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Chemical disinfectants and antiseptics — Quantitative surface test for the evaluation of fungicidal or yeasticidal activity of chemical disinfectants and antiseptics used in the veterinary area on non-porous surfaces without mechanical action — Test method and requirements (phase 2, step 2)

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National foreword

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

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Chemical disinfectants and antiseptics - Quantitative surface test for the evaluation of fungicidal or yeasticidal activity of chemical disinfectants and antiseptics used in the veterinary area on non-porous surfaces without mechanical action - Test method and requirements (phase 2, step 2)

Antiseptiques et désinfectants chimiques - Essai quantitatif de surface pour l'évaluation de l'activité fongicide ou levuricide des antiseptiques et des désinfectants chimiques utilisés dans le domaine vétérinaire sur des surfaces non poreuses sans action mécanique - Méthode d'essai et prescriptions (phase 2, étape 2)

Chemische Desinfektionsmittel und Antiseptika - Quantitativer Oberflächenversuch zur Bestimmung der fungiziden oder levuroziden Wirkung chemischer Desinfektionsmittel und Antiseptika für den Veterinärbereich auf nicht-porösen Oberflächen ohne mechanische Wirkung - Prüfverfahren und Anforderungen (Phase 2, Stufe 2)

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Foreword

This document (EN 16438:2014) 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of an identical text or by endorsement, at the latest by August 2014 and conflicting national standards shall be withdrawn at the latest by August 2014.

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Introduction

This European Standard specifies a surface test for establishing whether a chemical disinfectant or antiseptic has or does not have 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 utilisation concentration of the chemical disinfectant or antiseptic, found by this test corresponds to the chosen experimental conditions. However, for some applications the instructions 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 in hard water or – in the case of ready-to-use products– with water.

This European Standard applies to products for use 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 2 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 3 decimal log (lg) reduction from a water control, when tested in accordance with Table 1 and Clause 5 under simulated low level (3,0 g/l bovine albumin) or high level soiling (10 g/l yeast extract and 10 g/l bovine albumin) on a surface.

Table 1 — Obligatory and additional test conditions

Test Conditions	Fungicidal or yeasticidal activity on non-porous surfaces without mechanical action in the veterinary area
Test organism Fungicidal activity a) obligatory	*Aspergillus brasiliensis (conidiospores)* and *Candida albicans (vegetative cells)*
Test organism Yeasticidal activity a) obligatory	*Candida albicans (vegetative cells)*
b) additional	any relevant test organism
Test temperature a) obligatory	10 °C ± 1 °C
b) additional	4 °C ± 1 °C; 20 °C ± 1 °C; 40 °C ± 1 °C
Contact time a) obligatory	60 min ± 10 s
b) additional*	5 min ± 10 s; 30 min ± 10 s; 120 min ± 10 s
Interfering substance a) obligatory low level soiling high level soiling	3,0 g/l bovine albumin 10 g/l yeast extract plus 10 g/l bovine albumin
b) additional	any relevant substance
The obligatory contact times for surface disinfectants stated in Table 1 were chosen to enable comparison of standard conditions. *The recommended contact time for the use of the product is within the responsibility of the manufacturer. NOTE For the additional conditions, the concentration defined as a result can be lower than the one obtained under the obligatory test conditions.	

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

A test suspension of fungal conidiospores or yeast and interfering substance is inoculated onto the test surface and dried. After a drying time, an aliquot of the product under test is transferred to the surface, in a manner which covers the dried film. The surface is maintained at a specified temperature for a defined period of time specified in Clause 4 and 5.5.1.1. At the end of that contact time the surface is transferred to a neutraliser so that the action of the disinfectant is immediately neutralised. The numbers of surviving organisms which can be recovered from the surface is determined quantitatively.

The number of fungi or yeast on a surface treated with water in place of the disinfectant is also determined and the reduction is calculated.

The test is performed using the conidiospores of *Aspergillus brasiliensis* (formerly niger) and the vegetative cells of *Candida albicans* (fungicidal activity) or only the vegetative cells of *Candida albicans* (yeasticidal

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

5.2 Materials and reagents

5.2.1 Test organism

The fungicidal activity shall be evaluated using the following strains as the test organisms[1)]:

Aspergillus brasiliensis (formerly *Aspergillus niger*) ATCC 16404

Candida albicans ATCC 10231

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 organisms is 30 °C ± 1 °C (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 organism.

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.

Ready-to-use medium may be used if it complies with the required specification.

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

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.

5.2.2.2 Water

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

Sterilise in the autoclave [5.3.2.1 a)].

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

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

NOTE 3 See 5.2.2.6 for the procedure to prepare hard water.

5.2.2.3 Malt extract agar (MEA)

Malt extract agar, consisting of:

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

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

Sterilise in the autoclave [5.3.2.1a)]. After sterilisation, 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 neutralisation (5.5.1.2 and 5.5.1.3), it may be necessary to add neutraliser to the MEA. Annex B gives guidance on the neutralisers that may be used. It is recommended not to use a neutraliser that causes opalescence in the agar.

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 ml

Sterilise in the autoclave [5.3.2.1 a)]. After sterilisation, the pH of the diluent shall be equivalent to 7,0 ± 0,2 when measured at 20 °C ± 1 °C.

5.2.2.5 Neutraliser

The neutraliser 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 neutralisers 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.

5.2.2.6 Hard water for dilution of products

For the preparation of 1 000 ml of hard water, the procedure is as follows:

— prepare solution A: dissolve 19,84 g magnesium chloride ($MgCl_2$) and 46,24 g calcium chloride ($CaCl_2$) in water (5.2.2.2) and dilute to 1 000 ml. Sterilise by membrane filtration (5.3.2.7) or in the autoclave [5.3.2.1 a)]. Autoclaving if used – may cause a loss of liquid. In this case make up to 1 000 ml with water (5.2.2.2) under aseptic conditions. Store the solution in the refrigerator (5.3.2.8) for no longer than one month.

— prepare solution B: dissolve 35,02 g sodium bicarbonate ($NaHCO_3$) in water (5.2.2.2) and dilute to 1 000 ml. Sterilise by membrane filtration (5.3.2.7). Store the solution in the refrigerator (5.3.2.8) for no longer than one week;

— place 600 ml to 700 ml of water (5.2.2.2) in a 1 000 ml volumetric flask (5.3.2.12) and add 6,0 ml 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 375 mg/l of calcium carbonate ($CaCO_3$) in the test tube.

5.2.2.7 Interfering substance

5.2.2.7.1 General

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

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

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

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

5.2.2.7.2 Low level soiling (bovine albumin solution)

Dissolve 0,6 g of bovine albumin V (suitable for microbiological purposes) in 90 ml of water (5.2.2.2) in a 100 ml volumetric flask. Make up to the mark with water (5.2.2.2).

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

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

5.2.2.7.3 High level soiling (mixture of bovine albumin solution with yeast extract)

Dissolve 10 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 sterilise in an autoclave [5.3.2.1 a)]. Allow to cool to 20 °C ± 5 °C.

Pipette 25 ml of this solution into a 50 ml volumetric flask and add 10 ml of water (5.2.2.2). Dissolve 1 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) sterilise by membrane filtration and keep in a refrigerator (5.3.2.8) and use within one month.

The final concentration in the test procedure (5.5.2) is 10 g/l yeast extract and 10 g/l bovine albumin.

5.2.3 Test surface

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

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

The surface shall not be allowed to dry to any extent. The discs shall only be handled with forceps. Rinse the discs with flowing water for a further 10 s to ensure complete removal of the surfactant. To supply a satisfactory flow of water, a fluid dispensing pressure vessel with suitable hose and connectors or other suitable method can be used and regulated to supply approximately 2 000 ml per min. Place the clean discs in a bath containing 95 % 2-propanol for 15 min. Remove the discs and dry by evaporation.

5.3 Apparatus and glassware

5.3.1 General

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

a) by moist heat, in an autoclave [5.3.2.1 a)];

b) by dry heat, in a hot air oven [5.3.2.1 b)].

5.3.2 Usual microbiological laboratory equipment [4)]

and, in particular, the following:

5.3.2.1 Apparatus for sterilisation (moist and dry heat)

a) for moist heat sterilisation, an autoclave capable of being maintained at 121 $^{+3}_{0}$ °C for a minimum holding time of 15 min;

b) for dry heat sterilisation, 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 bath, capable of being controlled at 10 °C ± 1 °C, 20 °C ± 1 °C, and 45 °C ± 1 °C (to maintain melted MEA. If pour plate technique is used), and at additional temperatures ± 1 °C (5.5.1).

5.3.2.3 Incubator, capable of being controlled at 30 °C ± 1 °C and 37 °C ± 1 °C (for drying surfaces)

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

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3) Decon concentrate is obtained from Decon Laboratories Ltd, Conway Street, Hove, East Sussex, BN3 3LY, UK Tel. 01273 756598. Studies have shown that this method of cleaning is satisfactory. A suitable 'Generic' will be specified at a later stage.

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

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® [5)] mixer;

b) Mechanical shaker.

5.3.2.7 Membrane filtration apparatus, constructed of a material compatible with the substances to be filtered with a filter holder of at least 50 ml volume and suitable for use with filters of diameter 47 mm to 50 mm and 0,45 µm pore size for filtration of hard water (5.2.2.6) and bovine albumin (5.2.2.7.2, 5.2.2.7.3).

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

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

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

5.3.2.11 Glass beads, diameter: 3 mm to 4 mm.

5.3.2.12 Volumetric flasks

5.3.2.13 Temperature controlled cabinet, capable of being controlled at 10 °C ± 1 °C.

5.3.2.14 Glass screw top container, with a base diameter of 4 cm - 5 cm.

5.3.2.15 Fritted filter, with porosity of 40 µm to 100 µm according to **ISO 4793**.

5.3.2.16 Flasks with vented caps.

5.3.2.17 Microscope capable of x 400 magnification.

5.3.2.18 Vacuum desicator capable of achieving a vacuum of 20-25 in. mercury.

5.4 Preparation of test organism suspensions and product test solutions

5.4.1 Test organism suspension (test and validation suspension)

NOTE Test and validation suspension are the same in this standard.

5.4.1.1 General

For each test organism, one suspension shall be prepared: this is used as the fungal “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.

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5) Vortex ® is an example of a suitable product available commercially. This information is given for the convenience of users of this 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 on to 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* (previously *A.niger*) (5.2.1), use only the first subculture grown on MEA (5.2.2.3) in Petri dishes or flasks with ventilated caps (5.3.2.16) 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 homogenisation. At the end of incubation, all the cultures shall show a dark brown or black surface. Cultures with the appearance of rare and small white or grey areas may be used (see Figure 1).

Figure 1 — *A. brasiliensis* ATCC 16404 after 7 days of incubation at 30°C (correct appearance)

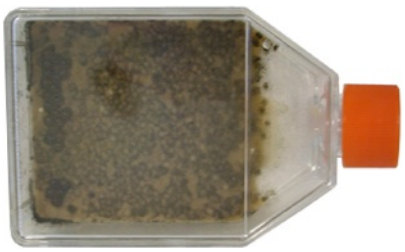

Figure 2 — *A. brasiliensis* ATCC 16404 after 7 days of incubation at 30°C (incorrect appearance)

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 suspension shall be noted, giving the reasons in the test report.

5.4.1.4 Test suspension (N)

NOTE Test and validation suspension are the same in this European Standard.

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 by rubbing the loop against the wet wall of the flask to dislodge the cells before immersing in the diluent. Shake the flask for 3 min using a mechanical shaker [5.3.2.6 b)]. Aspirate the suspension from the glass beads and transfer to another tube.

b) Adjust the number of cells in the suspension to $1{,}5 \times 10^8$ cfu/ml [6] to $5{,}0 \times 10^8$ cfu/ml using diluent (5.2.2.4), estimating the number of cfu by any suitable means. Maintain this test suspension in the waterbath at the test temperature θ [5.5.1.1] 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^{-6} and 10^{-7} dilutions of the test suspension using diluent (5.2.2.4). Mix [5.3.2.6 a)].

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

   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 °C ± 1 °C

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

For incubation and counting, see 5.4.1.5.

5.4.1.4.2 *Aspergillus brasiliensis*

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

a) Take the working culture (5.4.1.3.2) and suspend the conidiospores in 10 ml of sterile 0,05 % (w/v) polysorbate 80 in solution in water (5.2.2.2). Using a glass rod or spatula, detach the conidiospores from the culture surface. Transfer the suspension into a flask and gently shake by hand for one minute together with 5 g of glass beads (5.3.2.11). Filter the suspension through a fritted filter (5.3.2.15);

b) Carry out a microscopic examination under x 400 magnification (5.3.2.17) immediately after the preparation to show:

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[6] cfu/ml = colony forming unit(s) per millilitre

1) the presence of a high concentration (at least 75 % of spiny spores) of characteristic mature conidiospores 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* strain used or to the media. Therefore use another strain, preferably form another supplier, and/or try another medium;

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 second fritted filtration.

If mycelia are still present, discard the suspension.

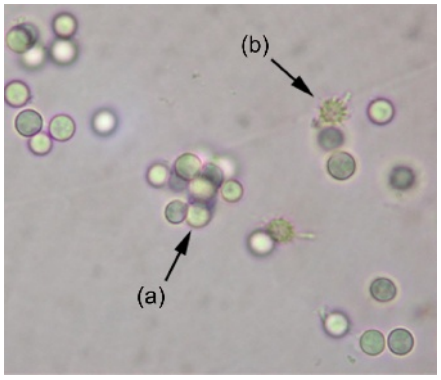

Figure 3 — Observation of conidiospores under light microscope: presence of smooth (a) and spiny (b) spores (insufficient spiny spores)

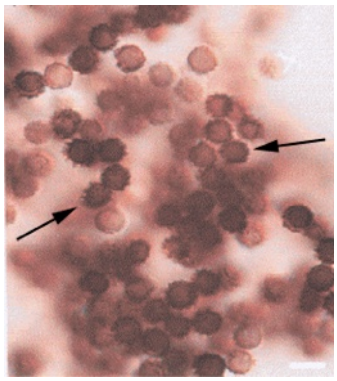

Figure 4 — Observation of conidiospores under light microscope: High concentration of characteristic mature spores with spiny aspect (sufficient spiny spores)

c) Adjust the number of conidiospores in the suspension to $1,5 \times 10^8$ cfu/ml to $5,0 \times 10^8$ cfu/ml using the diluent (5.2.2.4), estimating the number of cfu by any suitable means. Use the suspension within 48 h. It can be stored up to 2 days in the refrigerator and shall then be checked just before the test for absence of germinated conidiospores [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).

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 (5.4.1.5) 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^{-6} and 10^{-7} dilutions of the test suspension using diluent (5.2.2.4). Mix [5.3.2.6 a)].

Take a sample of 1,0 ml of each dilution 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 melted MEA (5.2.2.3), cooled to 45 °C ± 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 5.4.1.5.

5.4.1.5 Incubation and counting of the test suspension

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

a) Incubate (5.3.2.3) the plates for 42 h to 48 h at 30 °C ± 1 °C. Discard any plates that are not countable for any reason. Count the plates and determine the number of cfu.

b) Only for *Aspergillus brasiliensis*, incubate the plates for a further 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;

c) 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;

d) Calculate the numbers of cfu per 0,025 ml in the test suspension N using the method given in 5.6.2.3. Verify according to 5.7.

NOTE 0,05 ml of an equal parts mixture of the test suspension and interfering substance is added to the surface therefore 0,025 ml of the suspension is added.

5.4.2 Product test solutions

Product test solutions shall be prepared in hard water (5.2.2.6) at minimum three different concentrations to include one concentration in the active range and one concentration in the non-active range. 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 (5.2.2.6). Subsequent dilutions (lower concentrations) shall be prepared in volumetric flasks (5.3.2.12) on a volume/volume basis in hard water (5.2.2.6).

For liquid products, dilutions of the product shall be prepared with hard water 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.

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.

c) interfering substance:

The obligatory interfering substance to be tested is 3,0 g/l bovine albumin (5.2.2.7.2) for low level soiling or 10 g/l bovine albumin plus 10 g/l yeast extract (5.2.2.7.3) for high level soiling 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 Neutralisation

To determine a suitable neutraliser carry out the validation of the dilution-neutralisation method (5.5.2.4 and 5.5.2.5 in connection with 5.5.2.6) using a suitable neutraliser, chosen according to laboratory experience and/or published data.

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

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

5.5.1.3 General instructions for validation and control procedures

The neutralisation and/or removal of the fungicidal and/or yeasticidal 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 (water

control, neutraliser control and method validation) shall be performed at the same time with the test and with the same neutraliser used in the test.

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

5.5.1.4 Equilibration of temperature

Prior to testing, equilibrate the inoculated and dried stainless steel discs and all reagents (product test solutions (5.4.2), hard water (5.2.2.6)) to the test temperature of θ [5.5.1.1 a)] using the water bath (5.3.2.2) and/or the temperature controlled cabinet (5.3.2.13) controlled at θ. Check that the temperature of the reagents is stabilised at θ.

The neutraliser (5.2.2.5) and diluents (5.2.2.4) shall be equilibrated at a temperature of 20 °C ± 1 °C.

5.5.2 Test procedure (Dilution-neutralisation method)

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

NOTE Graphical representations of the method are given in Annex C.

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

a) To prepare the microbial test suspension pipette 1,0 ml of the interfering substance (5.2.2.7) into a tube. Add 1,0 ml of the test suspension (5.4.1.4). Start the stopwatch (5.3.2.5) immediately, mix [5.3.2.6 a)] and place the tube in a water bath or temperature controlled cabinet at the chosen test temperature θ (5.5.1.1) for 2 min ± 10 s.

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

b) Place the test surfaces (5.2.3) in a sterile Petri dish (5.3.2.10) and ensure that the dish is in a horizontal position. Prepare the test surfaces by inoculating 0,05 ml of the microbial test suspension [5.5.2.2 a)] on to each test surface.

   Dry the surfaces using either of the following methods:

   1) In open Petri dishes in an incubator (5.3.2.3) at 37 °C ± 1 °C until they are visibly dry.

   2) In open Petri dishes in a vacuum desicator (5.3.2.18) at room temperature under a vacuum of 20-25 in. mercury until they are visibly dry.

   It is understood that drying of the test surfaces will occur at different rates due to the ambient conditions of the laboratory and the design of the incubator (e.g. with or without fan). For this reason, no time duration is given and the minimum required time for surfaces to become visibly dry should be established for each laboratory. The drying time should not exceed 60 min and if it does, alternative drying conditions shall be used.

c) For the disinfectant test (Na), pipette 0,1 ml of each product test solution (5.4.2) to be tested onto separate dried surfaces ensuring that the dried inoculum is totally covered by the test product.

Place surfaces in a temperature controlled cabinet (5.3.2.13) at chosen test temperature θ and contact time t (5.5.1.1).

d) At the end of t, transfer each of the surfaces (Na) to a separate container (5.3.2.14) containing 10 ml of neutraliser (5.2.2.5) together with sufficient glass beads (5.3.2.11) to support the surface. The surfaces should be placed with the inoculated surface downwards in contact with the beads. Place the containers in a horizontal shaking device [5.3.2.6 b)] or place on a horizontal surface and shake in a horizontal manner by hand for 5 min ± 10 s. The shaking should be sufficiently vigorous to ensure that the test surface moves constantly over the beads. Ensure that the beads are able to move freely.

After the neutralisation time of 5 min ± 10 s prepare a series of 10-fold dilutions from 10^{-1} to 10^{-2} of the neutralised mixture in the diluent (5.2.2.4). Take a 1,0 ml sample of the neutralised mixture and each of the dilutions in duplicate and inoculate using pour plate or spread plate technique.

1) When using the pour plate technique, pipette each 1,0 ml sample into separate Petri dishes and add 15 ml to 20 ml of melted 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).

For incubation and counting, see 5.5.2.6.

e) Recover the test surface (“Na”), let the neutraliser drain off and rinse with 10 ml of water (5.2.2.2). Transfer to a Petri dish (5.3.2.10) containing 10 ml of solidified MEA (5.2.2.3) and place on top of the agar test side uppermost. Add 10 ml of MEA (5.2.2.3) melted and cooled to 45 °C ± 1 °C.

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

g) Perform the procedure a) to f) applying the other obligatory and – if appropriate – other additional experimental conditions (5.5.1.1).

5.5.2.3 Water control “Nw”

The procedure for determining the water control is as follows:

NOTE The control A - Experimental conditions control A (Validation of the selected experimental conditions or verification of the absence of any lethal effect in the test conditions) differs from other standard due to the fact it is directly determined in the water control “Nw”.

a) Prepare an inoculated test surface as in 5.5.2.2 a) and b).

b) For the water control (Nw), pipette 0,1 ml of hard water (5.2.2.6) or water (5.2.2.2) in the case of ready-to-use products onto the test surface ensuring that the dried inoculum is totally covered by the water.

Place the surface in a temperature controlled cabinet at the chosen test temperature θ (5.5.1.1).

c) At the end of t, transfer the surface “Nw” into a container (5.3.2.14) containing 10 ml of neutraliser (5.2.2.5) together with sufficient glass beads (5.3.2.11) to support the surface. The surface should be placed with the inoculated surface downwards in contact with the beads. Place the container in a horizontal shaking device [5.3.2.6 b)] or place on a horizontal surface and shake in a horizontal manner by hand for 5 min ± 10 s. The shaking should be sufficiently vigorous to ensure that the test surface moves constantly over the beads. Ensure that the beads are able to move freely.

After a neutralisation time of 5 min ± 10 s prepare a series of 10-fold dilutions from 10^{-1} to 10^{-5} of the neutralised mixture in the diluent (5.2.2.4). Take a 1,0 ml sample of the 10^{-3} to 10^{-5} dilutions in duplicate and inoculate using the pour plate or the spread plate technique [5.5.2.2 d)].

For incubation and counting, see 5.5.2.6.

5.5.2.4 Neutraliser control "B" - verification of the absence of toxicity of the neutraliser

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

a) Prepare an inoculated test surface as in 5.5.2.2 a) and b).

b) Pipette 10 ml of neutraliser (5.2.2.5) into a container (5.3.2.14) with sufficient glass beads (5.3.2.11) to support the surface. Then add 0,1 ml of hard water (5.2.2.6) or water (5.2.2.2) in the case of ready-to-use products. Mix [5.3.2.6 a)] and leave in contact for 5 min ± 10 s in a water bath (5.3.2.2) controlled at 20 °C ± 1 °C.

c) Transfer the inoculated and dried test surface into the container and place the inoculated surface downwards in contact with the beads (5.3.2.11). Place the container in a horizontal shaking device [5.3.2.6 b)] or place on a horizontal surface and shake in a horizontal manner by hand for 5 min ± 10 s. The shaking should be sufficiently vigorous to ensure that the test surface moves constantly over the beads. Ensure that the beads are able to move freely and that there are sufficient beads to support the surface.

d) After a neutralisation time of 5 min ± 10 s prepare a series of 10-fold dilutions of the neutralised mixture "B" in the diluent (5.2.2.4) to produce 10^{-1} to 10^{-5} dilutions. Take a sample of the 10^{-3} to 10^{-5} dilutions in duplicate and inoculate using the pour plate or the spread plate technique [5.5.2.2 d)].

For incubation and counting, see 5.5.2.6.

5.5.2.5 Method validation C - dilution-neutralisation validation

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

a) Prepare an inoculated test surface as in 5.5.2.2 a) and b).

b) Pipette 10 ml of neutraliser (5.2.2.5) into a container (5.3.2.14) with sufficient glass beads (5.3.2.11) to support the surface. Then add 0,1 ml of the highest product concentration used in the test (5.5.2.2). Mix [5.3.2.6 a)] and place the container in a water bath (5.3.2.2) or temperature controlled cabinet (5.3.2.13) controlled at θ for t. Just before the end of t, mix [5.3.2.6 a)] again.

c) At the end of t transfer the inoculated test surface into the container and place the inoculated surface downwards in contact with the beads (5.3.2.11).

   Place the container in a horizontal shaking device [5.3.2.6 b)] or place on a horizontal surface and shake in a horizontal manner by hand for 5 min ± 10 s. The shaking should be sufficiently vigorous to ensure that the test surface moves constantly over the beads. Ensure that the beads are able to move freely and that there are sufficient beads to support the surface.

d) After 5 min ± 10 s prepare a series of 10-fold dilutions of the neutralised mixture "C" in the diluent (5.2.2.4) to produce 10^{-1} to 10^{-5} dilutions. Take samples of 1,0 ml of the 10^{-3} to 10^{-5} dilutions in duplicate and inoculate using pour plate or spread plate technique [5.5.2.2 d)].

For incubation and counting, see 5.5.2.6.

5.5.2.6 Incubation and counting of the test, control and validation neutralisers

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

a) incubate (5.3.2.3) the plates for 42 h to 48 h at 30 °C ± 1 °C. 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. 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/test surface in the test mixture Na, in the water control mixture Nw and in the validation mixtures B and C using the method given in 5.6.2.4. Verify according to 5.7.3.

5.5.3 Observation of the test surface agar

Incubate the agar plates with the test surfaces (5.2.3) at 30 °C ± 1 °C for 42 h to 48 h. Examine for fungal colonies. Count and record the number of colonies. For *Aspergillus brasiliensis* re-incubate for a further 20 h to 24 h. Re-count the plates and record. Verify according to 5.7.3.

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 represents the fungal suspension and the validation suspension, Na represents the fungicidal test mixture, Nw (control test mixture), B (neutraliser control), C (method validation) represent the different control test mixtures.

NOTE The control A - Experimental conditions control A (Validation of the selected experimental conditions or verification of the absence of any lethal effect in the test conditions) differs from other standard due to the fact it is directly determined in the water control.

N represents the number of cells counted per 0,025 ml of the fungal test suspension and the validation suspension; Na, Nw and B, C represent the number of cells counted per test surface in the different test mixtures.

Table 2 — Number of cells counted per ml or per surface in the different test suspensions

	Number of cells per 0,025 ml at the beginning of the contact time (time = 0)	**Number of survivors per test surface at the end of the contact time t**
Test	*N*	*Na*
Controls	*N*	*Nw, B, C*

5.6.1.2 Vc values

All experimental data are reported as Vc values:

- a Vc value is the number of colony-forming units counted per 1,0 ml sample.

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, Na, Nw, B and C. The third step is the calculation of the reduction R (5.8).

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.

NOTE The lower limit (14) is based on the fact that the variability is increasing the smaller the number counted in the sample 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 (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) and for all countings of the method, 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 1,0 ml sample has been used to determine the Vc value, the count 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).

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

N is the lg number of cells per 0,025 ml in the microbial test suspension (5.4.1.4) at the beginning of the contact time (time “zero” = 0). It is less than the number in the fungal test suspension due to dilution by adding equal parts interfering substance and using 0,05 ml of this mixture for the inocula.

If two subsequent dilutions of N show duplicate Vc values within the counting limits calculate as the weighted mean using the formula:

$$N = \frac{0{,}025 \times c}{(n1 + 0{,}1n2) \times d}$$

where

- c is the sum of the Vc values taken into account;
- n1 is the number of Vc values taken into account in the lower dilution, i.e. 10^{-6};
- n2 is the number of Vc values taken into account in the higher dilution, i.e. 10^{-7};
- d is the dilution factor corresponding to the lower dilution (10^{-6}).

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/0,025ml is expressed by a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

EXAMPLE

$$N = \frac{(168 + 213 + 20 + 25) \times 0{,}025}{2{,}2 \times 10^{-6}} = \frac{426 \times 0{,}025}{2{,}2} \times 10^{6} = 4{,}84 \times 10^{6}$$

5.6.2.4 Calculation of Na and Nw

Na is the number of survivors per test surface (5.5.2.2) at the end of the contact time and before neutralisation. It is tenfold higher than the Vc values due to the addition of neutraliser.

Nw is the number of survivors per water control surface (5.5.2.3) at the end of the contact time and before neutralisation. It is tenfold higher than the Vc values due to the addition of neutraliser.

Calculate the mean for each dilution step Na and Nw using following formula:

$$Na\,(or Nw) = \frac{c \times 10}{n \times d}$$

where

- c is the sum of Vc values taken into account;
- n is the number of Vc values taken into account;
- d is the dilution taken into account.

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: 0, 0 (pour plates, from the neutraliser with test surface, 10^{0})

$$Na = \frac{(<14 + 14)}{2} \times 10 = <140 = <1{,}40 \times 10^{2}; \quad \lg\left(<1{,}40 \times 10^{2}\right) = <2{,}15$$

b) duplicate Vc values > 165, > 165 (pour plate *Aspergillus brasiliensis*, from the neutraliser with test surface, 10^{0})

$$Na = \frac{(>165 + 165)}{2} \times 10 = >1650 = >1{,}65 \times 10^{3}; \quad \lg\left(>1{,}65 \times 10^{3}\right) = >3{,}20$$

c) duplicate Vc values: > 330, > 330, 300, 300 (spread plates 2 per 1 ml sample, *Candida albicans* from the neutraliser with test surface, 10^{0})

$$Na = \frac{(> 660 + 600)}{2} \times 10 = > 6300 = > 6{,}30 \times 10^3; \quad \lg(> 6{,}3 \times 10^3) = > 3{,}80$$

d) Use maximum 2 subsequent dilutions for calculating *Na* as a weighted mean using the following formula:

$$Na = \frac{c \times 10}{2{,}2 \times 10^z}$$

where

c is the sum of Vc values taken into account;

z is the dilution factor corresponding to the lower dilution.

Exceptions and rules for special cases:

d1 If one or both duplicate Vc values in three or more subsequent dilutions of Na are within the counting limits (Na-2: 17, 23; Na-1: 120, 135; Na0: 308, 330) the whole test is invalid (5.7.1).

5.6.2.5 Calculation of B and C

B and C are the number of survivors on the neutraliser control (5.5.2.4) and method validation (5.5.2.5) at the end of the defined times 5 min (B) and t (C). It is tenfold higher than the Vc values due to the addition of neutraliser.

Calculate B and C using the following formula:

$$B\,(or\,C) = \frac{c \times 10}{n \times d}$$

where

c is the sum of Vc values taken into account;

n is the number of Vc values taken into account;

d is the dilution taken into account.

If two subsequent dilutions of B or C show duplicate Vc values within the counting limits calculate B or C as the weighted mean using the following formula:

$$B\,(or\,C) = \frac{c \times 10}{2{,}2 \times 10^z}$$

where

c is the sum of Vc values taken into account;

z is the dilution factor corresponding to the lower dilution.

5.7 Verification of methodology

5.7.1 General

A test is valid if:

— all results meet the criteria of 5.7.3 and 5.7.4;

— the requirements of 5.8.2 are fulfilled;

— it is not invalidated by a result described under 5.6.2.4 d).

5.7.2 Control of weighted mean counts

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

EXAMPLE

For N: 10^{-7} dilution: 168 + 215 cfu/ml, 10^{-8} dilution: 20 + < 14 cfu/ml; (168 + 215) / (20 + 14) = 383/34 = 11, 26

= between 5 and 15.

When the counts obtained on plates are out of the limits fixed for the determination of Vc values [5.6.2.2 b)], check for the weighted mean as mentioned above but 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 $3{,}75 \times 10^6$ and $1{,}25 \times 10^7$ ($6{,}57 \leq \lg N \leq 7{,}10$);

NOTE N is calculated for 0,025 ml.

b) lg Nw ≥ 5,27;

c) B ≥ 0,5 x Nw;

d) C ≥ 0,5 x Nw;

e) ≤ 14 cfu are observed on the treated surface for concentrations that pass the test;

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

5.7.4 Microscopic observation

At least 75 % spiny conidiospores in the *Aspergillus brasiliensis* conidiospore suspension (5.4.1.4.2).

5.8 Expression of results and precision

5.8.1 Reduction

The reduction (R) is the ratio between = Nw and Na.

The reduction is usually expressed as a logarithm.

lg R = lg Nw – lg Na

For each test organism record the number of cfu/test surface in the test procedure for fungicidal and/or yeasticidal activity of the product Na (5.5.2.2) and in the control procedure Nw (5.5.2.3).

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

For validation of the dilution-neutralisation method record the number of cfu/test surface in the neutraliser toxicity control B (5.5.2.4) and the dilution-neutralisation control C (5.5.2.5).

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) shall demonstrate a 3 lg or more reduction and at least one concentration shall demonstrate a lg reduction of less than 3.

5.8.3 Fungicidal or yeasticidal concentration

5.8.3.1 Fungicidal concentration

For *Aspergillus brasiliensis* and *Candida albicans*, record the lowest concentration of the product which passes the test (lg R ≥ 3). The lowest concentration of the product active on *Aspergillus brasiliensis* and *Candida albicans* is the fungicidal concentration determined according to this European Standard.

5.8.3.2 Yeasticidal concentration

The lowest concentration of the product which passes the test with *Candida albicans* (lg R ≥ 3) is the yeasticidal concentration determined according to this European Standard.

5.8.4 Precision, repetitions

Repetition of the test for a precision of ± 1 lg in reduction: 4 repetitions in the best case, 6 repetitions in the worst case is recommended. The number of repetitions shall be decided according to the required level of precision, taking into account the intended use of the test results.

NOTE 1 Recommendation is based on the precision of suspension test methodology determined by statistical analysis based on data provided by a collaborative study.

Repetition means the complete test procedure with separately prepared test- and validation suspensions. The repetitions may be restricted to the limiting test organism. The mean of the results of the repetitions - not each single result - shall demonstrate at least a 3 lg reduction and shall also be calculated and recorded.

NOTE 2 To calculate the mean all lg values are converted to real numbers, e.g. 3,2 lg to about 1 580. The mean is the arithmetic mean of these converted numbers.

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 standard may differ (Clause 4). A product can only pass the test if the requirements of 5.8.2 are fulfilled.

5.9.2 Fungicidal or yeasticidal activity for general purposes

The product shall be deemed to have passed the EN 16438 standard if it demonstrates in a valid test at least a 3 lg reduction within 60 min or less at 10 °C with the chosen interfering substance under the conditions defined by this European Standard when the test organisms are *Aspergillus brasiliensis* and/or *Candidia albicans*.

5.9.3 Qualification for certain fields of application

The fungicidal concentration for specific purpose is the concentration of the tested product for which at least a 3 lg reduction is demonstrated in a valid test under the additional chosen test conditions. The product shall be deemed to have passed the EN 16438 standard under the obligatory test conditions and the fungicidal or yeasticidal concentration for specific purposes may be lower than the one determined for general purposes.

For more details, see EN 14885.

5.10 Test report

The test report shall refer to this European Standard (EN 16438).

The test report shall, at least, state 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) expiry date;

   6) storage conditions;

   7) product diluent recommended by the manufacturer for use;

   8) active substance(s) and their concentration(s) (optional);

   9) appearance of the product;

d) test method and its validation:

   full details of the test for validation of the neutraliser shall be given;

e) experimental conditions:

   1) date(s) of test (period of analysis);

   2) diluent used for product test solution (hard water or water);

3) product test concentrations (= desired test concentrations according to 5.4.2);

4) appearance of product dilutions;

5) contact time(s);

6) test temperature(s);

7) interfering substance(s);

8) temperature of incubation;

9) neutraliser;

10) identification of the fungal strains used;

11) drying time of the inoculated test surfaces;

f) test results:

1) controls and validation;

2) evaluation of fungicidal activity;

3) number of repetitions per test organism;

g) special remarks;

h) conclusion;

i) locality, date and identified signature.

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

Annex A
(informative)

Referenced strains of national collections

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

Abbreviations:

ATCC	American Type Culture Collection
CBS	Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversiry Centre
CIP	Collection de l'Institut Pasteur
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen
IMI	International Mycological Institute
NCTC	National Collection of Type Cultures

Annex B
(informative)

Examples of neutralisers of the residual antimicrobial activity of chemical disinfectants and antiseptics

Important — Neutralisers of the residual antimicrobial activity of chemical disinfectants and antiseptics shall be validated according to the prescriptions of the standard

Antimicrobial agent	**Chemical compounds able to neutralise residual antimicrobial activity**	**Examples of suitable neutralisers** [a)]
Quaternary ammonium compounds and fatty amines Amphoteric compounds	Lecithin, Saponin, Polysorbate 80, Sodium dodecyl sulphate, 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 sulphate, 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.*
Oxidising 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 neutraliser 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 1 000 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 neutralisation 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

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

NOTE 2 The concentration of the various neutralising compounds or of the neutraliser as such may not be adequate to neutralise high concentrations of the products.

Annex C
(informative)

Graphical representations of dilution-neutralisation method

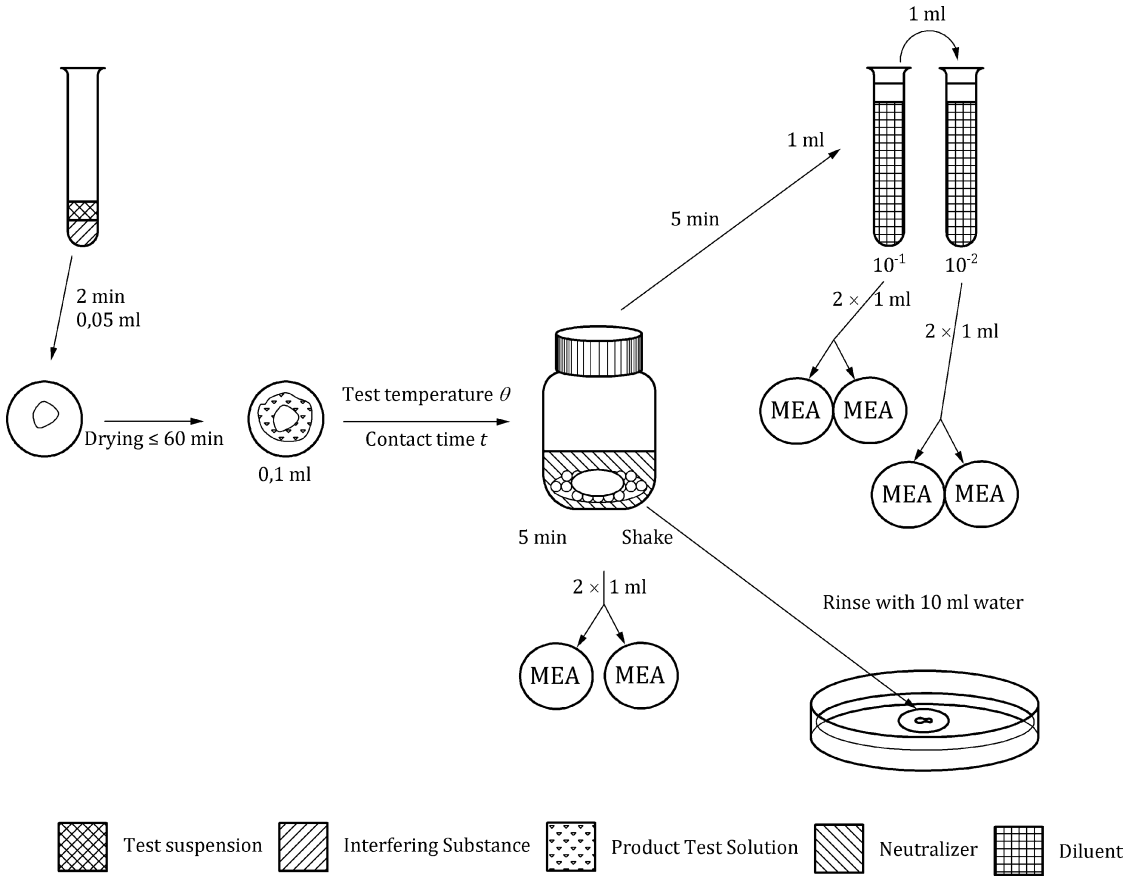

Figure C.1 — Test (Na)

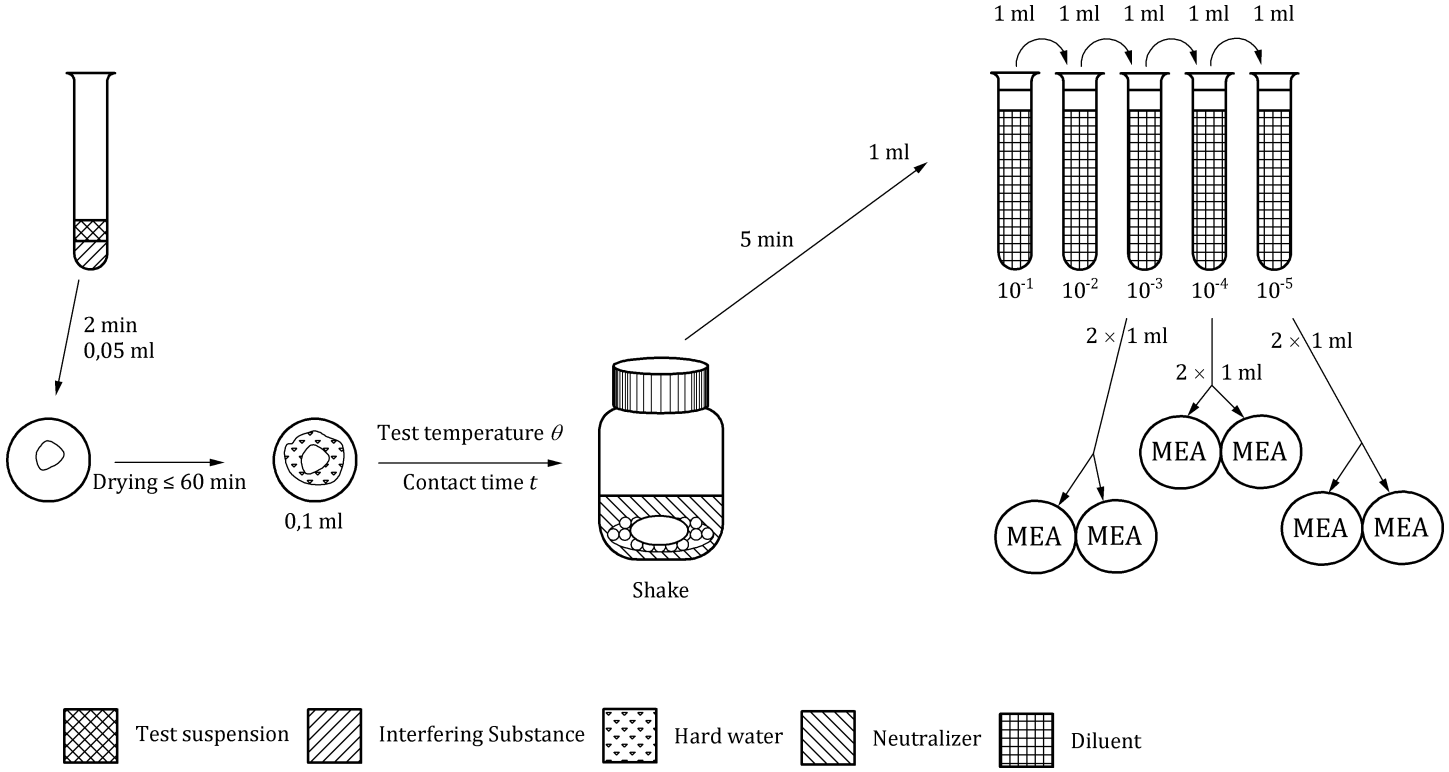

Figure C.2 — Water control (Nw)

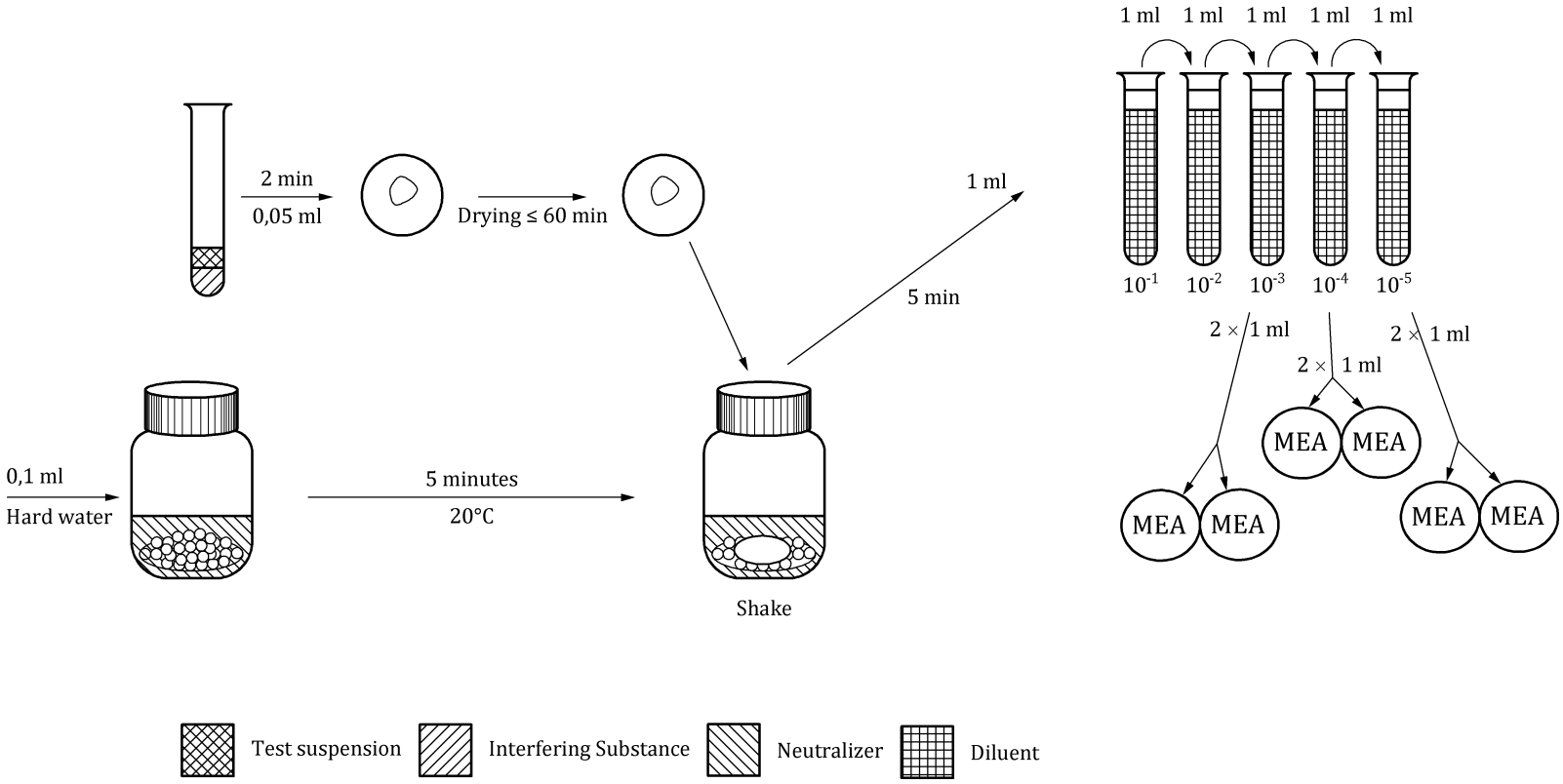

Figure C.3 — Neutraliser toxicity control B

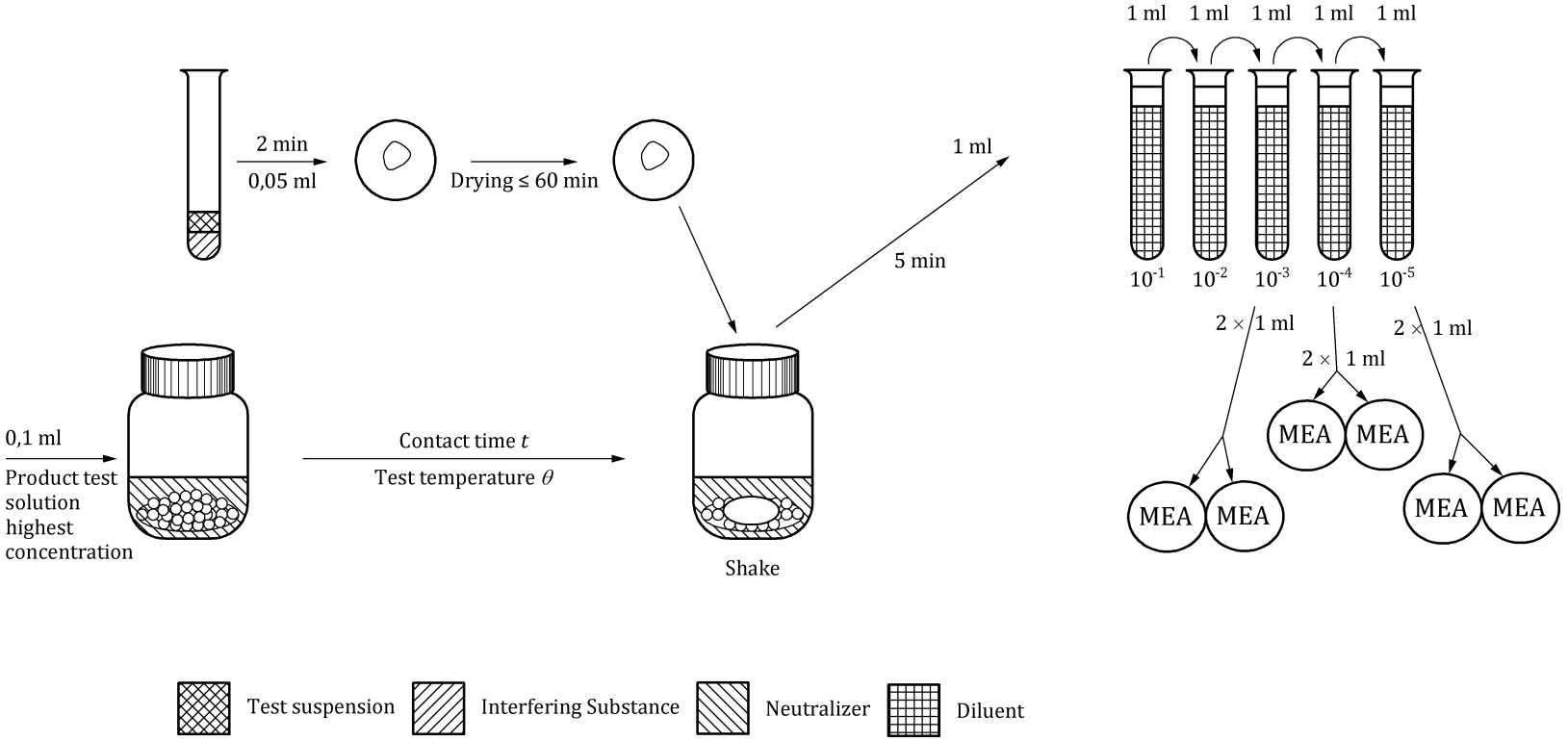

Figure C.4 — Method validation C

Annex D
(informative)

Example of a typical test report

NOTE 1 All names and examples in Annex D are fictious apart from those used in the standard.

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

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TEST REPORT

YEASTICIDAL/FUNGICIDAL ACTIVITY

(obligatory and additional conditions)

Client: Multi Formulations Inc., Mannheim/Euroland

Disinfectant-sample

Name of the product: Z **Batch number** 06-47-013

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

Period of testing

Date of delivery of the product 2012-10-09 **Date of tests**: see “Test results” (attached)

Experimental conditions

Product diluent: hard water **Concentrations of the product tested**: see “Test results” (attached)

EN 16438:2014 (E)

Obligatory conditions:

test organisms	*Aspergillus brasiliensis* ATCC 16404, *Candida albicans* ATCC 10231
test temperature	10 °C
contact time:	60 min
Interfering substance	10 g/l yeast extract + 10 g/l bovine albumin = high level soiling
incubation temperature	30 °C
Counting procedure	Spread plate
Drying time of inoculated test surfaces	45 min

Special remarks regarding the results:

1) All controls and validation were within the the basic limits.

2) At least one concentration of the product demonstrated a lg reduction of less than 3 lg.

Conclusion:

For the product Z (batch 06-47-013) the fungicidal concentration for general purposes determined according to EN 16438 (obligatory conditions) under high soiling conditions is:

1,0 % (v/v)

Antiseptville, 2010-10-23

Alexandra May, MD, PhD, Scientific Director

Test results (fungicidal quantitative carrier test, non-porous surfaces)

EN 16438.....(Phase 2, step 2) Product-name: Z Batch No: 06-47-013

Remarks:

Pour plate □ Spread plate ☒ Number of plates 3 /1ml

Neutraliser: Lecithin 3,0 g/l in diluent

Test temperature: 10 °C interfering substance: 10 g/l yeast extract + 10 g/l bovine albumin

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

Test organism: *Aspergillus brasiliensis* ATCC 16404 Drying time on carrier: 45 min (not > 60 min)

Internal lab. No: QS..*58/00* Date of test: 2010-10-19 Responsible person: Fang Signature: Fang

Validation and controls

Presence of at least 75 % of *A.brasiliensis* spiny spores in the spore suspension ☒yes □ no

Water control (Nw)			Neutraliser toxicity control (B)			Method validation control (C)		
	10^{-3}	10^{-4}		10^{-3}	10^{-4}		10^{-3}	10^{-4}
Vc1	40	3	Vc1	34	3	Vc1	26	6
Vc2	36	2	Vc2	28	4	Vc2	24	3
Nw = 40 +36/2 × 10/10^{-3} = 3,8 × 10^5 lg (3,8 × 10^5) = 5,60			B = 34 +28/2 × 10/10^{-3} = 3,1 × 10^5 lg (3,1 × 10^5) = 5,49			C = 26 + 24/2 × 10/10^{-3} = 2,5 × 10^5 lg (2,5 × 10^5) = 5,40		
lgNw ≥ 5,27? ☒yes □ no			B ≥ 0,5 x Nw? ☒ yes □ no			C ≥ 0,5 x Nw? ☒yes □ no		

Test suspension and Test

Test and Validation suspension (N)	N	Vc1	Vc2
	10^{-6}	60,57,50	50,50,50
	10^{-7}	18	16
N = (167 + 150 + 18 + 16) × 0,025 /2,2 × 10^{-6} = 3,99 × 10^6 ; lg (3,99 × 10^6) = 6,60			
6,57 <= lg N <= 7,10? ☒ yes □ no			

EN 16438:2014 (E)

Conc of the product %	Dilution	Counts per plate		Vc1	Vc2	lgNa	lgR (lg Nw = 5,60)	Contact time (min)
0,25	10^{0}	> 150 + > 150 + > 150	> 150 + > 150 + > 150	>450	>450	> 4,65	< 0,95	60
	10^{-1}	> 150 + > 150 + > 150	145 + 150 + 149	> 450*	444*			
0,5	10^{0}	> 150 + > 150 + > 150	> 150 + > 150 + > 150	>450	>450	4,48	1,12	60
	10^{-1}	96 + 110 + 92	106 + 111 + 95	298*	312*			
1,0	10^{0}	3 +0 +2	1 +1 +3	< 14*	< 14*	< 2,15	> 3,45	60
	10^{-1}	0 +0 +0	0 +0 +0	< 14	< 14			

* used for calculation

Explanations:

Vc = count per ml (one plate or more)

R = reduction (lg R = lg Nw – lg Na)

Bibliography

[1] EUROPEAN PHARMACOPOEIA (EP). Edition 1997 supplement 2000, Water for injections

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