BRITISH STANDARD

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Chemical disinfectants — Quantitative suspension test for the evaluation of sporicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas — Test method and requirements (phase 2, step 1)

The European Standard EN 13704:2002 has the status of a British Standard

ICS 71.100.35

National foreword

This British Standard is the official English language version of EN 13704:2002

The UK participation in its preparation was entrusted to Technical Committee IH/57, Chemical disinfectants and antiseptics, which has the responsibility to:

— aid enquirers to understand the text;

— present to the responsible European committee any enquiries on the interpretation, or proposals for change, and keep the UK interests informed;

— monitor related international and European developments and promulgate them in the UK.

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

Cross-references

The British Standards which implement international or European publications referred to in this document may be found in the BSI Standards Catalogue under the section entitled "International Standards Correspondence Index", or by using the "Find" facility of the BSI Standards Electronic Catalogue.

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English version

Chemical disinfectants - Quantitative suspension test for the evaluation of sporicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas - Test method and requirements (phase 2, step 1)

Désinfectants chimiques - Essai quantitatif de suspension pour l'évaluation de l'activité sporicide des désinfectants chimiques utilisés dans le domaine de l'agro-alimentaire, dans l'industrie, dans les domaines domestiques et en collectivité - Méthode d'essai et prescriptions (phase 2, étape 1)

Chemische Desinfektionsmittel - Quantitativer Suspensionsversuch zur Bestimmung der sporiziden Wirkung chemischer Desinfektionsmittel in den Bereichen Lebensmittel, Industrie, Haushalt und öffentliche Einrichtungen - Prüfverfahren und Anforderungen (Phase 2, Stufe 1)

This European Standard was approved by CEN on 11 November 2001.

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the Management Centre or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the Management Centre has the same status as the official versions.

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

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Contents

Foreword

This European Standard 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 2002, and conflicting national standards shall be withdrawn at the latest by August 2002.

In this standard the annexes A and B are normative. The annexes C, D, E, F, G and H are informative.

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

Introduction

This European Standard describes a suspension test method for establishing whether a chemical disinfectant has or does not have a sporicidal activity in the fields described in clause 1.

The laboratory test closely simulates practical conditions of application. Chosen conditions (contact time, temperature, in suspension, etc.) reflect parameters which are found in practical situations including conditions which can influence the action of disinfectants. Each utilization concentration found from this test corresponds to defined experimental conditions.

The conditions are intended to cover general purposes and to allow reference between laboratories and product types.

However for some applications the recommendations of use of a product can differ and therefore additional test conditions need to be used.

1 Scope

This European Standard specifies a test method (phase 2/step 1) (see annex H) and the minimum requirements for sporicidal activity of chemical disinfectant products that form a homogeneous, physically stable preparation in hard water and that are used in food, industrial, domestic and institutional areas, excluding areas and situations where disinfection is medically indicated and excluding products used on living tissues except those for hand hygiene in the above considered areas.

This European Standard applies at least to the following :

a) processing, distribution and retailing of :

   1) food of animal origin :

      — milk and milk products ;

      — meat and meat products ;

      — fish, seafood, and related products ;

      — eggs and egg products ;

      — animal feeds ;

      — etc. ;

   2) food of vegetable origin :

      — beverages ;

      — fruits, vegetables and derivatives (including sugar, distillery, etc.) ;

      — flour, milling and baking ;

      — animal feeds ;

      — etc. ;

b) institutional and domestic areas :

   — catering establishments ;

   — public areas ;

   — public transports ;

   — schools ;

   — nurseries ;

   — shops ;

   — sports rooms ;

   — waste containers (bins, etc.) ;

   — hotels ;

- dwellings ;
- clinically non sensitive areas of hospitals ;
- offices ;
- etc. ;

c) other industrial areas :

- packaging material ;
- biotechnology (yeast, proteins, enzymes, etc.) ;
- pharmaceutical ;
- cosmetics and toiletries ;
- textiles ;
- space industry, computer industry ;
- etc.

Using this European Standard, it is not possible to determine the sporicidal activity of undiluted product as some dilution is always produced by adding the inoculum and interfering substance. Products can only be tested at a concentration of 80 % or less.

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

2 Normative references

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text, and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this European Standard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies (including amendments).

EN 1040, *Chemical disinfectants and antiseptics – Basic bactericidal activity – Test method and requirements (phase 1).*

3 Terms and definitions

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

3.1
product (for chemical disinfection and/or antisepsis)
chemical agent or formulation used as a chemical disinfectant or antiseptic [EN 1040]

3.2
sporicide (in this standard)
product which kills spores of the genus *Bacillus*

NOTE The adjective derived from « sporicide » is « sporicidal ».

3.3
sporicidal activity (in this standard**)**
capability of the product to produce at least a reduction of 10^3 in the number of bacterial spores belonging to reference strain of *Bacillus subtilis* under conditions defined by this European Standard

3.4
clean conditions
conditions representative of surfaces which have received a satisfactory cleaning programme and/or are known to contain minimal levels of organic and/or inorganic materials

4 Requirements

The product, when diluted in hard water and tested in accordance with clause 5 under simulated clean conditions (0,3 g/l bovine albumin see 3.4) according to its practical applications and under the required test conditions (20 °C, 60 min, 1 selected reference strain), shall demonstrate at least a 10^3 reduction in viable counts.

The sporicidal activity shall be evaluated using the test organism *Bacillus subtilis*.

The determined sporicidal concentration of the test product is suggested as being suitable for practical situations of use.

Where appropriate, additional specific sporicidal activity shall be determined under other conditions of time, temperature and additional strains (see 5.2.1 and 5.5.1) in accordance with 5.5.1 in order to take into account intended specific use conditions.

NOTE For these additional conditions, the concentration defined as a result can be lower than the one obtained under the initial test conditions of 20 °C, 60 min, 1 selected reference strain.

5 Test method

5.1 Principle

5.1.1 A test suspension of bacterial spores in a solution of interfering substance, simulating clean conditions, is added to a prepared sample of the product under test diluted in hard water. The mixture is maintained at 20 °C ± 1 °C for 60 min ± 10 s (required test conditions).

At this contact time, an aliquot is taken ; the sporicidal 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 number of surviving bacterial spores in each sample are determined and the reduction in viable counts is calculated.

5.1.2 The test is performed using spores of *Bacillus subtilis.* Additional and optional exposure times, temperatures and strains are specified.

5.2 Materials and reagents

5.2.1 Test organisms

The sporicidal activity shall be evaluated by using spores of the following strain :

— *Bacillus subtilis* ATCC 6633 [1)].

If required for specific applications, additional strains may be chosen from, for example :

— *Bacillus cereus* ATCC 12826 [1)] ;

— *Clostridium sporogenes* CIP 7939 [1)].

NOTE 1 See annex G for corresponding strain numbers in some other culture collections.

NOTE 2 See annex C for particular culture and handling conditions for *Clostridium sporogenes*.

If additional strains are used, they shall be incubated under optimum growth conditions (temperature, time, atmosphere) and noted in the test report.

If the additional strains selected do not correspond to the specified strains, their suitability for supplying inocula of sufficient concentration shall be verified. If the additional strains tested 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 under a reference for 5 years.

5.2.2 Culture media and reagents

5.2.2.1 General

The reagents shall be of analytical grade and/or appropriate for microbiological purposes.

NOTE 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.

5.2.2.2 Water

The water shall be free from substances that are toxic or inhibiting to the bacterial spores or to the bacteria. It shall be freshly glass distilled water and not demineralized water.

Sterilize in the autoclave (see 5.3.1).

NOTE 1 If the water is sterilized during the sterilization of the reagents, this is not necessary.

NOTE 2 If distilled water of adequate quality is not available, water for injectable preparation (European Pharmacopoeia) can be used.

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1) ATCC 6633 and ATCC 12826 are the collection numbers of strains supplied by the American Type Culture Collections. CIP 7939 is the collection number of spores supplied by the Collection de l'Institut Pasteur. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of the product named. Corresponding strains supplied by other culture collections may be used if they can be shown to lead to the same results.

5.2.2.3 Glucose Yeast Extract Agar (GYA)

For counting of viable *Bacillus* spores :

Amino-acids, without vitamins, obtained by acid hydrolysis of casein	1,0 g
Soluble starch	1,0 g
Glucose	2,5 g
Yeast extract	5,0 g
$FeSO_4$	0,1 g
$MnSO_4$, H_2O	0,000 1 g
Agar	15,0 g
Water (see 5.2.2.2)	1 000,0 ml

Sterilize in the autoclave (see 5.3.1). After sterilization the pH of the medium shall be equivalent to 6,8 ± 0,2 when measured at 20 °C.

5.2.2.4 Neutralizer

The neutralizer shall be validated for the product under test in accordance with annex D. The neutralizer shall be sterile.

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

5.2.2.5 Rinsing liquid (for membrane filtration)

The liquid shall be sterile, compatible with the filter membrane and capable of filtration through the filter membrane under the test conditions described in annex B.

NOTE Information on rinsing liquids that have been found to be suitable for some categories of products is given in annex E.

5.2.2.6 Hard water for dilution of products

Hard water for dilution of products shall be prepared as follows :

— Solution A : Dissolve 19,84 g anhydrous magnesium chloride ($MgCl_2$) or an equivalent of hydrated magnesium chloride and 46,24 g anhydrous calcium chloride ($CaCl_2$) or an equivalent of hydrated calcium chloride in water (see 5.2.2.2) and dilute to 1000 ml.

  Sterilize in the autoclave (see 5.3.1). Store the solution at 2 °C - 8 °C for no longer than one month.

— Solution B : Dissolve 35,02 g sodium bicarbonate ($NaHCO_3$) in water (see 5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration (see 5.3.2.7). Store the solution at 2 °C - 8 °C for no longer than one week.

Hard Water :

For the preparation of 1 litre, place at least 600 ml water (see 5.2.2.2) in a 1000 ml volumetric flask (see 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 (see 5.2.2.2).

The pH of the hard water shall be 7,0 ± 0,2.

If necessary adjust the pH by using a solution of approximately 40 g/l (about 1 mol/l) of sodium hydroxide (NaOH) or approximately 36,5 g/l (about 1 mol/l) of hydrochloric acid (HCl).

The hard water shall be freshly prepared under aseptic conditions and used within 12 hours.

NOTE When preparing the product test solutions (see 5.4.2) the addition of the product to this hard water produces a different final water hardness in each test tube.

In any case the final hardness is lower than 300 mg/kg 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 sterile and prepared at 10 times its final concentration in the test.

5.2.2.7.2 Bovine albumin solution

Bovine albumin solution for the test conditions shall be prepared as follows :

— dissolve 0,30 g of bovine albumin (Cohn fraction V for Dubos medium) in 100 ml of water (see 5.2.2.2) ;

— sterilize by membrane filtration.

The final concentration of the bovine albumin in the test procedure (see 5.5.2) is 0,3 g/l.

5.3 Apparatus and glassware

5.3.1 General

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

a) in the autoclave (see 5.3.2.1) by maintaining it at (121^{+3}_{0}) °C for a minimum holding time of 15 min ;

b) in the dry heat sterilizer (see 5.3.2.1) by maintaining it at 180 °C for a minimum holding time of 30 min, at 170 °C for a minimum holding time of 1 h, or at 160 °C for a minimum holding time of 2 h.

5.3.2 Usual microbiological laboratory equipment [2] and, in particular, the following :

5.3.2.1 Apparatus for sterilization

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

b) for dry heat sterilization, a hot air oven capable of being maintained at 180 °C for a minimum holding time of 30 min, at 170 °C for a minimum holding time of 1 h, or at 160 °C a minimum holding time of 2 h.

5.3.2.2 Water baths, capable of being controlled at 20 °C ± 1 °C, 45 °C ± 1 °C and at additional test temperatures ± 1 °C (see 5.5.1).

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

5.3.2.4 pH-meter, having an accuracy of calibration of ± 0,1 pH units at 25 °C.

5.3.2.5 Stopwatch

5.3.2.6 Vortex mixer (electromechanical agitator, i.e. Vortex® mixer [3])

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

5.3.2.7 Membrane filtration apparatus (if this method is used), constructed of a material compatible with the product under test, with a filter holder which shall have a usable volume 50 ml minimum, and suitable for use with filters of diameter 47 mm to 50 mm, of 0,45 µm pore size.

The vacuum source used shall give an even filtration flow rate. In order to obtain a uniform distribution of the microorganisms over the membrane and in order 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 Containers : Test tubes or flasks of suitable capacity.

5.3.2.9 Graduated pipettes of nominal capacities 10 ml and 1 ml and 0,1 ml. Calibrated automatic pipettes may be used.

5.3.2.10 Petri dishes 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 Glass Roux bottles with straight neck

5.3.2.14 Microscope, preferably, a phase-contrast type, with magnification of at least x 400.

5.4 Preparation of spore test suspension and test solutions

5.4.1 Spore suspensions

5.4.1.1 Stock spore suspension of test organism

Bacillus subtilis spore stock suspension for the specific purposes of this standard can be purchased from a national culture collection or prepared by the testing laboratory.

If the *Bacillus subtilis* spore stock suspension is prepared in the testing laboratory, the suspension shall be prepared according to annex A.

As part of good laboratory practice (GLP), laboratories may want to include an internal reference product (e.g : sodium hypochlorite) to check the resistance of the spores.

For the preparation of the stock spore suspensions of additionnal strains (see 5.2.1) refer to :

— annex A for *Bacillus cereus* ATCC 12826 ;

— annex C for *Clostridium sporogenes* CIP 7939.

5.4.1.2 Spore test suspension

To prepare the spore test suspension, dilute the spore stock suspension (see 5.4.1.1) with water (see 5.2.2.2). The number of spores in the test suspension must be adjusted to 1,5 x 10^6 to 5 x 10^6 cfu/ml, estimating the number of units by any suitable mean.

Maintain the suspension test in the water bath at 20 °C ± 1 °C and use within 2 h.

Microscopic examination under 400 x magnification shall be carried out immediately after the preparation and just before the test, to show the absence of vegetative cells and germinative spores.

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3) 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.

If there is any evidence of spore germination, the suspension shall be discarded.

For counting of the spore test suspension prepare 10^{-4} and 10^{-5} dilutions of the test suspension (see 5.4.1.3) using water (see 5.2.2.2). Mix (see 5.3.2.6). Take a sample of 1,0 ml of each dilution in duplicate and transfer each 1,0 ml sample into separate Petri dishes (see 5.3.2.10) and add 12 ml to 15 ml melted GYA (see 5.2.2.3), cooled to 45 °C ± 1 °C.

5.4.1.3 Counting of spore test suspension

Incubate the Petri dishes at 30 °C ± 1 °C (see 5.3.2.3) for 3 days. Determine the higher number of colonies V_c for each plate. Calculate the number of cfu/ml [4] (see 5.4.1.3).

in the test suspension (N) using the method given in 5.6.1.1.

5.4.2 Product test solution

Details of samples of the product as received shall be recorded.

Product test solutions shall be prepared in hard water (see 5.2.2.6) at three different concentrations to include one concentration in the active range and one concentration in the non-active range. The concentration of the product test solution shall be 1,25 times the required test concentration.

For solid products, dissolve the product as received by weighing at least 1 g ± 10 mg of the product in a volumetric flask and filling up with hard water (see 5.2.2.6). Subsequent dilutions shall be prepared in volumetric flasks (see 5.3.2.12) on a volume/volume basis in hard water (see 5.2.2.6).

For liquid products, dilutions of the product shall be prepared in hard water (see 5.2.2.6) on a volume/volume basis using volumetric flasks (5.3.2.12).

For products supplied in a ready to use state, water (see 5.2.2.2) shall be used to prepare dilutions.

When the product is diluted in hard water it shall give a physically homogeneous stable preparation.

The product test solutions and dilutions of it shall be prepared freshly and used within 60 min.

NOTE If the product is of low stability this period should be shortened.

The concentration of the product stated in the test report shall be the test concentration. Record the test concentration in terms of mass per volume or volume per volume.

5.5 Procedure

5.5.1 Choice of experimental conditions

The selection of contact temperature and contact time shall be carried out according to the practical use considered for the product (see clause 4) as follows :

a) temperature : θ in °C :

- the temperature to be tested is 20 °C ± 1 °C ;
- the additional temperatures may be chosen from 4 °C ± 1 °C or 10 °C ± 1 °C or 40 °C ± 1 °C or 75 °C ± 1 °C ;

b) contact time : t in min :

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[4] cfu/ml = Colony forming unit per ml.

— the contact time to be tested is 60 min ± 10 s ;

— the additional contact times may be chosen from 5 min ± 10 s or 15 min ± 10 s or 30 min ± 10 s ;

c) strains :

— strains shall be as stated in 5.2.1 ;

d) interfering substance :

— the interfering substance to be tested is the bovine albumin solution (see 5.2.2.7) under clean conditions (0,3 g/l bovine albumin).

The product shall not cause the formation of any precipitate in the experimental conditions used.

Each selected experimental condition (θ, t, strains) shall be validated in accordance with annex B.

5.5.2 Test procedure for assessing the sporicidal effect of the product

5.5.2.1 General

The method of choice is the dilution-neutralization method. To determine a suitable neutralizer the following procedure shall be adopted. Carry out the validation of the dilution neutralization method (B.4.1) using a suitable neutralizer, chosen according to laboratory experience and published data.

If this neutralizer is not valid, repeat the validation test using an alternative neutralizer containing a combination of polysorbate 80 30 g/l, saponin 30 g/l, L-histidine 1 g/l, lecithin 3 g/l, sodium thiosulphate 5 g/l in either water (see 5.2.2.2) or in phosphate buffer 0,0025 mol/l. If both neutralizers are found to be invalid, the membrane filtration may be used in place of the dilution-neutralization method.

The inactivation of the sporicidal activity of the product shall be validated for each of the tested strains and for each of the chosen experimental conditions (see 5.5.1).

5.5.2.2 Dilution-neutralization method

5.5.2.2.1 General

a) if θ is lower or equal to 40 °C ± 1 °C :

Prior to testing, equilibrate all reagents (product test solutions, spore test suspension, interfering substance) to the test temperature of θ°C ± 1 °C using the water bath (see 5.3.2.2) controlled at θ°C ± 1 °C. Check that the temperature of the reagents is stabilized at θ°C ± 1 °C. The neutralizer and water (see 5.2.2.2) shall be equilibrated at a temperature of 20 °C ± 1 °C.

b) if θ is higher than 40 °C ± 1 °C :

Prior to testing, equilibrate the product test solutions to the test temperature of θ°C ± 1 °C using the water bath (see 5.3.2.2) controlled at θ°C ± 1 °C. Check that the temperature of the reagents is stabilized at θ°C ± 1 °C. The neutralizer, the spore test suspension, the interfering substance and the water (see 5.2.2.2) shall be equilibrated at a temperature of 20 °C ± 1 °C.

5.5.2.2.2 Test procedure for sporicidal activity of products

Pipette 1,0 ml of interfering substance (see 5.2.2.7) into a test tube. Add 1,0 ml of the spore test suspension containing 1,5 x 10^6 to 5 x 10^6 cfu/ml [5] (see 5.4.1.3).

Start the stopwatch immediately, mix (see 5.3.2.6) and place the test tube in the water bath at θ °C ± 1 °C for 2 min ± 10 s. At the end of the contact time, add 8,0 ml of one of the product test solutions. Restart the stopwatch immediately, mix (see 5.3.2.6) and place the test tube in a water bath controlled at θ °C ± 1 °C for the appropriate contact time t ± 10 s.

NOTE When adding spore suspension, care should be taken to avoid touching the upper part of the test tube sides.

Just before the end of the contact time, mix (see 5.3.2.6). At the end of the contact time pipette 1,0 ml of the test mixture into a tube containing 8,0 ml neutralizer (see 5.2.2.4) and 1,0 ml water (see 5.2.2.2). Mix (see 5.3.2.6) and place in a water bath controlled at 20 °C ± 1 °C.

After a neutralization time of 5 min ± 10 s, immediately take a sample of 1,0 ml of neutralized mixture (neutralizer, product test solution, interfering substance, spore test suspension) in duplicate and transfer each 1,0 ml sample into separate Petri dishes (see 5.3.2.10) and quickly add 12 ml to 15 ml melted GYA (see 5.2.2.3), cooled to 45 °C ± 1 °C.

Perform this procedure using the other product test solutions.

5.5.2.2.3 Counting of the test mixture

Incubate the Petri dishes at 30 °C ± 1 °C (see 5.3.2.3) for 3 days.

Determine the higher number of colonies V_c for each plate.

Calculate the number of cfu/ml [5] in the test mixture (N_a) using the method given in 5.6.1.2.

For calculating the viable count of the test mixture, the dilution factor is 10^{-1}.

5.5.2.3 Membrane filtration method

5.5.2.3.1 General

a) if θ is lower or equal to 40 °C ± 1 °C :

Prior to testing, equilibrate all reagents (product test solutions, spore test suspension, interfering substance) to the test temperature of θ° C ± 1 °C using the water bath (see 5.3.2.2) controlled atθ° C ± 1 °C. Check that the temperature of the reagents is stabilized at θ °C ± 1 °C. The rinsing liquid (see 5.2.2.2) shall be equilibrated at a temperature of 20 °C ± 1 °C.

b) if θ is higher than 40 °C ± 1 °C :

Prior to testing, equilibrate the product test solutions to the test temperature of θ° C ± 1 °C using the water bath (see 5.3.2.2) controlled at θ° C ± 1 °C. Check that the temperature of the reagents is stabilized atθ° C ± 1 °C. The rinsing liquid, the spore test suspension, the interfering substance and the water (see 5.2.2.2) shall be equilibrated at a temperature of 20 °C ± 1 °C.

5.5.2.3.2 Test procedure for sporicidal activity of products

Pipette 1,0 ml of interfering substance (see 5.2.2.7) into a test tube. Add 1,0 ml of the spore test suspension (see 5.4.1.3).

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[5] cfu/ml = Colony forming unit per ml.

Start the stopwatch immediately, mix (see 5.3.2.6) and place the test tube in the water bath at θ °C ± 1 °C for 2 min ± 10 s. At the end of the contact time, add 8,0 ml of one of the product test solutions. Restart the stopwatch immediately, mix (see 5.3.2.6) and place the test tube in a water bath controlled at θ °C ± 1 °C for the appropriate contact time t ± 10 s.

Just before the end of the chosen contact time, mix (see 5.3.2.6). At the chosen contact time pipette two samples of 0,1 ml of the test mixture and transfer each sample into a separate membrane filtration apparatus equipped with a membrane and containing 50 ml of the rinsing liquid (see 5.2.2.5). Filter immediately. The time required for transfer and filtration should not exceed 1 min. If greater than 1 min, this time shall be recorded in the test report. Rinse with at least 150 ml but not more than 500 ml of rinsing liquid (see 5.2.2.5). If the rinsing liquid is not water, complete the procedure by filtering 50 ml of water (see 5.2.2.2). Then transfer the membranes to the surface of two separate GYA plates.

NOTE When transferring, care should be taken to ensure that the spores are on the upper side of the membrane when placed on the GYA and to avoid trapping air between the membrane and agar surface.

Perform this procedure using the other product test solutions.

5.5.2.3.3 Counting of test mixture

Incubate the Petri dishes at 30 °C ± 1 °C (see 5.3.2.3) for 3 days.

Determine the higher number of colonies V_c for each plate.

Calculate the number of cfu/ml [6)] in the test mixture (N_a) using the method given in 5.6.1.2.

5.5.3 Validation of dilution neutralization and membrane filtration method

The dilution-neutralization and membrane filtration methods shall be validated for each of the test organisms according to annex B.

The validation test (see annex B) shall also be carried out at the same time as the test procedure (see 5.5) using only the highest concentration and the same conditions (spore test suspension, product test solution and neutralizer or rinsing liquid) as used in the test (see 5.5.2.2 or 5.5.2.3).

5.6 Calculation and expression of results

5.6.1 Calculation of viable counts (cfu/ml) [7)]

5.6.1.1 Spore test suspension

Viable counts of the spore test suspension (see 5.4.1.3) should be calculated according to the principles described as follows :

a) only colony counts which are less than 300 cfu/set of plates should be used for calculation of viable counts. For a result to be valid at least one set of plates has to contain 15 or more colonies ; viable counts should be calculated using at least one pair of the set of plates, where one or both are containing more than 15 colonies and both contain less than 300 colonies. If the set of plates from 2 dilutions fall within this range calculate the number of cfu/ml as the weighted mean count. If the set of plates from only one dilution fall within this range calculate the arithmetic mean.

For calculation of the weighted mean count, use the following formula (1) :

---

6) cfu/ml = Colony forming unit per ml.

7) cfu/ml = Colony forming unit per ml.

$$\text{cfu/ml} = \frac{c}{(n_1 + 0{,}1\, n_2)\, d} \qquad (1)$$

where

c is the sum of the colonies counted on all the plates taken into account ;

n_1 is the number of the sets of plates taken into account at the first dilution ;

n_2 is the number of the sets of plates taken into account at the second dilution ;

d is the dilution factor corresponding to the first dilution taken into account.

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.

b) as a result the number of cfu/ml is expressed by a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

EXAMPLE $\dfrac{168+215+14+25}{(2+0{,}1\,\text{x}\,2)10^{-4}} = \dfrac{422}{2{,}2\,\text{x}\,10^{-4}} = 1{,}9182\ \text{x}\ 10^{6} = 1{,}9\ \text{x}\ 10^{6}\ (\text{in cfu/ml})$

5.6.1.2 Test procedure and test for validation

For the test procedure (see 5.5.2.2.2 and 5.5.2.3.2) and test for validation (see B.2, B.4.1 and B.4.2) the viable count should be calculated using the following method. Only colony counts which are less than 300 cfu per plate should be used for calculation of viable counts. Viable counts should be calculated using colony counts from both plates. When at least one plate contains 15 or more colonies, use the following formula (2) for calculation of the viable counts in cfu/ml :

$$\frac{c}{n \times d \times V} \qquad (2)$$

where

c is the sum of the colonies counted on both plates ;

n is the number of plates taken into account ;

d is the dilution factor corresponding to the dilution taken into account. For the dilution-neutralization test procedure (see 5.5.2.2.) and the spore suspension (see B.2) the dilution factor is 10^{-1} ;

V is the sample volume. For the dilution neutralization and validation procedures (see 5.5.2.2.3 and B.4.1) and the spore suspension (see B.2) the sample volume is 1,0 ml. For the membrane filtration test and validation procedure (see 5.5.2.3.3 and B.4.2) the sample volume is 0,1 ml.

For the test procedure (see 5.5) where the number of cfu on all plates counted is lower than 15, record the viable count of the test mixture as lower than $1{,}5 \times 10^2$ cfu/ml ($< 1{,}5 \times 10^2$ cfu/ml). Where the number of cfu on all plates counted is higher than 300 record the viable count of the test mixture as higher than 3×10^3 cfu/ml ($> 3 \times 10^3$ cfu/ml).

5.6.2 Verification of methodology

For each test organism check that :

a) N is between 1,5 x 10^6 cfu/ml and 5 x 10^6 cfu/ml ;

b) N_v is between 6 x 10^2 and 3 x 10^3 cfu/ml ;

c) B is equal to or greater than 0,05 times N_v ;

d) C is equal to or greater than 0,5 times B ;

e) A is equal to or greater than 0,05 times N_v.

where

N is the number of cfu/ml of the spore test suspension (see 5.4.1.3) ;

N_v is the number of cfu/ml of the spore suspension (see B.2) ;

B is the number of cfu/ml of the neutralizer toxicity validation (see B.4.1.2 b)) or of the filtration control (see B.4.2.2 b)) ;

C is the number of cfu/ml of the dilution-neutralization validation (see B.4.1.2 c)) or of the membrane filtration test control (see B.4.2.2 c)) ;

A is the number of cfu/ml of the experimental conditions validation (see B.4.1.2 a) or B.4.2.2 a)).

5.6.3 Expression of results

For the test organism record the number of cfu/ml in the spore test suspension (N) (see 5.4.1.3) and after the test procedure for sporicidal activity of the product (N_a) (see 5.5.2.2.3 or 5.5.2.3.3).

For the validation of neutralization (see annex B) record the number of cfu/ml (N_v) in the spore suspension (see B.2).

For validation of the dilution neutralization method (see B.4.1) record the number of cfu/ml in the neutralizer toxicity control (B), the dilution neutralization control (C) and the experimental conditions control (A).

For validation of the membrane filtration method (see B.4.2), record the number of cfu/ml in the filtration control (B), the filtration test control (C) and the experimental conditions control (A).

For each test organism and product test concentration calculate and record the reduction in viability as follows :

$$R = \text{reduction in viability} = \frac{N \times 10^{-1}}{N_a} \qquad (3)$$

5.7 Conclusion

Sporicidal activity for general purposes is characterized by the concentration of the tested product for which criteria 5.6.1 and 5.6.2 are met and for which a 10^3 or more reduction in viability is demonstrated under the required test conditions : 60 min ± 10 s, 20 °C ± 1 °C and clean conditions (see clause 4), and when the test organisms are spores of *Bacillus subtilis*.

Sporicidal activity for specific purpose is characterized by the concentration of the tested product for which criteria 5.6.1 and 5.6.2 are met and for which a 10^3 or more reduction in viability is demonstrated, when the test organisms are spores of *Bacillus subtilis* and if required (see clause 4) additional test organisms, under additional conditions : t in min and θ in °C.

It is accepted that for certain applications, this suspension test may provide sufficient information for the particular application and that additional surface test may not be relevant (see annex H).

For applications where this suspension test without surface test is used to support use recommendations, the justification for omitting surface test should be given.

5.8 Test report

The test report shall refer to this European Standard.

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

a) identification of the laboratory ;

b) identification of the sample :

   1) name of the product ;

   2) batch number ;

   3) manufacturer ;

   4) date of delivery ;

   5) storage conditions ;

   6) product diluent recommended by the manufacturer for use ;

   7) active substance(s) and its (their) concentration(s) (optional) ;

c) test method and its validation :

   If the dilution-neutralization method is used, full details of the tests for validation of the neutralizer shall be given.

   If the membrane filtration method is used, full details of the procedure which was carried out in order to justify the use of the membrane filtration method shall also be given.

d) experimental conditions :

   1) period of analysis ;

   2) product diluent used during the test ;

   3) product test concentrations ;

   4) appearance product dilutions ;

   5) contact time(s) ;

   6) test temperature(s) ;

   7) stability of the mixture (interfering substance and product diluted in hard water) ;

   8) temperature of incubation ;

   9) neutralizer or rinsing liquid ;

   10) identification of the spore strains used ;

   11) preparation of the spore suspension (dates of preparation, origin and sensitivity control) ;

12) details of any operation not specified in this European Standard, and any operation regarded as optional as well as any incidents likely to have affected the results ;

e) test results :

1) validation tests ;

2) evaluation of sporicidal activity (see Table F.1) ;

f) conclusion ;

g) locality, date and identified signature.

NOTE An example of a typical test report is given in annex F.

Annex A
(normative)

Preparation of *Bacillus* spore stock suspensions

A.1 Material and reagents

Those defined in 5.2 and the following :

A.1.1 Tryptone Glucose Broth (TGB)

For preparation of the inoculum of *Bacillus* :

Yeast extract	2,5 g
Tryptone	5,0 g
Glucose	1,0 g
Water (see 5.2.2.2)	1 000,0 ml

Distribute in test tubes at a rate of 10 ml per tube. Sterilize in the autoclave (see 5.3.1). After sterilization the pH of the medium shall be equivalent to 7,2 ± 0,2 when measured at 20 °C.

A.1.2 Yeast extract Agar (MYA)

For preparation of *Bacillus* spores :

Meat extract	10,0 g
Yeast extract	2,0 g
$MnSO_4$, H_2O	0,04 g
Agar	15,0 g
Water (see 5.2.2.2)	1 000,0 ml

Distribute in Roux flasks. Sterilize in the autoclave (see 5.3.1). After sterilization the pH of the medium shall be equivalent to 7,0 ± 0,2 when measured at 20 °C.

A.1.3 Centrifuge, capable of 10 000 g acceleration

A.2 Preparation of *Bacillus* spore stock suspensions

Seed the TGB medium (see A.1.1) with approximately 10^6 spores from a batch previously prepared. Incubate at 30 °C in order to obtain a culture in the exponential growth stage (about 24 h) containing approximately 10^7 bacillus per millilitre. Transfer 2 ml or 3 ml of this culture into a Roux flask containing MYA (see A.1.2) and tilt it several times so that the inoculum comes into contact with all of the surface of the agar. Remove excess inoculum.

Incubate the Roux flasks at 30 °C ± 1 °C. After the third day of incubation, note the state of the culture under a microscope, preferably a phase-contrast type. If sporulation has not started, it is better to begin again. Otherwise, continue the incubation until the plant cells have dissolved (8 to 10 days). Recover the cultures and place them in suspension in water (see 5.2.2.2).

Carry out four successive flushing operations by centrifuging during 20 min (see A.1.3) in water (see 5.2.2.2). Transfer the last suspension to a flask with screw top and heat it for 10 min at 75 °C ± 1 °C.

Titrate the spores on GYA (see 5.2.2.3) after suitable dilution in water (see 5.2.2.2). Incubate the plates for 72 h at 30 °C ± 1 °C. Store at 5 °C ± 1 °C or store as a frozen stock suspension.

Annex B
(normative)

Validation of dilution-neutralization and membrane filtration methods

B.1 Principle

A neutralizer is chosen for each product in accordance with B.4.1 (see 5.5). If a suitable neutralizer cannot be found the membrane filtration method in accordance with B.4.2 is used.

B.2 Preparation of spore suspension

To prepare the spore suspension, dilute the spore test suspension (see 5.4.1.3) with water (see 5.2.2.2) to obtain the spore count of 6 x 10^2 cfu/ml to 3 x 10^3 cfu/ml.

For counting of the suspension prepare a 10^{-1} dilution with water (see 5.2.2.2). Mix (see 5.3.2.6). Take a sample of 1,0 ml of the 10^{-1} dilution in duplicate and transfer each 1,0 ml sample into separate Petri dishes (see 5.3.2.10) and add 12 ml to 15 ml melted GYA (see 5.2.2.3), cooled to 45 °C ± 1 °C.

Incubate the Petri dishes at 30 °C ± 1 °C (see 5.3.2.3) for 3 days.

Determine the higher number of colonies V_c of each plate.

Calculate the number of cfu/ml in the test suspension (N_v) using the method given in 5.6.1.2 (dilution factor 10^{-1} and volume 1 ml).

B.3 Preparation of product test solution

Prepare (see 5.4.2) a product test solution of the highest strength used in the test (1,25 C).

B.4 Test for validation

B.4.1 Dilution-neutralization method

B.4.1.1 General

Perform the following procedure for each experimental conditions (strain, temperature, contact time) :

a) if θ is lower or equal to 40 °C ± 1 °C :

Prior to testing, equilibrate all reagents (product test solutions, spore test suspension, hard water (see 5.2.2.6) to the test temperature of θ°C ± 1 °C using the water bath (see 5.3.2.2) controlled atθ°C ± 1 °C. Check that the temperature of the reagents is stabilized at θ°C ± 1 °C. The neutralizer and the water (see 5.2.2.2) shall be equilibrated at 20 °C ± 1 °C.

b) if θ is higher than 40 °C ± 1 °C :

Prior to testing, equilibrate the product test solutions to the test temperature of θ °C ± 1 °C using the water bath (see 5.3.2.2) controlled at θ °C ± 1 °C. Check that the temperature of the reagents is stabilized at θ °C ± 1 °C. The neutralizer, the spores test suspension, the interfering substance and the hard water (see 5.2.2.6) shall be equilibrated at a temperature of 20 °C ± 1 °C.

B.4.1.2 Validation

a) Validation of selected experimental conditions (or verification of the absence of any lethal effect in the test conditions).

Place in a tube 1,0 ml of the interfering substance (see 5.2.2.7) and 1,0 ml of the diluted spore suspension containing 6 x 10^2 cfu/ml to 3 x 10^3 cfu/ml prepared in accordance with B.2.

Mix (see 5.3.2.6) for a few seconds and leave in the water bath at θ °C ± 1 °C for 2 min ± 10 s. At the end of this time, add 8,0 ml of hard water (see 5.2.2.6). Start the stopwatch at the beginning of the addition and mix (see 5.3.2.6) for a few seconds.

Leave in the water bath at θ °C ± 1 °C for the time t ± 10 s. Immediately before the end of the contact time, mix (see 5.3.2.6). At the end of the contact time, take a sample of 1,0 ml of the mixture in duplicate and transfer into separate Petri dishes (see 5.3.2.10). Add 12 ml to 15 ml melted GYA (see 5.2.2.3) cooled to 45 °C ± 1 °C.

Incubate the Petri dishes at 30 °C ± 1 °C (see 5.3.2.3) for 3 days.

Determine the higher number of colonies V_c for each plate.

Calculate the number of cfu/ml in the experimental conditions validation (A) using the method given in 5.6.1.2.

b) Neutralizer toxicity validation

Place in a test tube 8,0 ml of neutralizer (see 5.2.2.4) and 1,0 ml of water (see 5.2.2.2). Introduce 1,0 ml of the diluted spore suspension containing 6 x 10^2 cfu/ml to 3 x 10^3 cfu/ml prepared in accordance with B.2.

Start a stopwatch at the beginning of the addition and mix (see 5.3.2.6), leave in contact in the water bath at 20 °C ± 1 °C for 5 min ± 10 s. Immediately before the end of the contact time, mix (see 5.3.2.6). At the end of the contact time take a sample of 1,0 ml of the mixture in duplicate and transfer into separate Petri dishes (see 5.3.2.10). Add 12 ml to 15 ml melted GYA (see 5.2.2.3) cooled to 45 °C ± 1 °C.

Incubate the Petri dishes at 30 °C ± 1 °C (see 5.3.2.3) for 3 days. Determine the higher number of colonies V_c for each plate. Calculate the number of cfu/ml in the neutralizer toxicity conditions validation (B) using the method given in 5.6.1.2.

c) Dilution-neutralization validation

Place 1,0 ml of interfering substance (see 5.2.2.7) in a tube, add 1,0 ml of water (see 5.2.2.2) and then, starting a stopwatch, 8,0 ml of the product dilution prepared in B.3. Leave in contact in the water bath for the time t ± 10 s at a temperature θ °C ± 1 °C.

Then, transfer 1,0 ml of the mixture into a test tube containing 8,0 ml of neutralizer (see 5.2.2.4) previously kept in the water bath at 20 °C ± 1 °C. Leave in contact in this water bath for 5 min ± 10 s.

Add 1,0 ml of the spore suspension containing 6 x 10^2 cfu/ml to 3 x 10^3 cfu/ml prepared in accordance with B.2.

Start a stopwatch at the beginning of the addition and mix (see 5.3.2.6) for a few seconds. Leave in contact in the water bath at 20 °C ± 1 °C for 30 min ± 1 min.

Immediately before the end of the contact time, mix (see 5.3.2.6).

At the end of the contact time, take a sample of 1,0 ml of the mixture in duplicate and transfer into separate Petri dishes (see 5.3.2.10). Add 12 ml to 15 ml melted GYA (see 5.2.2.3) cooled to 45 °C ± 1 °C.

Incubate the Petri dishes at 30 °C ± 1 °C (see 5.3.2.3) for 3 days.

Determine the higher number of colonies V_c for each plate.

Calculate the number of cfu/ml in the dilution-neutralization validation (C) using the method given in 5.6.1.2.

B.4.2 Membrane filtration method

Perform the following procedure for each experimental conditions (strains, diluent, contact time, temperature).

B.4.2.1 General

a) if θ is lower or equal to 40 °C ± 1 °C :

Prior to testing, equilibrate all reagents (product test solutions, spore test suspension, hard water (see 5.2.2.6)) to the test temperature of θ°C ± 1 °C using the water bath (see 5.3.2.2) controlled at θ°C ± 1 °C. Check that the temperature of the reagents is stabilized at θ°C ± 1 °C. The rinsing liquid shall be equilibrated at 20 °C ± 1 °C.

b) if θ is higher than 40 °C ± 1 °C :

Prior to testing, equilibrate the product test solutions to the test temperature of θ°C ± 1 °C using the water bath (see 5.3.2.2) controlled at θ°C ± 1 °C. Check that the temperature of the reagents is stabilized at θ°C ± 1 °C. The rinsing liquid, the spores test suspension, the interfering substance and the hard water (see 5.2.2.6) shall be equilibrated at a temperature of 20 °C ± 1 °C.

B.4.2.2 Validation

a) Validation of selected experimental conditions (or verification of the absence of any lethal effect in the test conditions).

Place in a tube 1,0 ml of the interfering substance (see 5.2.2.7) and 1,0 ml of the diluted spore suspension suspension containing 6 x 10^2 cfu/ml to 3 x 10^3 cfu/ml prepared in accordance with B.2.

Mix (see 5.3.2.6) for a few seconds and leave in the water bath at θ °C for 2 min ± 10 s. At the end of this time, add 8,0 ml of hard water (see 5.2.2.6). Start a stopwatch at the beginning of the addition and mix (see 5.3.2.6) for a few seconds.

Leave in contact in the water bath at θ °C ± 1 °C for the time t ± 10 s. Immediately before the end of the contact time, mix (see 5.3.2.6). At the end of the contact time, sample in duplicate 1,0 ml of the mixture and transfer into two separate membrane filtration apparatus equipped with a membrane and containing 50 ml of the rinsing liquid (see 5.2.2.5). Filter and rinse with 50 ml of water (see 5.2.2.2) and then transfer the membranes to the surface of two separate GYA plates (see 5.2.2.3).

NOTE 1 When transferring membrane to GYA plates, care should be taken to ensure that the membrane is filtrate side uppermost and to avoid trapping air between the membrane and agar surface.

Incubate the Petri dishes at 30 °C ± 1 °C (see 5.3.2.3) for 3 days.

Determine the higher number of colonies V_c for each plate.

Calculate the number of cfu/ml in the experimental conditions control (A) using the method given in 5.6.1.2.

b) Validation of the filtration procedure

Sample in duplicate 0,1 ml of the diluted spore suspension suspension containing 6×10^2 cfu/ml to 3×10^3 cfu/ml prepared in accordance with B.2. Transfer into two separate membrane filtration apparatus equipped with a membrane and containing 50 ml of the rinsing liquid (see 5.2.2.5).

Filter and rinse with 50 ml of water (see 5.2.2.2) and then transfer the membranes to the surface of two separate GYA plates (see 5.2.2.3).

NOTE 2 When transferring membrane to GYA plates, ensure that the membrane is filtrate side uppermost and avoid trapping air between the membrane and agar surface.

Incubate the Petri dishes at 30 °C ± 1 °C (see 5.3.2.3) for 3 days.

Determine the higher number of colonies V_c for each plate.

Calculate the number of cfu/ml in the filtration control (B) using the method given in 5.6.1.2.

c) Validation of the membrane filtration method (or counting of the spores on the membranes which have previously been in contact with the mixture of product and interfering substance).

Place 1,0 ml of interfering substance (see 5.2.2.7) in a tube. Add 1,0 ml of the water (see 5.2.2.2) and then, starting a stopwatch, 8,0 ml of the product dilution prepared in B.3. Mix (see 5.2.3.6).

Leave in contact in the water bath for the time t ± 10 s at the temperature θ ± 1 °C.

Immediately before the end of the contact time, mix (see 5.3.2.6). At the end of the contact time sample in duplicate 0,1 ml of the mixture and transfer into two separate membrane filtration apparatus equipped with a membrane and containing 50 ml of the rinsing liquid (see 5.2.2.5).

Filter and rinse the membranes with at least 150 ml and not more than 500 ml per 50 ml or 100 ml measure of rinsing liquid (see 5.2.2.5).

Then cover the membranes with 50 ml of rinsing liquid (see 5.2.2.5) and add 0,1 ml of the diluted spore suspension containing 6×10^2 cfu/ml to 3×10^3 cfu/ml prepared in accordance with B.2.

Filter and rinse with 50 ml of water (see 5.2.2.2) and then transfer the membranes to the surface of two separate GYA plates (see 5.2.2.3).

NOTE 3 When transferring membrane to GYA plates, care should be taken to ensure that the membrane is filtrate side uppermost and to avoid trapping air between the membrane and agar surface.

Incubate the Petri dishes at 30 °C ± 1 °C (see 5.3.2.3) for 3 days.

Determine the higher number of colonies V_c for each plate.

Calculate the number of cfu/ml in the membrane filtration test validation (C) using the method given in 5.6.1.2.

B.5 Validation

Check that the test results comply with the relevant requirements of 5.6.1.2 or 5.6.2.

Annex C
(informative)

Preparation of *Clostridium sporogenes* spore stock suspension

C.1 Culture media and reagents

Those defined in 5.2 and the following :

C.1.1 Tryptone Broth (TB)

For preparation of the inoculum of *Clostridium* :

Tryptone	30,0 g
Glucose	5,0 g
Yeast extract	20,0 g
Sodium thioglycolate	1,0 g
Water (see 5.2.2.2)	1 000,0 ml

Sterilize in the autoclave (see 5.3.1). After sterilization the pH of the medium shall be equivalent to 7,4 ± 0,2 when measured at 20 °C.

C.1.2 Tryptone Agar (TA)

For preparation of *Clostridium* spores :

Tryptone	30,0 g
Glucose	5,0 g
Yeast extract	20,0 g
Sodium thioglycolate	1,0 g
Agar	15 g
Water (see 5.2.2.2)	1 000,0 ml

Sterilize in the autoclave (see 5.3.1). After sterilization the pH of the medium shall be equivalent to 7,4 ± 0,2 when measured at 20 °C.

C.1.3 Meat Glucose Yeast Agar (MGYA)

For counting of *Clostridium* spores :

Yeast extract	3,0 g
Peptic peptone of meat	10,0 g
Meat extract	10,0 g
Glucose	5,0 g
Sodium acetate	5,0 g
Soluble starch	1,0 g
Cystein	0,5 g
Agar	15 g
Water (see 5.2.2.2)	1 000,0 ml

Sterilize in the autoclave (see 5.3.1). After sterilization the pH of the medium shall be equivalent to 7,0 ± 0,2 when measured at 20 °C.

C.1.4 Sterile paraffin

Melting point 60 °C.

C.2 Apparatus and glassware

C.2.1 **Jars** for anaerobiosis with oxygen removal system : Sealed jars permitting anaerobiosis to be maintained.

C.2.2 **Incubator**, capable of being controlled at 37 °C ± 1 °C

C.2.3 **Centrifuge**, capable of 10 000 g acceleration

C.3 Preparation of regenerated media and incubation conditions

Media shall be "regenerated" by placing them in a bath of boiling water for 20 min. Cool the liquid media by placing it in a bath of cold water and solid media at 45 °C ± 1 °C. Seed immediately.

For the incubation of broths, pour a 1 to 2 cm layer of paraffin (see C.1.4), melted in a bath of boiling water and cooled to a temperature of approximately 65 °C beforehand, onto the surface of the liquid media.

For the incubation of agars, place the solid media (poured beforehand into a dish) in jars for anaerobiosis.

For counting of C*lostridium* spore suspensions or test mixtures, incubate Petri dishes 3 days at 37 °C ± 1 °C.

C.4 Preparation of *Clostridium* spore stock suspension

The following procedure generally provides good sporulation. Any other procedure with the same culture media may be used.

Using approximately 10^6 spores from a batch prepared previously, sow the regenerated (see C.3) medium TB (see C.1.1) then cover it with paraffin (see C.1.4). Incubate for 48 h at 37 °C ± 1 °C in order to obtain a culture in the exponential growth stage containing approximately 10^7 cells per millilitre.

Transfer 2 ml or 3 ml of this culture into a Roux flask containing TA (see C.1.2) and tilt the flask several times so that the inoculum comes into contact with all of the surface of the agar-agar. Remove excess inoculum. Incubate the Roux flask at 37 °C ± 1 °C with anaerobiosis. After 3 days of incubation, note the state of the culture under the microscope, preferably a phase-contrast type. If sporulation has not started, it is better to begin again. Otherwise, continue the incubation until the plant cells have lysed (8 to 10 days). Recover the cultures and place them in suspension in water (see 5.2.2.2).

Carry out 4 successive flushing operations by centrifuging in water during 20 minutes (see 5.2.2.2). Transfer the last suspension into a flask with screw top and heat it for 10 min at 75 °C ± 1 °C.

Titrate the spores on MGYA (see C.1.3) after suitable dilution in water (see 5.2.2.2). Incubate the plates for 48 h at 37 °C ± 1 °C. Store at 5 °C ± 1 °C or store as a frozen stock suspension.

Annex D
(informative)

Neutralizers

Any of the following neutralizers may be used :

— lecithin 3 g/l ; polysorbate 80 [8)] 30 g/l ; sodium thiosulphate 5 g/l ; L-histidine 1 g/l ; saponine 30 g/l in diluent (see 5.2.2.4) or in phosphate buffer 0,25 mol/l at 1 % (V/V) ;

— 30 g/l polysorbate 80 [8)] ; lecithin 3 g/l ; L-histidine 1 g/l ;

— glycine as a function of concentration of product ;

— sodium thiosulphate at 5 g/l ;

— catalase or peroxidase : One unit of these enzymes catalyzes the decomposition of 1 μmol of hydrogen peroxide per minute at 25 °C and at pH 7 ;

— polysorbate 80 [8)] 30 g/l ; saponin 30 g/l ; L-histidine 1 g/l ; L-cysteine 1 g/l.

NOTE The above list is not exhaustive and other media may be used.

[8)] Analytical quality, non-hydrolized in accordance with European Pharmacopaeia volume 1.TWEEN 80® 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.

Annex E
(informative)

Rinsing liquids

Any of the following rinsing liquids may be used :

— water (see 5.2.2.2) ;

— aqueous solution of 0,1 % (V/V) polysorbate 80 [9] ;

— aqueous solution of 0,5 % (V/V) polysorbate 80 [9] ;

— aqueous solution of 0,5 % (V/V) polysorbate 80 [9] and 0,7 g/l lecithin ;

— neutralizer (see 5.2.2.4) ;

— buffer solutions.

NOTE The above list is not exhaustive and other liquids can be used.

[9] Analytical quality, non-hydrolized in accordance with European Pharmacopaeia volume 1.TWEEN 80® 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.

Annex F
(informative)

Example of a typical test report

Sporicidal activity in general use conditions

a) Identification of the test laboratory ;

b) Identification of the sample ;

Name of the product	Z
Batch number	91-71-51
Manufacturer	Centipede Formulations Inc
Date of delivery	1991-02-11
Storage conditions	room temperature and darkness
Product diluent recommended by the manufacturer for use	Potable water
Active substance(s) and its (their) concentration(s) (optional)	Not indicated

c) Test method and its validation :

Method	Dilution-neutralization
Neutralizer	glycine

d) Experimental conditions :

Period of analysis	1991-02-20 to 1991-03-12
Product diluent used during the test	sterile hard water 300 mg/kg $CaC0_3$
Product test concentrations	0,5 ; 0,75 ; 1 % (V/V)
Appearance product dilutions	colourless, clear product solution,
Contact time	t = 60 min ± 10 s
Test temperature	θ = 20 °C ± 1 °C
Stability of the mixture, (interfering substance and product diluted in hard water)	
Precipitate absent throughout the test	
Temperature of incubation	37 °C ± 1 °C
Identification of the spore strains used	*Bacillus subtilis* ATCC 6633
Spore origin	Institut Pasteur

e) Test results

See tables.

f) Conclusion

According to EN (date of edition), the batch 91.71.51 of product Z when diluted at 1 % (V/V), in hard water, possesses sporicidal activity in sixty minutes at 20 °C under clean condition (0,3 g/l bovine albumin) for reference strain of spores of *Bacillus subtilis* ATCC 6633.

g) Locality, date and identified signature

NOTE The test product, batch No. and manufacturer are given as imaginary examples only.

Table F.1 — Test results

Test organisms	**Validation test**				**Spore test suspension (5.4.1.4)**	**Test procedure at concentration % (v/v) (5.5.2)**			
	Spore suspension (B.2)	Experimental conditions (B.4.1.2a) and (B.4.2.2a)	Neutralizer toxicity control (B.4.1.2b) or filtration control (B.4.2.2b)	Dilution-Neutralization control (B4.1.2c) or filtration test control (B4.2.2c)			0,50	0,75	1,00
Bacillus	V_c : 195 ;154	V_c : 159 ;163	V_c : 168 ; 183	V_c : 155 ;161	10^{-4} : 157 ; 172	V_c	> 300 ; > 300	83 ; 102	0 ; 7
subtilis					10^{-5} : 21 ; 16	N_a	> 3 x 10^3	9,3 x 10^2	< 1,5 x10^2
ATCC 6633	N_v : 1,7 x 10^3	A : 1,6 x 10^2	B : 1,8 x10^2	C : 1,6 x 10^2	N : 1,7 x10^8	R	< 5,6 10^5	1,8 x 10^2	>1,1 x 10^3

V_c = viable count

N = number of cfu/ml of the spore test suspension (5.4.1.3)

N_v = number of cfu/ml of the spore suspension (B.2)

R = reduction in viability

N_a = number of cfu/ml in the test mixture (5.5.2.2.3 or 5.5.2.3.3)

A = number of cfu/ml of the experimental conditions validation (B.4.1.2 a)) or (B.4.2.2 a))

B = number of cfu/ml of the neutralizer toxicity validation (B.4.1.2 b)) or of the filtration validation (B.4.2.2 b))

C = the number cfu/ml of the dilution-neutralization validation (B.4.1.2 c)) or of the membrane filtration test validation (B.4.2.2 c))

Annex G
(informative)

Referenced strains in national collections

G.1 *Bacillus subtilis*

ATCC	6633
DSM	347
NCTC	10400
CCM	19999
IAM	1069
NCIB	8054
CIP	52.62

G.2 *Bacillus cereus*

ATCC	12826
CIP	105151

G.3 *Clostridium sporogenes*

CIP	79.39

Annex H
(informative)

Information on the application and interpretation of European Standards on chemical disinfectants and antiseptics

CEN/TC 216 would like to draw the attention of the reader of this standard to the agreements which were reached concerning the relationship between this standard and future standards.

This information should be followed when using the European Standards on chemical disinfectants and antiseptics.

H.1 General guidelines for the application and interpretation of test methods in accordance with European Standards for chemical disinfectants and antiseptics

a) All "use recommendations" for chemical disinfectant and antiseptic products should be supported by results of bactericidal, fungicidal, sporicidal and virucidal EN tests which are appropriate to the intended field and method of application ;

b) to achieve this, chemical disinfectant and antiseptic products should be subjected to a specified programme of testing which will include phase 1, phase 2 step 1 and phase 2 step 2 tests, except for situations as given in points e), f) and g) ;

c) "use recommendations" may be supported by results of phase 3 tests which are appropriate to the intended field and method of application ;

d) the various steps and phases are defined as follows :

   — phase 1 suspension tests for the basic activity of the product ;

   — phase 2 step 1 suspension tests under conditions representative of practical use ;

   — phase 2 step 2 other laboratory tests e.g. handwash, handrub and surface tests simulating practical conditions ;

   — phase 3 field tests under practical conditions ;

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

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

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

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

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

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

h) all bactericidal, fungicidal and sporicidal claims for "bioactive substances" should be supported by appropriate phase 1 tests.

H.2 Guide to interpretation of tests for chemical disinfectants and antiseptics

A separate standard (or standards) which will be used as a "Guide to interpretation of tests for chemical disinfectants and antiseptics" will be prepared after the standard test methods have been agreed ; the purpose of this standard will be to specify in detail the relationship of the various tests to one another and to use recommendations.

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