SLOVENSKI STANDARD
oSIST prEN 13624:2010

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Kemična razkužila in antiseptiki - Kvantitativni suspenzijski preskus za ocenjevanje fungicidnega delovanja ali delovanja kemičnih razkužil in antiseptikov na kvasovke v humani medicini - Preskusna metoda in zahteve (faza 2, stopnja 1)

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

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

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

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

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

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Foreword

This document (prEN 13624:2010) has been prepared by Technical Committee CEN/TC 216 “Chemical disinfectants and antiseptics”, the secretariat of which is held by AFNOR.

This document is currently submitted to the CEN Enquiry.

This document will supersede EN 13624:2003.

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

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

Introduction

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

This laboratory test takes into account practical conditions of application of the product including contact time, temperature, test organisms and interfering substances, i. e. conditions which may influence its action in practical situations.

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 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 when diluted with hard water, or - in the case of ready-to-use products - with water. Products can only be tested at a concentration of 80 % or less (97 % with a modified method for special cases) as some dilution is always produced by adding the test organisms and interfering substance.

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

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

— in hospitals, in community medical facilities and in dental institutions;

— in clinics of schools, of kindergartens and of nursing homes;

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

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

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

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

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 12353, *Chemical disinfectants and antiseptics — Preservation of test organisms used for the determination of bactericidal, mycobactericidal, sporicidal and fungicidal activity*

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

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

3 Terms and definitions

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

4 Requirements

The product shall demonstrate at least a 4 decimal log (lg) reduction (for hygienic and surgical hand wash at least a 2 lg reduction), when tested in accordance with table 1 and clause **5** under simulated clean conditions (0,3 g/l bovine albumin solution) or dirty conditions (3 g/l bovine albumin solution, plus 3 ml/l washed sheep erythrocytes). For instrument disinfectants recommended by the manufacturer to be used at higher temperatures the product may demonstrate the 4 lg reduction only at the recommended temperature.

Table 1 — Obligatory and additional test conditions

Test conditions	Hygienic handrub and handwash	Surgical handrub and handwash	Instrument disinfection	Surface disinfection
Test organism a) obligatory	*Candida albicans* (vegetative cells)	*Candida albicans* (vegetative cells)	a1) fungicidal activity: *Aspergillus niger* (spores) *Candida albicans* (eg. cells) a2) yeasticidal activity: *Candida albicans* (veg. cells)	a1) fungicidal activity: *Aspergillus niger* (spores) *Candida albicans* (eg. cells) a2) yeasticidal activity: *Candida albicans* (veg. cells)
b) additional	Any relevant test organism			
Test temperature a) obligatory	20 °C	20 °C	At the lowest recommended temperature, min. 20 °C, max. 60 °C	20 °C
b) additional	according to the manufacturer's recommendation, but not higher than			
	20 °C	20 °C	60 °C	30 °C
Contact time a) obligatory	60 s	5 min	60 min	5 min or 60 min*
b) additional	according to the manufacturer's recommendation, but no longer than			
	60 s **	5 min	60 min	5 min or 60 min*
Interfering substance a) obligatory clean conditions	0,3 g/l bovine albumin solution (hygienic handrub)	0,3 g/l bovine albumin solution (surgical handrub)	0,3 g/l bovine albumin solution	0,3 g/l bovine albumin solution
dirty conditions	3,0 g/l bovine albumin solution plus 3,0 ml eryhtrocytes (hygienic handwash)	3,0 g/l bovine albumin solution plus 3,0 ml erythrocytes (surgical and handwash)	and/or 3,0 g/l bovine albumin solution plus 3,0 ml erythrocytes	and/or 3,0 g/l bovine albumin solution plus 3,0 ml erythrocytes
b) additional	—	—	any relevant substance	any relevant substance

* The obligatory contact times for surface disinfectants stated in table 1 are chosen on the basis of exacted practical application of the product. The referenced test conditions are by no means intended as requirements for the use of a product, nor as requirements for the evaluation and acceptance of products by regulatory authorities. The recommended contact time for the use of the product is within the responsibility of the manufacturer. Products intended to disinfect surfaces that are likely to come into contact with the patient and / or the medical staff, which could lead to the transmission of micro-organisms to the patient, and where the contact time of the product shall be limited for practical reasons, shall be tested with an obligatory contact time of 5 min. Products for other surfaces than stated above, could be tested with an obligatory contact time of 60 min.

** For hygienic handwash 75 s.

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

Table 2 — Obligatory and additional test conditions.

Any additional specific fungicidal or yeasticidal 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 the temperature and the contact time specified in clause **4** and

5.5.1.1. At the end of this contact time, an aliquot is taken; the fungicidal and/or the fungistatic action in this portion is immediately neutralized or suppressed by a validated method. The method of choice is dilution-neutralization. If a suitable neutralizer cannot be found, membrane filtration is used. The numbers of surviving fungi in each sample are determined and the reduction is calculated.

NOTE 1 Handwash products are always prediluted with hard water (**5.2.2.7**). The resulting solution is regarded as a ready-to-use product (**5.4.2).**

5.1.2 The test is performed using the vegetative cells of *Candida albicans* and the spores of *Aspergillus niger* (fungicidal activity) or only the vegetative cells of *Candida albicans* (yeasticidal activity) as test-organisms (clause **4**, table **1**).

5.1.3 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 organisms

The fungicidal activity shall be evaluated using the following strains as test organisms selected according to clause **4** (table 1)[1)]:

— *Candida albicans* ATCC 10231;

— *Aspergillus niger* ATCC 16404.

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

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

The required incubation temperature for these test organisms is (30 ± 1) °C.

If additional test organisms are used, they shall be incubated under optimum growth conditions (temperature, time, atmosphere, media) noted in the test report. If the additional test organisms selected do not correspond to the specified strains, their suitability for supplying the required inocula shall be verified. If these additional test organisms are not classified at a reference centre, their identification characteristics shall be stated. In addition, they shall be held by the testing laboratory or national culture collection under a reference for five years.

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

5.2.2 Culture media and reagents

5.2.2.1 General

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

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

NOTE 1 To improve reproducibility, it is recommended that commercially available dehydrated material is used for the preparation of culture media. The manufacturer's instructions relating to the preparation of these products should be rigorously followed.

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

5.2.2.2 Water

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

Sterilize in the autoclave [**5.3.2.1** a)]. Sterilization is not necessary if the water is used e. g. for preparation of culture media and subsequently sterilized.

NOTE 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	30,0 g
Soya peptone, papaic digest of Soybean Meal	3,0 g
Agar	15,0 g
Water **(5.2.2.2**)	to 1000,0 ml

Sterilize in the autoclave (**5.3.1**). After sterilization, the pH of the medium shall be equivalent to 5,6 ± 0,2 when measured at (20 ± 1) °C (**5.3.2.4**).

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

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 1000,0 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 ± 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**.

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 1000 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 1000 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 1000 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 1000 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 1000 ml with water (**5.2.2.2**). The pH of the hard water shall be 7,0 ± 0,2, when measured at (20 ± 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 hours.

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 Clean conditions (bovine albumin solution – low concentration)

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

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

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

5.2.2.8.3 Dirty conditions (Mixture of bovine albumin solutions – high concentration with sheep erythrocytes)

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

Sterilize by membrane filtration (**5.3.2.7**).

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

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

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

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

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

a) Clean conditions (**5.2.2.8.2**) – dissolve 1,50 g bovine albumin (instead of 0,3 g) in 100 ml of diluent;

b) Dirty conditions (**5.2.2.8.3**) – dissolve 15,0 g bovine albumin (instead of 3,0 g) in 85 ml of diluent (instead of 97 ml).

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

5.2.2.9 Sterile defibrinated sheep blood

The sterile defibrinated sheep blood can be acquired from a commercial supplier or prepared according to EN 14820 [2].

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 [2)]

and, in particular, the following:

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

5.3.2.1 Apparatus for moist and dry heat 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 (20 ± 1) °C, at (45 ± 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 either at (30 ± 1) °C.

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

NOTE A puncture electrode or a flat membrane electrode should be used for measuring the pH of the agar media (**5.2.2.3**).

5.3.2.5 Stopwatch.

5.3.2.6 Shakers

a) Electromechanical agitator, e.g. Vortex® mixer[3];

b) Mechanical shaker

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

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

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

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

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

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

5.3.2.12 Volumetric flasks.

5.3.2.13 Centrifuge (800 g_N and 2000g_N).

5.3.2.14 Roux bottles or similar flasks.

5.3.2.15 Fritted filters: Porosity of 40 µm to 100 µm (see **clause 2**: ISO 4793).

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3) Vortex® in 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 Preparation of test organism suspensions and product test solutions

5.4.1 Test organism suspensions (test and validation suspension)

5.4.1.1 General

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

5.4.1.2 Preservation and stock cultures of test organisms

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

5.4.1.3 Working culture of test organisms

5.4.1.3.1 Candida albicans (yeast)

In order to prepare the working culture of *Candida albicans* (**5.2.1**), subculture from the stock culture (**5.4.1.2**) by streaking onto MEA (**5.2.2.3**) slopes or plates and incubate (**5.3.2.3**). After 42 h to 48 h prepare a second subculture from the first subculture in the same way and incubate for 42 h to 48 h. From this second subculture a third subculture may be produced in the same way. The second and (if produced) **third** subculture are the working cultures.

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

Never produce and use a fourth subculture.

5.4.1.3.2 Aspergillus niger (mould)

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

5.4.1.3.3 Other test organisms (yeasts or moulds)

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

5.4.1.4 Test suspension (“*N*”)

5.4.1.4.1 *Candida albicans*

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 a tube.

b) Adjust the number of cells in the suspension to 1,5 x 10^7 cfu/ml[4)] to 5,0 x 10^7 cfu/ml using diluent (**5.2.2.4**) (1,5 x 10^8 cfu/ml to 5,0 x 10^8 cfu/ml in the case of the modified method – **5.5.4),** estimating the number of cfu by any suitable means. Maintain this test suspension in the water bath at 20 °C and use within 2 h. Adjust the temperature according to **5.5.1.1 a)** and **5.5.1.4** only immediately before the start of the test.

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

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

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

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

1) When using the pour plate technique, transfer 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.

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

For incubation and counting, see **5.4.1.6**.

5.4.1.4.2 *Aspergillus niger*

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

a) Take the working culture (**5.4.1.3.2**) and suspend the spores in 10 ml of sterile 0,05 % (*w/v*) polysorbate 80 solution in water (**5.2.2.2**). Using a glass rod or spatula, detach the conidiospores from the culture surface. Transfer the suspension into a flask and gently shake by hand for one minute together with 5 g of glass beads (**5.3.2.11**). Filter the suspension through a fritted filter (**5.3.2.14**);

b) Carry out a microscopic examination under x 400 magnification immediately after the preparation and just before the test, to show the absence of mycelia fragments and spore germination (check at least ten fields of view for absence of both).

If germinated spores are present, discard the suspension.

If mycelia are present, set up a washing process (centrifugation) as follows. Transfer the filtered suspension to centrifuge tubes. The filtered suspension is centrifuged (**5.3.2.13**) at 2 000 g_N for 20 min. The conidiospores are washed at least twice by resuspension in diluent (**5.2.2.4**) and subsequent centrifugation. If mycelia are still present, repeat the washing process.

c) Adjust the number of spores in the suspension to $1,5 \times 10^7$ cfu/ml to $5,0 \times 10^7$ cfu/ml ($1,5 \times 10^8$ cfu/ml to $5,0 \times 10^8$ cfu/ml in the case of the modified method – **5.5.4**) using the diluent (**5.2.2.4**), estimating the number of cfu by any suitable means. Use the suspension within 4 h. It can be stored up to 2 d in the refrigerator (**5.3.2.8**) and shall be checked again for absence of germinated spores [see b)] just before the test. In any case, adjust the temperature according to **5.5.1.4** only immediately before the start of the test (**5.5.2**, **5.5.3** or **5.5.4**).

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

Each laboratory should therefore produce calibration data to establish the relationship between the counts obtained using the counting device and the counts (**5.4.1.6**) obtained by the pour plate or the spread plate technique [**5.4.1.4.2** e)]. Experienced laboratories found a better fit to the required number of spores when the spore suspension count in the device was 10 % to 50 % higher than the number aimed at.

d) For counting, prepare 10^{-5} and 10^{-6} of the test suspension using diluent (**5.2.2.4**). Mix [**5.3.2.6 a**)].

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