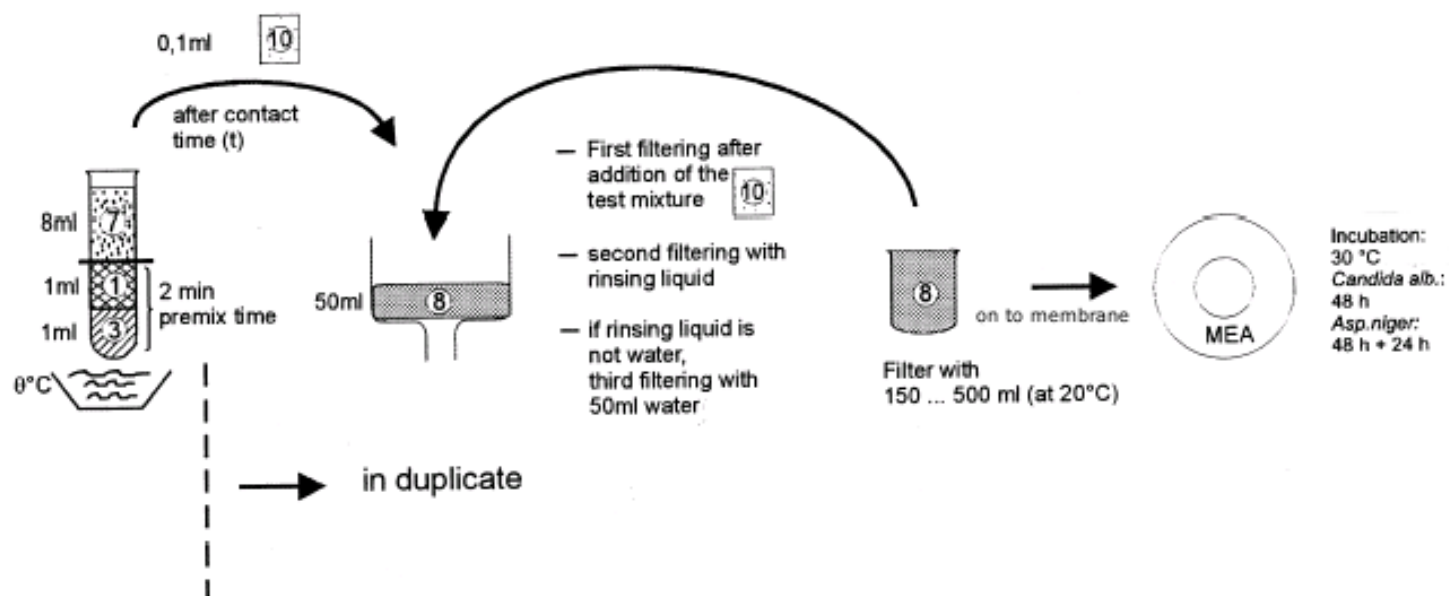
## Key

(1)	Test suspension (N)	(3)	Interfering substance	(5)	Neutralizer (20 °C)	(7)	Product test solution	(9)	Water
(2)	Validation suspension (Nv)	(4)	Hard water	(6)	Diluent	(8)	Rinsing liquid (20 °C)	(10)	Mixture



## C.2 — Membrane filtration method

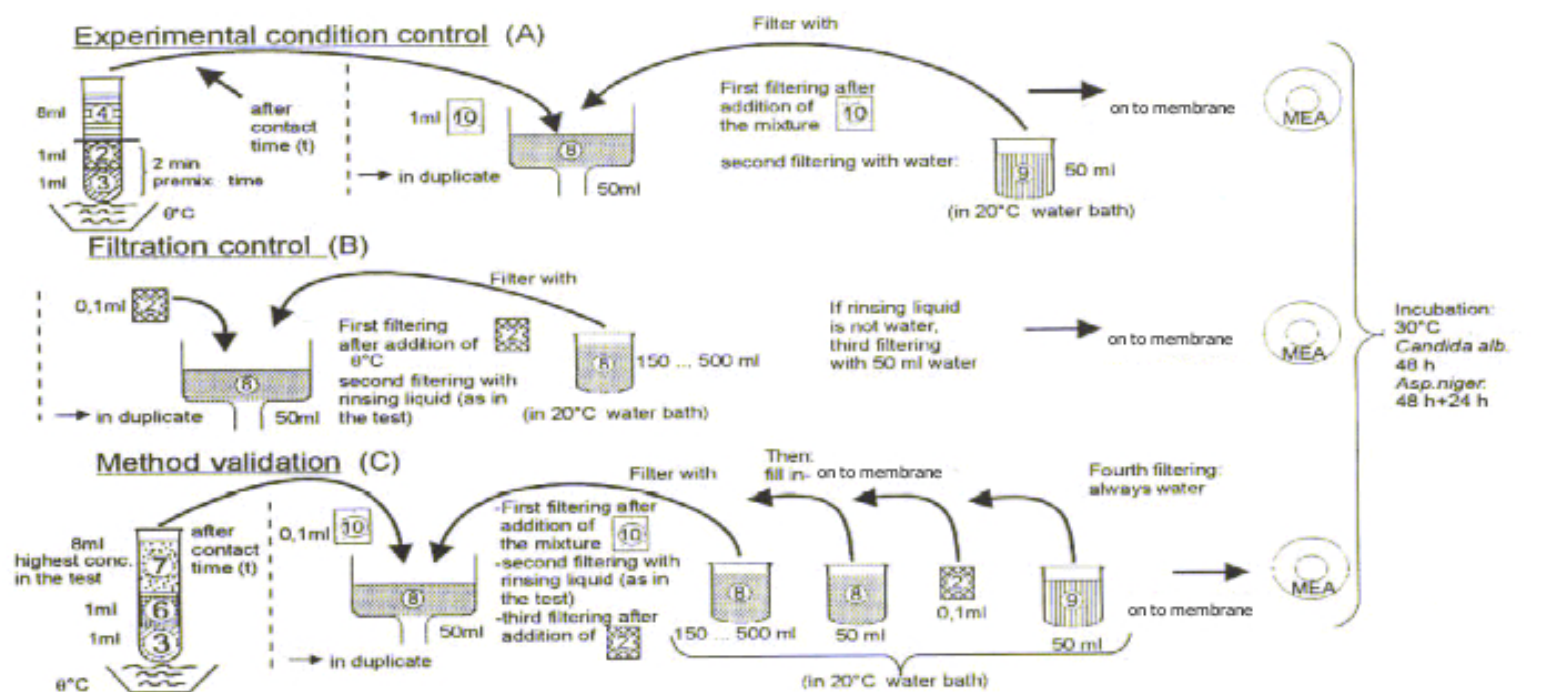
## Test (Na)



## Key

	Test suspension (N)		Interfering substance		Neutralizer (20 °C)		Product test solution		Water
	Validation suspension (Nv)		Hard water		Diluent		Rinsing liquid (20 °C)		Mixture

## Validation



### Key

1	Test suspension ( <i>N</i> )	3	Interfering substance	5	Neutralizer (20 °C)	7	Product test solution	9	Water
2	Validation suspension ( <i>N<sub>v</sub></i> )	4	Hard water	6	Diluent	8	Rinsing liquid (20 °C)	10	Mixture

## 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 Test reports for yeasticidal activity should be entitled "EN 1657, YEASTICIDAL ACTIVITY", and be presented in the same format.

NOTE 3 Only the test results of one replicate for *Aspergillus niger* and for *Candida albicans* are given as an example.

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## TEST REPORT

### EN 1657, FUNGICIDAL ACTIVITY (obligatory and additional conditions)

1. **Client** : Centipede Formulations Inc., Markkleeborg / Euroland

2. **Disinfectant-sample**

**Name of the product:** Z **Batch number** : 91-71-51

**Manufacturer** or – If not known – **supplier:** Centipede 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

3. **Period of testing**

**Date of delivery of the product:** 2005-07-16

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

4. **Experimental conditions**

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

**Obligatory conditions:** test-organisms: *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404 ; test temperature: 10 °C; contact time: 30 min; interfering substance: 3,0 g/l bovine albumin = low-level soiling;

Incubation temperature: 30 °C

**Additional conditions:** test organisms: *Penicillium xxx* and *Saccharomyces yyy*;

Test temperature: 20 °C; contact time: 15 min; interfering substance: 3,0 g/l bovine albumin = low-level soiling;

Incubation temperature: 30 °C

**Special remarks regarding the results:**

All controls and validation were within the basic limits.

At least one concentration of the product demonstrated a lg reduction of less than 4 lg.

No precipitate during the test procedure (test mixtures were homogeneous).

**5. Test results:** see attached sheets

**6. Conclusion:**

For the product Z (batch 91-71-51), the fungicidal concentration for general purposes determined according to the EN 1657 standard (obligatory conditions) under low-level soiling is:

1% (v/v)

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

For the product Z (batch 91-71-51), the fungicidal concentration for specific purposes determined according to the EN 1657 standard at 20 °C, with 15 min contact time, under low-level soiling using *Saccharomyces yyy* and *Penicillium xxx* as test organisms is :

0.5% (v/v).

Antiseptville, 2005-10-10

Alexandra May, MD, PhD, Scientific Director

### Test results (fungicidal suspension test)

EN....1657.....(Phase 2. step.1) Product-name :.....Z..... Batch No :...91-71-51.....

Remarks :

Dilution neutralization method ☒ Pour plate ☐ Spread plate ☐ Number of plates ..... / ml

Neutralizer :.....Lecithin 3,0 g/l in diluent

Membrane filtration method ☐ Rinsing liquid : .....

Test temperature : 10 °C Interfering substances : .....bovine albumin 3,0 g/l.....

Test organism : *Aspergillus niger* ATCC 16404.....Incubation temperature : 30 °C.....

Internal lab. no : QS 58/00

Date of test : 2005-08-05

Responsible person : Fang

Signature : Fang

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

#### Validation and controls

Validation suspension ( $N_{V_0}$ )			Experimental Conditions control (A)			Neutralizer or filtration control (B)			Method validation (C) Product conc. : 10 ml/l		
Vc1	84	$\bar{x}$ =	Vc1	85	$\bar{x}$ =	Vc1	81	$\bar{x}$ =	Vc1	79	$\bar{x}$ =
Vc2	87	85,5	Vc2	83	84	Vc2	85	83	Vc2	86	82,5
30 ≤ $\bar{x}$ of $N_{V_0}$ ≤ 160 ?			$\bar{x}$ of A is ≥ 0,5x $\bar{x}$ of $N_{V_0}$ ?			$\bar{x}$ of B is ≥ 0,5x $\bar{x}$ of $N_{V_0}$ ?			$\bar{x}$ of C is ≥ 0,5x $\bar{x}$ of $N_{V_0}$ ?		
<input checked="" type="checkbox"/> yes <input type="checkbox"/> no			<input checked="" type="checkbox"/> yes <input type="checkbox"/> no			<input checked="" type="checkbox"/> yes <input type="checkbox"/> no			<input checked="" type="checkbox"/> yes <input type="checkbox"/> no		

#### Test suspension and Test

Test-suspension ( $N$ and $N_0$ ) :		$N$	Vc1	Vc2	$\bar{x}$ wm = 147,27 × 10 <sup>5</sup> ; lgN = 7,17
		10 <sup>-5</sup>	139	154	$N_0 = N/10$ ; lgNo = 6,17
		10 <sup>-6</sup>	14	17	6,17 ≤ lg $N_0$ ≤ 6,70 ? <input checked="" type="checkbox"/> yes <input type="checkbox"/> no

Conc. of the product %	Vc1	Vc2	$Na = \bar{x} \times 10$	lgNa	lgR	Contact time (min)
0,50	> 165	> 165	> 1 650	> 3,22	< 2,95	30 min
0,75	57	65	610	2,79	3,38	30 min
1,00	0	7	< 140	< 2,15	> 4,02	30 min

Countings per plate for ( $N$ ) : 10<sup>-5</sup> = 60 + 79, 72 + 82, 10<sup>-6</sup> = 4 + 10, 2 + 15

Explanations:

Vc = count per ml (one plate or more)

$\bar{x}$  = average of Vc1 and Vc2 (1. + 2. duplicate)

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

R = reduction (lgR = lg $N_0$  - lgNa)

## Test results (fungicidal suspension test)

EN....1657.....(Phase 2. step.1) Product-name :.....Z..... Batch No :...91-71-51.....

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 : 10 °C Interfering substances :.....bovine albumin 3,0 g/l.....

Test organism : *Candida albicans* ATCC 10231.....Incubation temperature : 30 °C.....

Internal lab. no : QS 58/08

Date of test : 2005-08-27

Responsible person : Fang

Signature : Fang

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

## Validation and controls

Validation suspension ( $N_{V_0}$ )			Experimental Conditions control (A)			Neutralizer or filtration control (B)			Method validation (C) Product conc. : 7,5 ml/l		
Vc1	76	$\bar{x}$ =	Vc1	82	$\bar{x}$ =	Vc1	81	$\bar{x}$ =	Vc1	70	$\bar{x}$ =
Vc2	72	74	Vc2	81	81,5	Vc2	72	85,5	Vc2	76	73
30 ≤ $\bar{x}$ of $N_{V_0}$ ≤ 160 ?			$\bar{x}$ of A is ≥ 0,5x $\bar{x}$ of $N_{V_0}$ ?			$\bar{x}$ of B is ≥ 0,5x $\bar{x}$ of $N_{V_0}$ ?			$\bar{x}$ of C is ≥ 0,5x $\bar{x}$ of $N_{V_0}$ ?		
<input checked="" type="checkbox"/> yes <input type="checkbox"/> no			<input checked="" type="checkbox"/> yes <input type="checkbox"/> no			<input checked="" type="checkbox"/> yes <input type="checkbox"/> no			<input checked="" type="checkbox"/> yes <input type="checkbox"/> no		

## Test suspension and Test

Test-suspension (N and $N_0$ ) :	N	Vc1	Vc2	$\bar{x}$ wm = 259,54 × 10 <sup>5</sup> ; lgN = 7,41
	10 <sup>-5</sup>	241	283	$N_0 = N/10$ ; lgN <sub>0</sub> = 6,41
	10 <sup>-6</sup>	20	27	6,17 ≤ lgN <sub>0</sub> ≤ 6,70 ? <input checked="" type="checkbox"/> yes <input type="checkbox"/> no

Conc. of the product %	Vc1	Vc2	$N_a = \bar{x} \times 10$	lgN <sub>a</sub>	lgR	Contact time (min)
0,25	> 660	> 600	> 6 300	> 3,80	< 2,61	30 min
0,50	21	28	245	2,39	4,02	30 min
0,75	0	8	< 140	< 2,15	> 4,26	30 min

Countings per spread plate (2/ml) for: 1.  $N_{V_0}$  38 + 38, 34 + 38 2. A 60 + 22, 52 + 29 3. B 32 + 49, 32 + 40 4. C 34 + 36, 25 + 51 5. N 10<sup>-5</sup> 120 + 121, 130 + 153 6. N 10<sup>-6</sup> 10 + 10, 15 + 1 7.  $N_a$  0,25 % > 330 + > 330, 320 + 280 8.  $N_a$  0,5% 13 + 8, 7 + 21 9.  $N_a$  0,75 % 1 + 7

Explanations:

Vc = count per ml (one plate or more)

 $\bar{x}$  = average of Vc1 and Vc2 (1. + 2. duplicate) $\bar{x}$  wm = weighted mean of  $\bar{x}$ R = reduction (lgR = lgN<sub>0</sub> – lgN<sub>a</sub>)

## Annex E (informative)

### Precision of the test result

A collaborative study (ANDISTAND 1997 to 1999) was carried out to determine the precision of the test method within and between different laboratories. The study involved 12 laboratories from different European countries. Each laboratory replicated the test three times.

The tests were performed using the dilution neutralization or membrane filtration methods with sodium dichlorisocyanurate and phenol on *Aspergillus niger* and *Candida albicans* (as test organisms). The complete results and the statistical evaluation of this study are described in documents CEN/TC 216 HWG N 121 + N 121 Annexes + N 121 Corrigendum.

The agreement between laboratories, expressed in terms of fungicidal effect (reduction  $R = 4 \lg$ ), is very good at low and high concentrations, but less good at intermediate levels.

Uncountable data were replaced by theoretical fake values of reduction factor. When counts were below the lower counting limit (15 viable colonies), the reduction factor could vary between  $3,33 \times 10^4$  (the maximum value when data were countable) and  $5,00 \times 10^8$  (no surviving colonies), corresponding respectively to 4,52 and 6,70 log reduction. In order to simulate a possible variability, three theoretical reduction factors (one for each replicate) were chosen in this range: 4,5, 5,6 and 6,7 (in logarithmic terms).

When counts were above the upper counting limit (150 viable colonies), the reduction factor could vary between  $1,00 \times 10^0$  (no reduction) and  $1,00 \times 10^3$  (the minimum value when data were countable), giving the log reduction values of, respectively, 0,00 and 3,00. In this case, the three chosen fake theoretical reduction factors were 0,0, 1,5 and 3,0.

All these theoretical values were collected and are shown in Table E.1.

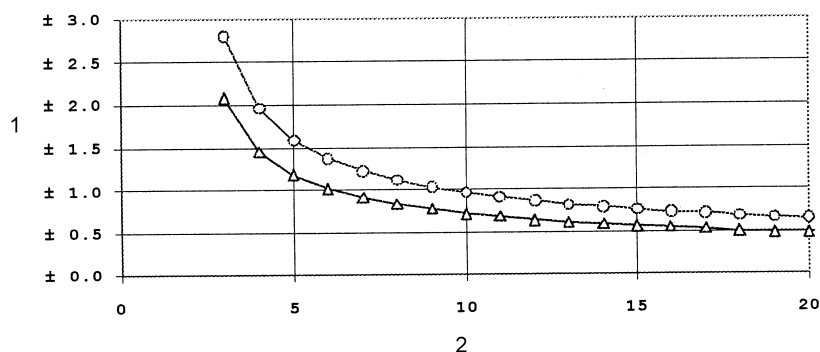
**Table E.1 — Theoretical reduction factors used for fungi (in logarithmic terms)**

	Below the lower limit (15)	Above the upper limit (150)
All three replicates are ...	4,5 – 5,6 – 6,7	0,0 – 1,5 – 3,0
Only two replicates are ...	4,5 – 6,7	0,0 – 3,0
Only one replicate is ...	5,6	1,5

Variance analyses were performed on the reduction factors in order to point out the tested conditions with significant obtained results and to estimate the two types of variability: "within" and "between" laboratories. The differences in reduction factors across the laboratories were essentially obtained with medium and high dilutions of the tested products. Some of those significant results were due to outliers, certain laboratories giving completely different reduction factor from most of the others. In other cases, it was more difficult to point out the outliers, but the significant differences were the consequences of the important "within lab" variability.

In most cases, the "between" estimated standard deviation, which varied (in logarithmic terms) from 0,89 to 2,42 for sodium dichlorisocyanurate and from 0,47 to 1,89 for phenol, was higher than the "within" value, which oscillated between 0,63 and 1,80. Nevertheless, in some cases, this ratio was inverted ("between" dispersion lower than "within" one), due probably to the techniques used for replacing uncountable data, which overestimated the "within" variability and reduced the "between" variation.

The "inside" variability was also used to estimate the precision of the obtained reduction factors. Two different hypotheses were taken into account: the first, which could be qualified as the "worst" case, used the maximum of the calculated "inside" variability ( $\sigma = 1,66$ ), while the second one was based on an average "inside" dispersion ( $\sigma = 1,23$ ). The estimated precision depended also on the sample size (number of replicates) and the confidence level (90 % probability used in the calculation). With three replicates, the "worst" case lead to a reduction factor precision of  $\pm 2,80$ , while the "mean" case was about  $\pm 2,07$ . In other words, if the precision target is fixed to  $\pm 1$  lg reduction, ten replicates were needed in the "worst" case and seven in the "mean" one (Figure E.1 or Table E.2).



### Key

1 Precision (in log terms)

2 Sample size (Number of Replicates)



Worst case



Mean case

**Figure E.1 — Precision of the reduction factor obtained with fungi (in logarithmic terms)**

**Table E.2 — Precision of the reduction factors obtained for fungi (in logarithmic terms)**

Replicates	Standard deviation ( $\sigma$ )								
	1,0	1,1	1,2	1,3	1,4	1,5	1,6	1,7	1,8
2	$\pm 4,46$	$\pm 4,91$	$\pm 5,36$	$\pm 5,80$	$\pm 6,25$	$\pm 6,70$	$\pm 7,14$	$\pm 7,59$	$\pm 8,04$
3	$\pm 1,69$	$\pm 1,85$	$\pm 2,02$	$\pm 2,19$	$\pm 2,36$	$\pm 2,53$	$\pm 2,70$	$\pm 2,87$	$\pm 3,03$
4	$\pm 1,18$	$\pm 1,29$	$\pm 1,41$	$\pm 1,53$	$\pm 1,65$	$\pm 1,77$	$\pm 1,88$	$\pm 2,00$	$\pm 2,12$
5	$\pm 0,95$	$\pm 1,05$	$\pm 1,14$	$\pm 1,24$	$\pm 1,33$	$\pm 1,43$	$\pm 1,53$	$\pm 1,62$	$\pm 1,72$
6	$\pm 0,82$	$\pm 0,90$	$\pm 0,99$	$\pm 1,07$	$\pm 1,15$	$\pm 1,23$	$\pm 1,32$	$\pm 1,40$	$\pm 1,48$
7	$\pm 0,73$	$\pm 0,81$	$\pm 0,88$	$\pm 0,95$	$\pm 1,03$	$\pm 1,10$	$\pm 1,18$	$\pm 1,25$	$\pm 1,32$
8	$\pm 0,67$	$\pm 0,74$	$\pm 0,80$	$\pm 0,87$	$\pm 0,94$	$\pm 1,00$	$\pm 1,07$	$\pm 1,14$	$\pm 1,21$
9	$\pm 0,62$	$\pm 0,68$	$\pm 0,74$	$\pm 0,81$	$\pm 0,87$	$\pm 0,93$	$\pm 0,99$	$\pm 1,05$	$\pm 1,12$
10	$\pm 0,58$	$\pm 0,64$	$\pm 0,70$	$\pm 0,75$	$\pm 0,81$	$\pm 0,87$	$\pm 0,93$	$\pm 0,99$	$\pm 1,04$
11	$\pm 0,55$	$\pm 0,60$	$\pm 0,66$	$\pm 0,71$	$\pm 0,77$	$\pm 0,82$	$\pm 0,87$	$\pm 0,93$	$\pm 0,98$
12	$\pm 0,52$	$\pm 0,57$	$\pm 0,62$	$\pm 0,67$	$\pm 0,73$	$\pm 0,78$	$\pm 0,83$	$\pm 0,88$	$\pm 0,93$
13	$\pm 0,49$	$\pm 0,54$	$\pm 0,59$	$\pm 0,64$	$\pm 0,69$	$\pm 0,74$	$\pm 0,79$	$\pm 0,84$	$\pm 0,89$
14	$\pm 0,47$	$\pm 0,52$	$\pm 0,57$	$\pm 0,62$	$\pm 0,66$	$\pm 0,71$	$\pm 0,76$	$\pm 0,80$	$\pm 0,85$
15	$\pm 0,45$	$\pm 0,50$	$\pm 0,55$	$\pm 0,59$	$\pm 0,64$	$\pm 0,68$	$\pm 0,73$	$\pm 0,77$	$\pm 0,82$

NOTE It cannot be excluded that the precision may be better or worse when other test organisms, products, and/or interfering substances are tested. However, it is likely that the precision in these cases will be in the same range.



## Annex F (informative)

### Information on the application and interpretation of European Standards on chemical disinfectants and antiseptics<sup>8)</sup>

#### F.1 Application and interpretation of test methods

General guidelines for the application and interpretation of test methods in accordance with European Standards for chemical disinfectants and antiseptics are as follows:

- a) all "use recommendations" for chemical disinfectant and antiseptic products should be supported by results of bactericidal, mycobactericidal, tuberculocidal, fungicidal, yeasticidal, sporicidal and virucidal European Standard tests that are appropriate to the intended field and method of application;
- b) to achieve this, chemical disinfectant and antiseptic products should be subjected to a specified programme of testing that will include phase 1, phase 2 step 1 and phase 2 step 2 tests, except for situations as given in points e), f) and g);
- c) "use recommendations" may be supported by results of phase 3 tests that are appropriate to the intended field and method of application;
- d) the various steps and phases are defined as follows:
  - phase 1                      suspension tests for the basic activity of the product;
  - phase 2 step 1            suspension tests under conditions representative of practical use;
  - phase 2 step 2            other laboratory tests e.g. handwash, handrub and surface tests simulating practical conditions;
  - phase 3                      field tests under practical conditions.
- e) it is accepted that for certain applications, the phase 2 step 1 and phase 2 step 2 tests may provide sufficient information for the particular application and that additional phase 1 tests may not be relevant.

For applications where phase 2 step 1 and phase 2 step 2 tests without phase 1 tests are used to support "use recommendations", the justification for omitting phase 1 tests should be given. Such applications will be indicated either in the European Standard itself or in the additional European Standard that specifies guidelines for the application and interpretation of the tests.

- f) it is accepted that for certain applications, the phase 2 step 1 suspension tests may provide sufficient information for the particular application and that additional phase 2 step 2 tests may not be relevant.

For applications where phase 2 step 1 tests without phase 2 step 2 tests are used to support use recommendations, the justification for omitting phase 2 step 2 tests should be given. Such applications

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<sup>8)</sup>CEN/TC 216 would like to draw the attention of the reader of this European Standard to the agreements which were reached concerning the relationship between this European Standard and future standards. The guidelines given in this annex should be followed when using the European Standards on chemical disinfectants and antiseptics.

will be indicated either in the European Standard itself or in the additional European Standard that specifies guidelines for the application and interpretation of the tests;

- g) it is accepted that for certain applications, the phase 2 step 2 together with phase 1 tests may provide sufficient information for the particular application and that additional phase 2 step 1 tests may not be relevant.

For applications where phase 2 step 2 tests without phase 2 step 1 tests are used to support product claims, the justification for omitting phase 2 step 1 tests should be given. Such applications will be indicated either in the European Standard itself or in the additional European Standard that specifies guidelines for the application and interpretation of the tests;

- h) All claims for "bioactive substances" should be supported by appropriate phase 1 tests.

## **F.2 Guide to interpretation of tests for chemical disinfectants and antiseptics**

A separate European Standard (or European Standards) that will be used as a guide to the interpretation of tests for chemical disinfectants and antiseptics will be prepared after the standard test methods have been agreed.

This European Standard will specify in detail the relationship of the various tests to one another and to "use recommendations".

## Bibliography

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