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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)

bsi.

National foreword

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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)

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European foreword

This document (EN 1657:2016) 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of an identical text or by endorsement, at the latest by October 2016, and conflicting national standards shall be withdrawn at the latest by October 2016.

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 1657:2005.

This European Standard was revised to harmonize the preparation of the fungal spore suspension with other fungicidal tests of CEN/TC 216 and to incorporate amendments applicable to all European Standards.

An additional requirement has been added for the *Aspergillus* spore suspension and therefore results obtained using EN 1657:2005 and not fulfilling this additional requirement will need to be confirmed by repeating the tests using EN 1657:2015.

The test conditions for teat disinfectants have been added.

According to the CEN/CENELEC Internal Regulations, the national standards organizations 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 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.

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

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

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

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, *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 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 Table 1 and Clause 5 under simulated low level soiling (3,0 g/l bovine albumin) or high level soiling (10 g/l yeast extract and 10 g/l bovine albumin) or 10 g/l skimmed milk for teat disinfectants or in additional test conditions.

Table 1 — Obligatory and additional test conditions

Test conditions	Fungicidal activity	Yeasticidal activity	Yeasticidal activity for teat disinfectants
Test organisms obligatory	*Aspergillus brasiliensis* *Candida albicans*	*Candida albicans*	*Candida albicans*
additional	any relevant test organism	any relevant test organism	any relevant test organism
Test temperature obligatory	10°C ± 1°C	10°C ± 1°C	30°C ± 1°C
additional	4°C ± 1°C; 20°C ± 1°C; 40°C ± 1°C;	4°C ± 1°C; 20°C ± 1°C; 40°C ± 1°C	20°C ± 1°C
Contact time obligatory	30 min ± 10 s	30 min ± 10 s	5 min ± 10 s for post-milking teat disinfectants 30 s ± 5 s for pre-milking teat disinfectants
additional	5 min ± 10 s; 60 min ± 10 s; 120 min ± 10 s[a]	5 min ± 10 s; 60 min ± 10 s; 120 min ± 10 s[a]	1 min ± 5 s
Interfering substance obligatory			
low level soiling high level soiling	3,0 g/l bovine albumin 10 g/l yeast extract plus 10 g/l bovine albumin	3,0 g/l bovine albumin 10 g/l yeast extract plus 10 g/l bovine albumin	10,0 g/l of reconstituted skimmed milk
additional	any relevant substance	any relevant substance	any relevant substance

The obligatory contact times for disinfectants stated in Table 1 were chosen to enable comparison of standard conditions.

NOTE For the additional conditions, the concentration defined as a result can be lower than the one obtained under the obligatory test conditions.

[a] The recommended contact time for the use of the product is within the responsibility of the manufacturer.

Any additional specific fungicidal activity shall be determined in accordance with 5.2.1 and 5.5.1.1 in order to take into account intended specific use 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°C ± 1 °C for 30 min ± 10 s or 30°C ± 1 °C for 5 min ± 10 s or 30 s± 5 s for teat disinfectants (obligatory test conditions). At the end of this contact time, an aliquot is taken, and the fungicidal/yeasticidal and/or the fungistatic/yeastistatic 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 brasiliensis* (fungicidal activity) or only the vegetative cells of *Candida albicans* (yeasticidal 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: [1)]

— *Candida albicans* ATCC 10231;

— *Aspergillus brasiliensis* ATCC 16404.

(formerly *A.niger*)

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 ± 1) °C (see 5.3.2.3). The same temperature shall be used for all incubations performed during a test and its control and validation.

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.

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 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)].

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.

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.

5.2.2.3 Malt extract agar (MEA)

Malt extract agar, consisting of:

Malt extract [a]	30,0 g
Agar	15,0 g
Water (5.2.2.2)	to 1 000,0 ml

[a] The malt extract should be of food grade (Cristomalt poudre from Difal is recommended) or equivalent that is not highly purified and not only based on maltose (Malt extract from OXOID is recommended [2)]).

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

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 ± 0,2 when measured at 20 °C ± 1 °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.

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2) 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. Equivalent products may be used if they can be shown to lead to the same results.

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. 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 ($NaHCO_3$) 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 ± 0,2, when measured at 20 °C ± 1 °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 ($CaCO_3$) 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 °C ± 1 °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.

5.2.2.8.4 Milk for teat disinfectants

Skimmed milk, guaranteed free of antibiotics and additives and reconstituted at a rate of 100 g powder per litre of water (5.2.2.2), shall be prepared as follows:

Prepare a solution of 100 g milk powder in 1 000 ml water (5.2.2.2). Heat for 30 min at 105°C ± 3 °C or 5 min at 121 °C ± 3 °C.

The final concentration of reconstituted milk in the test procedure (5.5) is 10,0 g/l.

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[3] 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 4°C ± 1 °C, 10 °C ± 1 °C, at 20 °C ± 1 °C, at 30°C ± 1 °C, 40°C ± 1 °C at 45 °C ± 1 °C (to maintain melted MEA in case of pour plate technique) and at additional test temperatures ± 1 °C (5.5.1).

5.3.2.3 Incubator, capable of being controlled at 30 °C ± 1 °C.

5.3.2.4 pH-meter, having an inaccuracy of calibration of no more than ± 0,1 pH units at 20 °C ± 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

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

5.3.2.6 Shakers

a) Electromechanical agitator, e.g. Vortex® mixer[4)]

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 microorganisms 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, 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 Flasks with ventilated caps.

5.3.2.15 Microscope capable of x 400 magnification

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.

[4)] Vortex® 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.

5.4.1.3 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 brasiliensis* (mould)

For *Aspergillus brasiliensis* (5.2.1), use only the first subculture grown on MEA (5.2.2.3) in Petri dishes or flasks with ventilated caps (5.3.2.15) and incubate for 7 days to 9 days at 30 °C ± 1°C. No further subculturing is needed. Do not stack the Petri dishes during the incubation to improve the temperature homogenization. At the end of incubation, all the cultures have to show a dark brown or black surface. Cultures with rare and small white or grey areas may be used (see Figure 1).

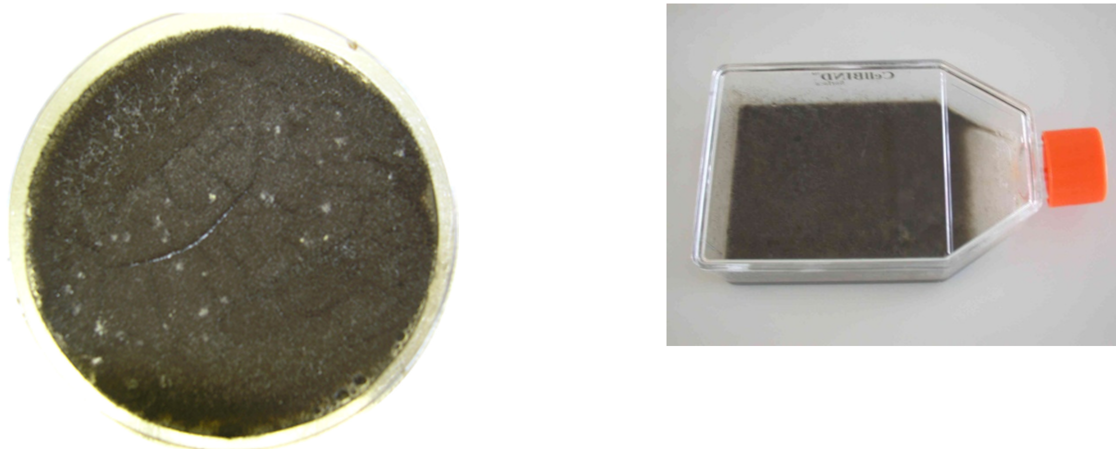

Figure 1 — Photo No 1: *A. brasiliensis* ATCC 16404 after 7 d of incubation at 30°C

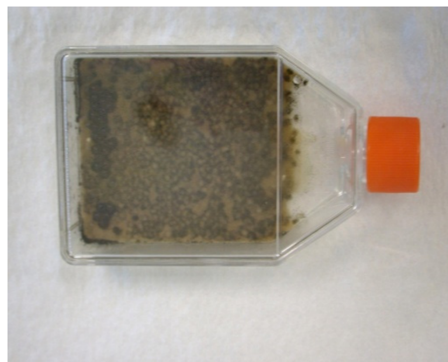

Figure 2 — Photo No.2: Example of inappropriate (not usable) culture of *A. brasiliensis* ATCC 16404 after 7 d of incubation at 30°C

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*

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[5)] 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 θ [5.5.1.1 a)] and use within 2 h.

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 colourimeter 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 ± 1) °C.

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

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 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 spores in 10 ml of sterile 0,05 % (w/v) polysorbate 80 solution in water (5.2.2.2). Using a sterile glass rod or spatula, detach the conidiospores from the culture surface. Transfer the suspension into a flask and gently shake by hand for one minute together with 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 (5.3.2.15) immediately after the preparation to show:

   1) the presence of a high concentration (at least 75 %) of characteristic mature spores i.e. spiny spores (versus smooth spores) [see Figures 3 and 4].

   If there are less than 75 % spiny conidiospores it may be due to the *Aspergillus brasiliensis* culture or the media used to produce the spores. In this situation, it will be necessary to obtain the culture from another cultrure collection and/or use a MEA from a different supplier..

   2) the absence of spore germination (check at least 10 fields of view).

   3) If germinated spores are present, discard the suspension.

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

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

If mycelia are still present, discard the suspension.

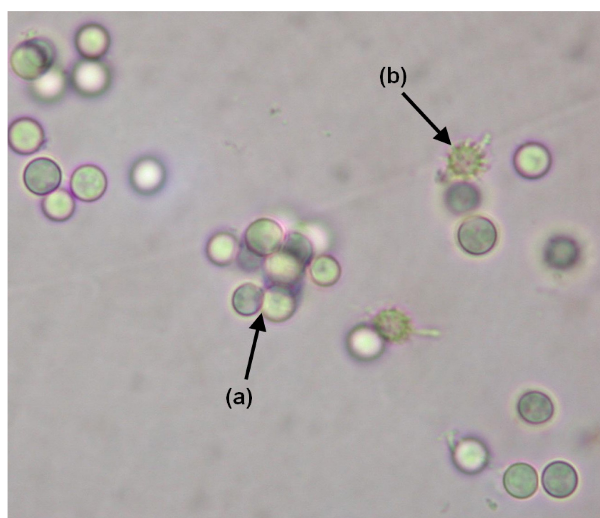

Figure 3 — Photo No 3: Observation of conidiospores under light microscope: presence of smooth (a) and spiny (b) spores (insufficient spiny spores)

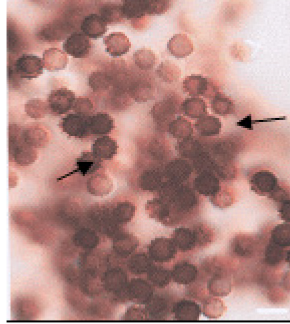

Figure 4 — Photo No 4: Observation of conidiospores under light microscope: High concentration of characteristic mature spores with spiny aspect (sufficient spiny spores)

c) Adjust the number of spores in the suspension to 1,5 × 10^7 cfu/ml to 5,0 × 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, maintain in a water bath controlled at 20°C ± 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 or 5.5.3).

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. 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 ± 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).

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 × 10^2 cfu/ml to 1,6 × 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 brasiliensis*, 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 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 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 g ± 10 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 microorganisms 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):

a) temperature (in °C):

   The obligatory and additional temperatures to be tested are specified in Clause 4, Table 1.

   The allowed deviation for each chosen temperature is ± 1 °C.

b) contact time t (in min):

   The obligatory and additional contact times to be tested are specified in Clause 4, Table 1.

   The allowed deviation for each chosen time is ± 10 s or ± 5 s for contact times of 1 min or less.

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 or skimmed milk 10 g/l for teat disinfectants (5.2.2.8.4) according to Clause 4, Table 1 and practical applications. Additional interfering substances may be tested according to specific fields of application.

d) test organisms:

   Aspergillus brasiliensis and *Candida albicans* (Clause 4, Table 1 and 5.2.1).

   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/or 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.

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.

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 θ [5.5.1.1 a)] using the water bath (5.3.2.2) controlled at θ. Check that the temperature of the reagents is stabilized at θ.

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 °C ± 1 °C.

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

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 [6)]

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 and separately for each experimental condition (5.5.1.1).

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 θ [5.5.1.1 a)] for 2 min ± 10 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 θ 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 ± 1)°C. After a neutralization time of 5 min ± 10 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 °C ± 1 °C.

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

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

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:

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 θ for 2 min ± 10 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 θ 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 ± 1) °C for 5 min ± 10 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 θ 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 ± 1) °C for 5 min ± 10 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 ± 1) °C for t. 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 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 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 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[7)]

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.

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

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 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 sample 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 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 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 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 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 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 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 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 Vc 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 Nv 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, Nv, N_0, Nv_0, Na 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* or 5 min (*B*) or 30 min (*C*)**
Test	*N* Test suspension	N_0 (= $N/10$)	Na (before neutralization or filtration)
Controls	*Nv* Validation suspension	Nv_0 (= $Nv/10$)	*A*, *B*, *C*

5.6.1.2 Vc values

All experimental data are reported as Vc values:

— in the dilution-neutralization method (test and controls), a Vc value is the number of colony-forming units counted per 1,0 ml sample;

— in the membrane filtration method, a Vc 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 control A.

5.6.2 Calculation

5.6.2.1 General

The first step in the calculation is the determination of the Vc values, the second the calculation of N, N_0, Na, Nv, Nv_0, A, B and C. The third step is the calculation of the reduction R (5.8.1).

5.6.2.2 Determination of Vc values

The Vc 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 Vc 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 (5.4.1.4), the validation suspension Nv (5.4.1.5) and for all countings of the dilution-neutralization method (5.5.2.6), determine and record the Vc values according to the number of plates used per 1 ml sample (5.6.1.2).

If more than one plate per 1 ml sample has been used to determine the Vc 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 Vc value as “> sum of the counts” (e.g. for “ >165, 132, 144”, report “> 441”).

If a Vc value is lower than 14, report the number (but substitute by “< 14” for further calculation in the case of Na).

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

c) Only Vc values within the respective counting limits are taken into account for further calculation, except in the case of Na (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).

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:

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

where

c is the sum of Vc values taken into account;

n_1 is the number of Vc values taken into account in the lower dilution, i.e. 10^{-5};

n_2 is the number of Vc 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

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

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 Na

Na 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 Vc 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 Na using the following formula:

$$Na = 10c / n \tag{2}$$

where

c is the sum of Vc values taken into account;

n is the number of Vc values taken into account.

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

EXAMPLES

a) duplicate Vc values: 2, 16

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

b) duplicate Vc values (pour plate, *Aspergillus brasiliensis*): > 165, > 165

$$Na = \frac{(>165+>165)\text{ x }10}{2} = >1\,650 = >1{,}6 \times 10^3$$

c) duplicate Vc values (membrane-filtration, *Aspergillus brasiliensis*): 40, > 55

$$Na = \frac{(40+>55)\text{x}10}{2} = >475 = >4{,}8 \times 10^2$$

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

$$Na = \frac{(>660+600)\text{x}10}{2} = >6\,300 = >6{,}3 \times 10^3$$

5.6.2.5 Calculation of Nv and Nv_0

Nv 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 Vc values due to the dilution step of 10^{-1} (5.4.1.5).

Nv_0 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 Vc values of Nv [5.4.1.6 c)] taken into account.Calculate Nv and Nv_0 using the following formulas:

$$Nv = 10c / n \quad (3)$$

$$Nvo = c / n \quad (4)$$

where

c is the sum of Vc values taken into account;

n is the number of Vc 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 Vc values of the mixtures A, B and C taken into account.

Calculate A, B and C using the following formula:

$$A, B, C = c/n \quad (5)$$

where

c is the sum of Vc values taken into account;

n is the number of Vc 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 *Vc* values [5.6.2.2b)], it is advised to check for the weighted mean as mentioned above but to use only the *Vc* 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 $5,0 \times 10^7$ ($7,17 \leq \lg N \leq 7,70$);

b) N_0 is between $1,5 \times 10^6$ and $5,0 \times 10^6$ ($6,17 \leq \lg N0 \leq 6,70$);

c) Nv_0 is between 30 and 160 ($3,0 \times 10^1$ and $1,6 \times 10^2$);

d) (Nv is between $3,0 \times 10^2$ and $1,6 \times 10^3$);

e) A, B, C are equal to or greater than 0,5 x Nv0;

f) control of weighted mean counts (5.7.2): quotient is not lower than 5 and not higher than 15.

5.7.4 Additional limits for *Aspergillus brasiliensis*

Check for 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 ($R = N_0/Na$) 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 formula:

$$\lg R = \lg N_0 - \lg N_a \quad (6)$$

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/yeasticidal concentration

5.8.3.1 Fungicidal concentration

For each test organism, record the lowest concentration of the product which passes the test (lg R ≥ 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 Yeasticidal concentration

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

5.8.4 Precision, replicates

See EN 14885, Clause 5 Precision of the test methods (Repetitions)

5.9 Interpretation of results – conclusion

5.9.1 General

According to the chosen experimental conditions (obligatory or obligatory and additional) the fungicidal or yeasticidal concentrations determined according to this European Standard may differ (Clause 4).A product can only pass the test if if the requirements of 5.8.2 are fulfilled.

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 brasiliensis*.

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.

5.9.6 Yeasticidal activity for teat disinfectants

The product shall be deemed to have passed the EN 1657 standard (yeasticidal activity) for post-milking teat disinfectants if it demonstrates in a valid test at least a 4 lg reduction within 5 min or less at 30 °C or for pre-milking teat disinfectants within 30 s or less at 30°C with skimmed milk interfering substance under the conditions defined by this European Standard when the test organism is *Candida albicans*.

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 yeasticidal 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

Candida albicans:	ATCC	10231
	CIP	4872
	DSM	1386
	CBS	6431
	NCTC	3179
Aspergillus brasiliensis:	ATCC	16404
Formerly *Aspergillus niger*	DSM	1988
	CBS	733.88
	CIP	1431.83
	NCTC	2275
	IMI	149007

Abbreviations:

ATCC	American Type Culture Collection
CBS	CBS-KNAW Fungal Biodeiversity Centre
CIP	Collection de l'Institut Pasteur
DSM	Deutsche Sammlung von Mikrooganismen und Zellkulturen
NCTC	National Collection of Type Cultures
IMI	International Mycological Institute

Annex B
(informative)
Suitable neutralizers and rinsing liquids

IMPORTANT Neutralizers of the residual antimicrobial activity of chemical disinfectants and antiseptics and rinsing fluids shall be validated according to the prescriptions of the standard

Table B.1

Antimicrobial agent	Chemical compounds able to neutralize residual antimicrobial activity	Examples of suitable neutralizers and of rinsing liquids (for membrane filtration methods) [a)]
Quaternary ammonium compounds and fatty amines Amphoteric compounds	Lecithin, Saponin, Polysorbate 80, Sodium dodecyl sulfate, Ethylene oxide condensate of fatty alcohol (non-ionic surfactants) [b)]	- Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3g/l. - Polysorbate 80, 30 g/l + sodium dodecyl sulfate, 4 g/l + lecithin, 3g/l. - Ethylene oxide condensate of fatty alcohol, 3 g/l + lecithin, 20g/l + polysorbate 80, 5g/l. - *Rinsing liquid: tryptone, 1g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*
Biguanides and similar compounds	Lecithin [c)], Saponin, Polysorbate 80	- Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3g/l. - *Rinsing liquid: tryptone, 1g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*
Oxidizing compounds (Chlorine, iodine, hydrogen peroxide, peracetic acid, hypochlorites, etc…)	Sodium thiosulphate [d)] Catalase [for hydrogen peroxide or products releasing hydrogen peroxide]	- Sodium thiosulphae, 3 g/l to 20 g/l + polysorbate 80, 30 g/l + lecithin, 3 g/l. - Polysorbate 80, 50 g/l + catalase 0,25g/l + lecithin 10 g/l. - *Rinsing liquid: sodium thiosulphate, 3 g/l*
Aldehydes	L-histidine Glycine	- Polysorbate 80, 30 g/l + lecithin, 3g/l + L-histidine, 1g/l (or + glycine, 1 g/l). - Polysorbate 80, 30 g/l + saponin, 30 g/l + L-histidine, 1 g/l (or + glycine, 1 g/l). - *Rinsing liquid: polysorbate 80, 5 g/l + L-histidine, 0,5 g/l (or + glycine, 1 g/l).*
Phenolic and related compounds: orthophenylphenol, phenoxyethanol, triclosan, phenylethanol, etc… Anilides	Lecithin Polysorbate 80 Ethylene oxide condensate of fatty alcohol[b)]	- Polysorbate 80, 30 g/l + lecithin, 3g/l. - Ethylene oxide condensate of fatty alcohol, 7 g/l + lecithin, 20 g/l + polysorbate 80, 4 g/l. - *Rinsing liquid: tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*
Alcohols	Lecithin, Saponin, Polysorbate 80 [e)]	- Polysorbate 80, 30 g/l + saponin, 30 g/l +lecithin, 3 g/l. - *Rinsing liquid: tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*

[a] 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_2PO_4) 34 g; distilled water (500 ml); adjusted to pH 7,2 ± 0,2 with sodium hydroxide (NaOH) 1 mol/l; distilled water up to 1000 ml].

[b] The carbon chain-length varies from C_{12} to C_{18} carbon atoms.

[c] Egg and soya; egg is preferable.

[d] The toxic effect of sodium thiosulphate differs from one microorganism to another.

[e] For the neutralization of short chain alcohols (less than C_5), simple dilution may be appropriate. Care should be taken if the alcohol-based products contain additional antimicrobial agents

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

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

Annex C
(informative)
Graphical representation of test procedures

C.1 Dilution-neutralization method

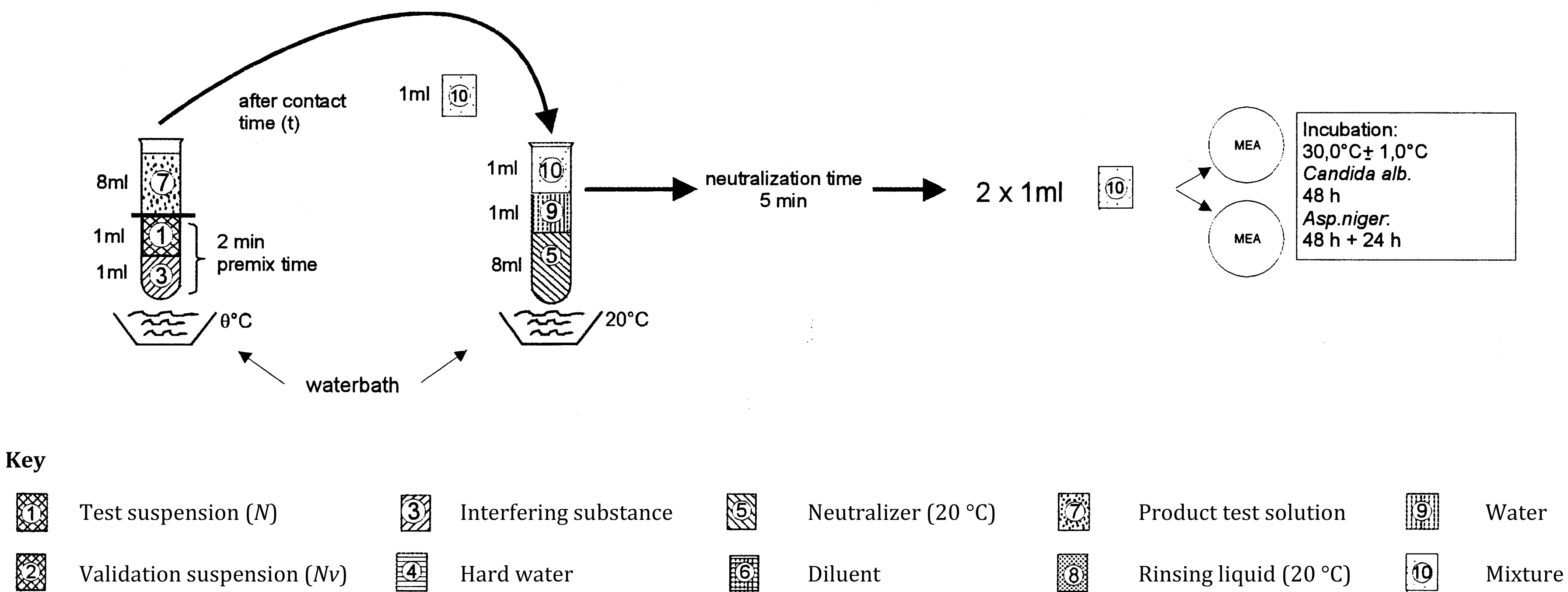
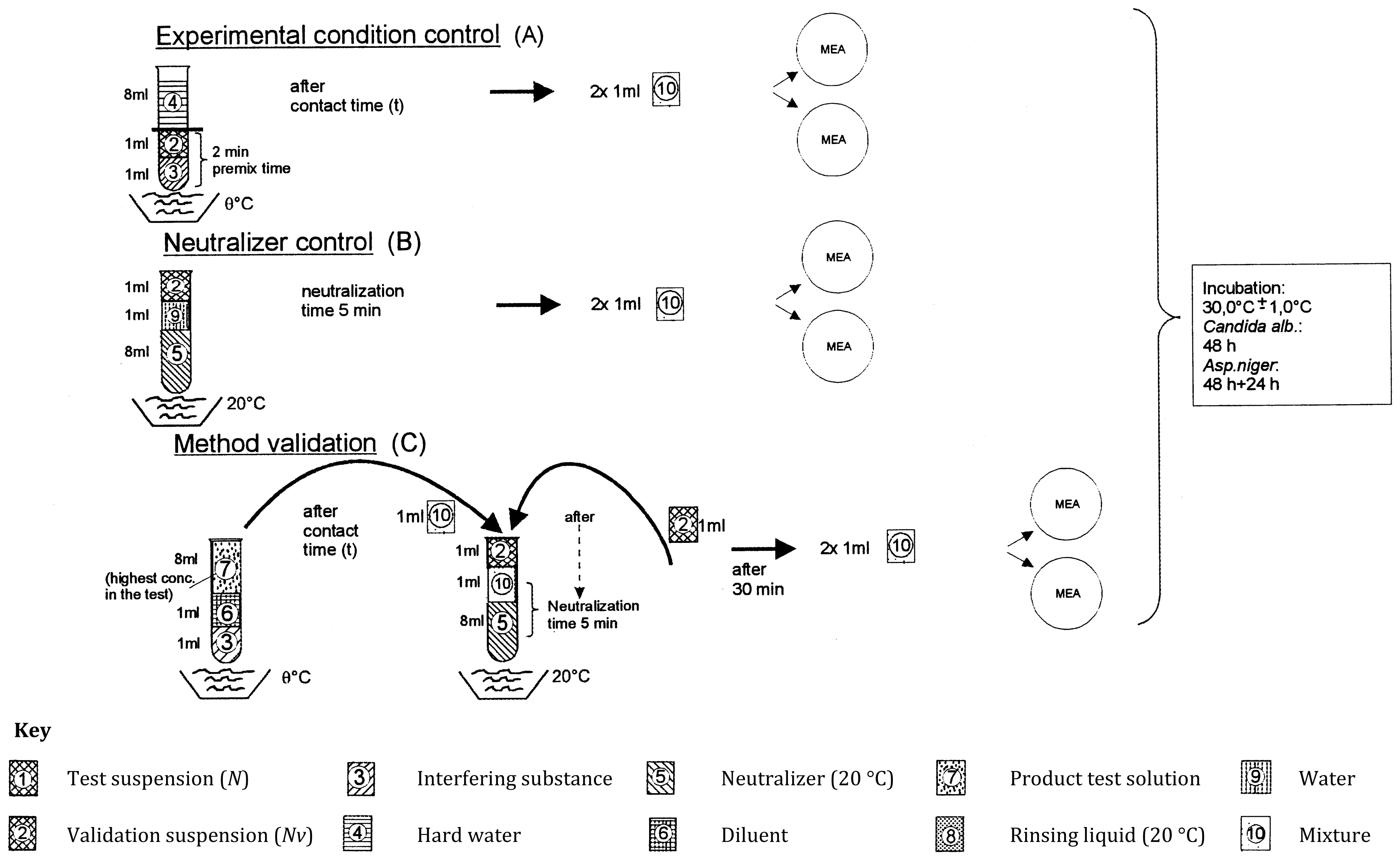


Figure C.1 — Test (Na)

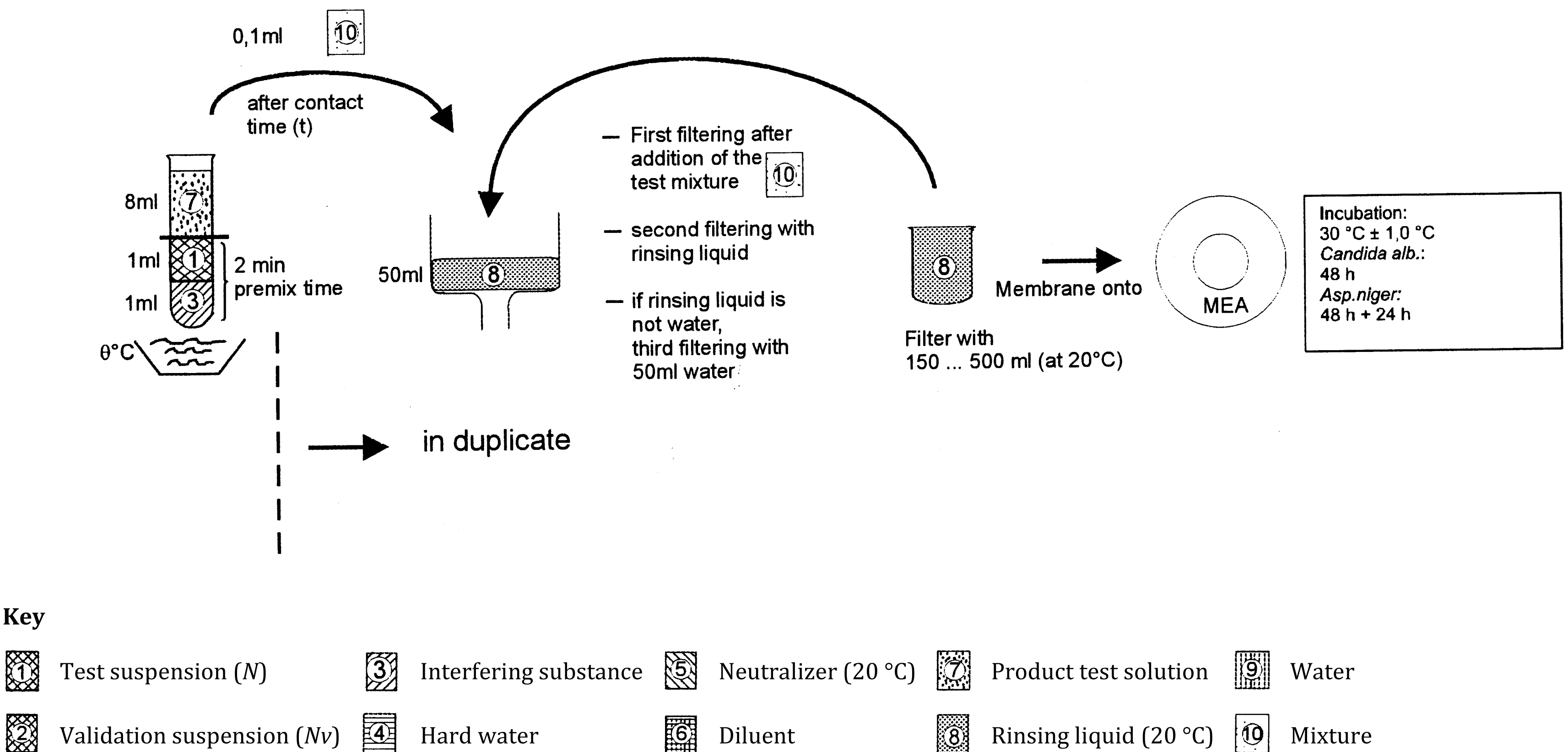


Key

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

Figure C.2 — Validation

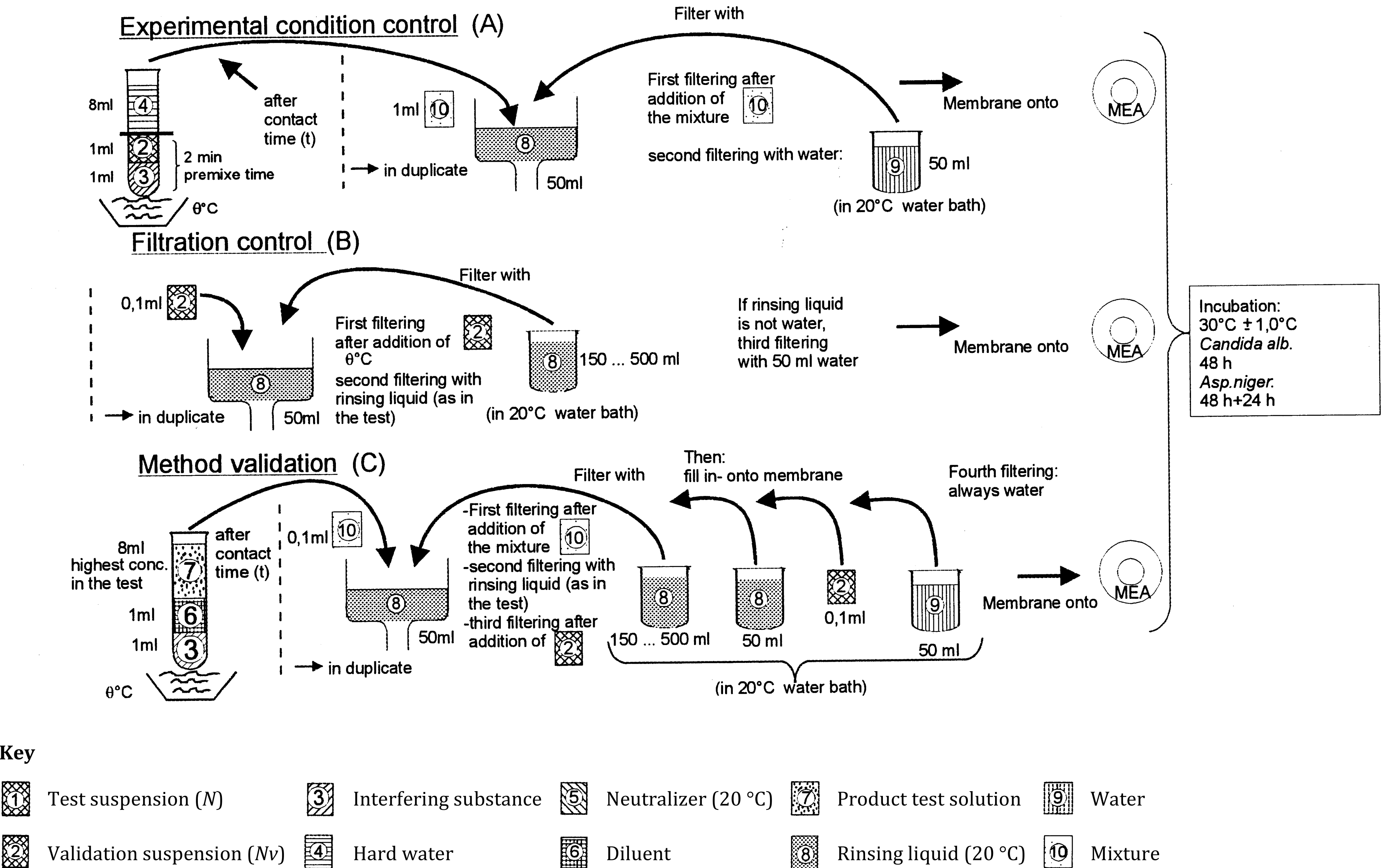
C.2 Membrane filtration method



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

Figure C.3 — Test (Na)



Key

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

Figure C.4 — 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* and for *Candida albicans* are given as an example.

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

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TEST REPORT

EN 1657, FUNGICIDAL ACTIVITY

(obligatory and additional conditions)

1. **Client**: Centipede Formulations Inc., Markkleeberg / 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: 2014–07–16 **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 brasiliensis 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 brasiliensis* 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 brasiliensis).*

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, 2014–10–10

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 brasiliensis 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

Concentration of spiny spores of *A. brasiliensis 75 % or more* in the conidiospore suspension

yes ☒ *no* ☐

Validation suspension (**Nv_0**)			Experimental Conditions control (***A***)			Neutralizer or filtration control (***B***)			Method validation (***C***) Product conc.: *10 ml/l*		
*Vc*1	*84*	$\overline{x}$ =	*Vc*1	*85*	$\overline{x}$ =	*Vc*1	*81*	$\overline{x}$ =	*Vc*1	*79*	$\overline{x}$ =
*Vc*2	*87*	85,5	*Vc*2	*83*	*84*	*Vc*2	*85*	*83*	*Vc*2	*86*	*82,5*
30 ≤ $\overline{x}$ of Nv_0 ≤ 160 ? ☒ *yes* ☐ *no*			$\overline{x}$ of *A* is ≥ 0,5x $\overline{x}$ of Nv_0 ? ☒ *yes* ☐ *no*			$\overline{x}$ of *B* is ≥ 0,5x $\overline{x}$ of Nv_0? ☒ *yes* ☐ *no*			$\overline{x}$ of *C* is ≥ 0,5x $\overline{x}$ of Nv_0 ? ☒ *yes* ☐ *no*		

Test suspension and Test

Test-suspension (***N*** **and** N_0):	***N***	*Vc*1	*Vc*2	$\overline{x}$ wm = *147,27* × 10^5 ; lgN = *7,17*
	10^{-5}	*139*	*154*	N_0 = *N*/10 ; lgNo = *6,17*
	10^{-6}	*14*	*17*	**6,17 ≤ lgN_0 ≤ 6,70 ?** ☒ ***yes*** ☐ ***no***

Conc.of the product %	*Vc*1	*Vc*2	***Na*** **=** $\overline{x}$ x10	**lg*Na***	**lg*R***	Contact time (min)
0,50	*> 165*	*> 165*	*> 1 650*	*> 3,22*	*< 2,95*	*30 min*
0,75	*57*	*65*	*610*	*2,78*	*3,39*	*30 min*
1,00	*0*	*7*	*< 140*	*< 2,15*	*> 4,02*	*30 min*

Countings per plate for (*N*): 10^{-5} = 60 + 79, 72 + 82, 10^{-6} = 4 + 10, 2 + 15

Explanations:

Vc = count per ml (one plate or more)

$\overline{x}$ = average of *Vc*1 and *Vc*2 (1. + 2. duplicate)

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

R = reduction (lg*R* = lgN_0 – lg*Na*)

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 . Two / 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 (Nv_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*	*76,5*	$Vc2$	*76*	*73*
$30 \leq \bar{x}$ of $Nv_0 \leq 160$? ☒ *yes* ☐ *no*			$\bar{x}$ of A is $\geq 0,5 \mathrm{x} \bar{x}$ of Nv_0 ? ☒ *yes* ☐ *no*			$\bar{x}$ of B is $\geq 0,5 \mathrm{x} \bar{x}$ of Nv_0? ☒ *yes* ☐ *no*			$\bar{x}$ of C is $\geq 0,5 \mathrm{x} \bar{x}$ of Nv_0 ? ☒ *yes* ☐ *no*		

<u>Test suspension and Test</u>

<u>Test-suspension</u> (**N and N_0**):	***N***	$Vc1$	$Vc2$	$\bar{x}$ wm = *259,54* × 10^5 ; lgN = *7,41*
	10^{-5}	*241*	*283*	$\mathbf{N_0}$ = N/10 ; lgNo = *6,41*
	10^{-6}	*20*	*27*	**6,17 ≤ lgN_0 ≤ 6,70** ? ☒ ***yes*** ☐ ***no***

Conc.of the product %	$Vc1$	$Vc2$	***Na*** **=** $\bar{x}$ x10	**lg*Na***	**lg*R***	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.** Nv_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^{-5} 120 + 121, 130 + 153 **6.** *N* 10^{-6} 10 + 10, 15 + 12 **7.** *Na* 0,25 % > 330 + > 330, 320 + 280 **8.** *Na* 0,5 % 13 + 8, 7 + 21 **9.** *Na* 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 ($\lg R = \lg N_0 - \lg Na$)

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.

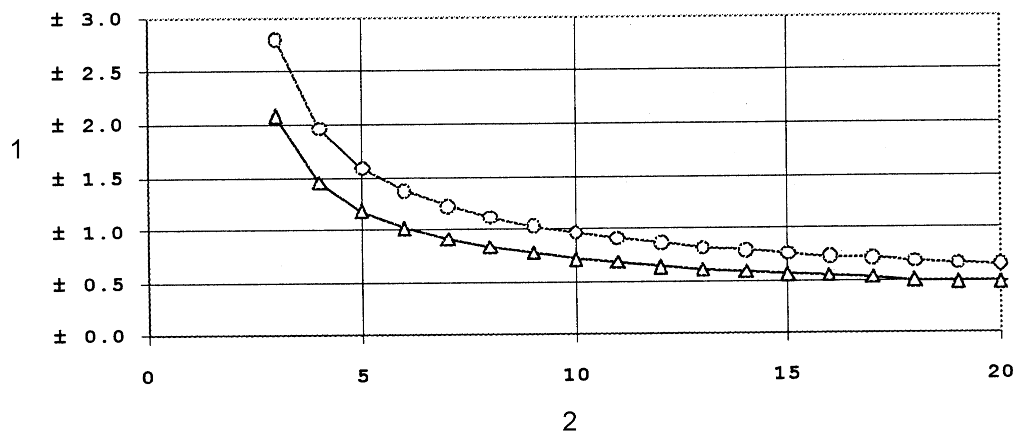
Table E.1 — Theoretical reduction factors used for fungi (in logarithmic terms)

	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 (σ = 1,66), while the second one was based on an average “inside” dispersion (σ = 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 ± 2,80, while the “mean” case was about ± 2,07. In other words, if the precision target is fixed to ± 1 lg reduction, 10 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 (σ)								
	1,0	**1,1**	**1,2**	**1,3**	**1,4**	**1,5**	**1,6**	**1,7**	**1,8**
2	***± 4,46***	***± 4,91***	***± 5,36***	***± 5,80***	***± 6,25***	***± 6,70***	***± 7,14***	***± 7,59***	***± 8,04***
3	***± 1,69***	***± 1,85***	***± 2,02***	***± 2,19***	***± 2,36***	***± 2,53***	***± 2,70***	***± 2,87***	***± 3,03***
4	***± 1,18***	***± 1,29***	***± 1,41***	***± 1,53***	***± 1,65***	***± 1,77***	***± 1,88***	***± 2,00***	***± 2,12***
5	±0,95	***± 1,05***	***± 1,14***	***± 1,24***	***± 1,33***	***± 1,43***	***± 1,53***	***± 1,62***	***± 1,72***
6	±0,82	±0,90	±0,99	***± 1,07***	***± 1,15***	***± 1,23***	***± 1,32***	***± 1,40***	***± 1,48***
7	±0,73	±0,81	±0,88	±0,95	***± 1,03***	***± 1,10***	***± 1,18***	***± 1,25***	***± 1,32***
8	±0,67	±0,74	±0,80	±0,87	±0,94	***± 1,00***	***± 1,07***	***± 1,14***	***± 1,21***
9	±0,62	±0,68	±0,74	±0,81	±0,87	±0,93	±0,99	***± 1,05***	***± 1,12***
10	±0,58	±0,64	±0,70	±0,75	±0,81	±0,87	±0,93	±0,99	***± 1,04***
11	±0,55	±0,60	±0,66	±0,71	±0,77	±0,82	±0,87	±0,93	±0,98
12	±0,52	±0,57	±0,62	±0,67	±0,73	±0,78	±0,83	±0,88	±0,93
13	±0,49	±0,54	±0,59	±0,64	±0,69	±0,74	±0,79	±0,84	±0,89
14	±0,47	±0,52	±0,57	±0,62	±0,66	±0,71	±0,76	±0,80	±0,85
15	±0,45	±0,50	±0,55	±0,59	±0,64	±0,68	±0,73	±0,77	±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.

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[1] European Pharmacopoeia (EP), Water for injections

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