BRITISH STANDARD

BS EN
14347:2005

Chemical disinfectants and antiseptics — Basic sporicidal activity — Test method and requirements (phase 1, step 1)

The European Standard EN 14347:2005 has the status of a British Standard

ICS 11.080.20; 71.100.35

National foreword

This British Standard is the official English language version of EN 14347:2005.

The UK participation in its preparation was entrusted to Technical Committee CH/216, Chemical disinfectants and antiseptics, which has the responsibility to:

— aid enquirers to understand the text;

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

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This British Standard was published under the authority of the Standards Policy and Strategy Committee on 5 April 2005

Summary of pages

This document comprises a front cover, an inside front cover, the EN title page, pages 2 to 39 and a back cover.

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Amendments issued since publication

Amd. No.	Date	Comments

ISBN 0 580 45807 5

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 14347

March 2005

ICS 11.080.20; 71.100.35

English version

Chemical disinfectants and antiseptics - Basic sporicidal activity - Test method and requirements (phase 1, step 1)

Désinfectants chimiques - Activité sporicide de base - Méthode d'essai et exigences

Chemische Desinfektionsmittel und Antiseptika - Sporizide Wirkung (Basistest) - Prüfverfahren und Anforderungen (Phase 1, Stufe 1)

This European Standard was approved by CEN on 8 December 2004.

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Ref. No. EN 14347:2005: E

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Foreword

This document (EN 14347:2005) has been prepared by Technical Committee CEN/TC 216 “Chemical disinfectants and antiseptics”, the secretariat of which is held by AFNOR.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by September 2005, and conflicting national standards shall be withdrawn at the latest by September 2005.

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Introduction

This document describes a suspension test for establishing whether a chemical disinfectant or antiseptic has or does not have a sporicidal activity in the areas described in the scope.

If a product complies with the test requirements, it can be considered as possessing a sporicidal activity. The acceptibility of a product as a chemical disinfectant or antiseptic for a defined purpose cannot be determined from this test method. Chemical disinfectants or antiseptics are subjected to further testing by relevant tests according to European Standards to evaluate their activity under conditions appropriate to their intended use.

There is no evidence that the strains used in this document are virulent.

1 Scope

This document specifies a test method (phase 1) and the minimum requirements for sporicidal activity of chemical disinfectant or antiseptic products that form a homogeneous, physically stable preparation when diluted with water. Products can only be tested at a concentration of 80 % or less as some dilution is always produced by adding the test organisms.

This document applies to products that are used in agricultural (but not crop protection), domestic service, food hygiene and other industrial fields, institutional, medical and veterinary applications.

NOTE 1 This method cannot be applied for testing sporicidal activity of a product against spores of *Clostridium sp.*

NOTE 2 This document does not evaluate the activity of a product for an intended use. More specific test methods described in European Standards (see Introduction) are used for further assessement of the efficacy of chemical disinfectants and antiseptics for a defined purpose.

NOTE 3 This method corresponds to a phase 1 test (Annex E).

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 microbial strains used for the determination of bactericidal and fungicidal activity*

EN 14079, *Non-active medical devices – Performance requirements and test methods for absorbent cotton gauze and absorbent cotton and viscose gauze*

3 Terms and definitions

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

3.1
product (for chemical disinfectants and/or antiseptics)
chemical agent or formulation used as chemical disinfectant or antiseptic

3.2
sporicide
product which kills dormant bacterial spores of relevant test organisms under defined conditions

NOTEThe adjective derived from "sporicide" is "sporicidal"

3.3
sporicidal activity
capability of a product to produce a reduction in the number of viable bacterial spores of relevant test organisms under defined conditions

3.4
sporistatic activity
capability of a product to inhibit the germination of dormant bacterial spores under defined conditions

4 Requirements

The product, when diluted with water (5.2.2.2) and tested in accordance with Clause 5 under the obligatory test conditions (20 °C; 30 min, 60 min or 120 min), shall demonstrate at least a decimal log (lg) reduction in viable counts of 4 and ONT (original neutralization tube) shows visible growth. It is possible to test also the product as delivered (highest test concentration is 80 %).

The sporicidal activity shall be evaluated using the dormant spores of: *Bacillus subtilis* and *Bacillus cereus.*

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

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

5 Test method

5.1 Principle

5.1.1 A test suspension of bacterial spores is added to a sample of the product as delivered and/or diluted with water. The mixture is maintained at 20 °C ± 1 °C for a specific contact time chosen from one of the following 30 min ± 10 s, 60 min ± 10 s or 120 min ± 60 s. At the end of this contact time, an aliquot is taken and transferred into a original neutralization tube (ONT). This tube serves for neutralizing the sporicidal and/or the sporistatic action in this portion and, after incubation, is an indicator for a successful neutralization in the actual test. The numbers of surviving bacterial spores in each sample are determined (by counting the sporeforming bacteria) and the reduction is calculated.

5.1.2 The test is performed using dormant spores of *Bacillus subtilis* and *Bacillus cereus* as test-organisms.

5.1.3 Additional and optional contact times, temperatures and test organisms are specified. Further test organisms can be used.

5.2 Materials and reagents

5.2.1 Test organisms

The sporicidal activity shall be evaluated using the dormant spores of the following organisms[1)]:

— *Bacillus subtilis subsp. spizizenii* ATCC 6633

— *Bacillus cereus* ATCC 12826

1) The ATCC numbers are the collection numbers of strains supplied by the American Type Culture Collection (ATCC). This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of the product named.

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 test organisms are not classified at a reference center, their identification characteristics shall be stated. In addition, they shall be held by the testing laboratory or national culture collection under a reference for 5 years.

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

5.2.2 Culture media and reagents

5.2.2.1 General

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

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

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

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

5.2.2.2 Water

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

Sterilize in the autoclave [5.3.2.1 a)].

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

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

5.2.2.3 Tryptone Soya Agar (TSA)

Tryptone, pancreatic digest of casein	15,0 g
Soya peptone, papaic digest of Soybean meal	5,0 g
Sodium chloride (NaCl)	5,0 g
Agar	15,0 g
Water (5.2.2.2)	to 1 000,0 ml

Sterilize in the autoclave [5.3.2.1 a)]. After sterilization the pH of the medium shall be equivalent to $7,2 \pm 0,2$ when measured at 20 °C.

NOTE In special circumstances (problems with neutralization - see 5.5.1.2 and 5.5.1.3) it may be necessary to add neutralizer to TSA (Annex B.3).

5.2.2.4 Blood agar[2)]

Protease peptone	15,0 g
Liver digest	2,5 g
Yeast extract	5,0 g
Sodium chloride (NaCl)	5,0 g
Agar	12,0 g
Water (5.2.2.2)	to 1 000,0 ml
Defibrinated sterile sheep blood	70 ml to 100 ml

Sterilize in the autoclave excluding the sheep blood [5.3.2.1 a)]. After sterilization cool down to 45 °C to 50 °C and add the sterile defibrinated sheep blood and mix thoroughly. Fill into Petri dishes.

5.2.2.5 Neutralizer

The neutralizer shall be validated for the product being tested in accordance with 5.5.2.4. The neutralizer shall be sterile.

NOTE 1 Information on neutralizers that have been found to be suitable for some categories of products is given in Annex B.

NOTE 2 The neutralizer has to form a homogenous, clear, not cloudy preparation when added to TSB [5.2.2.6 b)].

5.2.2.6 Tryptone Soya Broth (TSB)

a) Composition of Tryptone Soya Broth (TSB)

Tryptone, pancreatic digest of casein	17,0 g
Soya peptone, papaic digest of Soybean meal	3,0 g
Sodium chloride (NaCl)	5,0 g
Dipotassiumhydrogenphosphat (K_2HPO_4)	2,5 g
Water (5.2.2.2)	to 1 000,0 ml

Sterilize in the autoclave [5.3.2.1 a)]. After sterilization the pH of the medium shall be equivalent to $7,3 \pm 0,2$ when measured at 20 °C.

b) Trypone Soya Broth (TSB) with neutralizer.

TSB [5.2.2.6 a)]. An adequate neutralizer shall be added according to its chemical properties before or after autoclaving (5.2.2.5). TSB plus neutralizer should be filled into glass tubes both in portions of 9 ml and 10 ml.

2) OXOID blood agar base in an example of a suitable product available commercially.

5.2.2.7 Sporulation Agar (Manganese-Sulfate-Agar):

Peptone USP 10,0 g

Yeast extract 2,0 g

Manganese sulfate ($MnSO_4$) 0,04 g

Agar 15,0 g

Water (5.2.2.2) to 1 000,0 ml

Sterilize in autoclave [5.3.2.1 a)] at 121 °C ± 1 °C for 20 min. Fill 150 ml into 1 000 ml Roux-bottle (5.3.2.15).

5.3 Apparatus and glassware

5.3.1 General

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

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

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

5.3.2 Usual microbiological laboratory equipment [3] and in particular, the following:

5.3.2.1 Apparatus for sterilization:

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

a) 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 °C ± 1 °C, at 45 °C ± 1 °C (to maintain melted TSA in case of pour plate technique) and at additional test temperatures ± 1 °C (5.5.1.1).

5.3.2.3 Incubator, capable of being controlled either at 36 °C ± 1 °C or at 37 °C ± 1 °C and at 30 °C ± 1 °C.

The same temperature shall be used for all incubations performed during a test and its control and validation.

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

For measuring the pH of the agar media (5.2.2.3, 5.2.2.4 and 5.2.2.7) a puncture electrode or a flat membrane electrode shall be used.

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

5.3.2.5 Stopwatch

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

5.3.2.7 Containers: Test tubes, culture bottles or flasks of suitable capacity.

5.3.2.8 Graduated pipettes of nominal capacities 10 ml and 1 ml and 0,1 ml.

Calibrated automatic pipettes may be used.

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

5.3.2.10 Glass beads (Diameter: 3 mm to 4 mm).

5.3.2.11 Volumetric flasks.

5.3.2.12 Orbital mechanical shaker.

5.3.2.13 Centrifuge, capable of 3 000 g and capable of being controlled at 10 °C to 15 °C.

5.3.2.14 Screw capped tubes of suitable capacity.

5.3.2.15 1 000 ml-glass-Roux bottles with straight neck.

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

5.3.2.17 Gauze (EN 14079).

5.3.2.18 Glass funnel (Diameter: 20 cm to 30 cm).

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

5.4 Preparation of bacterial spore suspensions and product test solutions

5.4.1 Test organism suspensions (Test and validation suspension)

Two different bacterial spore suspensions of the test organism have to be prepared: the “test suspension” to perform the test and the “validation suspension” to perform the controls and method validation.

5.4.1.1 Stock culture of test organisms

Stock cultures shall be kept in accordance with the requirements of EN 12353.

NOTE A freeze dried sample of the test organism is obtained from a culture collection.

This sample is cultured, prepared for storage, filled into storage vessels and placed in the deep freeze according to EN 12353. From the deep freeze samples a stock culture is prepared and subsequently used to prepare working cultures for the test procedure.

Defrost a deep freeze sample at room temperature. Inoculate a blood agar plate (5.2.2.4) with this suspension and incubate (5.3.2.3). Use the culture as stock culture and control culture for purity by visual examination. From the stock culture the bacterial spore suspension can be prepared (5.4.1.2).

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4) Vortex® in an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of this product.

5.4.1.2 Working culture of the test organisms (Sporulation) - Bacterial spore suspension

Inoculate 50 ml of TSB [5.2.2.6 a)] in a 100 ml culture flask with one single colony of the stock culture (5.4.1.1) and incubate for 24 h on an orbital mechanical shaker (5.3.2.12) at 36 °C $\pm$ 1 °C (or 37 °C $\pm$ 1 °C).

10 ml of this preculture are transferred into 1 000 ml Roux-bottles with straight necks (5.3.2.15) containing 150 ml of sporulation agar (5.2.2.7). The liquid culture is distributed on the agar surface with the help of sterile glass beads, diameter 3 mm to 4 mm, incubated in the Roux-bottles for 2 days at 36 °C $\pm$ 1 °C (or 37 °C $\pm$ 1 °C) and then for 21 days at 30 °C $\pm$ 1 °C. During incubation close the bottle with a sterile plug (cellulose tissue).

For harvesting the spores 10 ml of water (5.2.2.2) are pipetted into the Roux-bottle and the spores are washed off and resuspended with the help of the glass beads left back in the bottle. Aspirate the suspension from the glass beads and transfer to a 100 ml culture flask. Another 10 ml of water (5.2.2.2) are pipetted into the Roux-bottle and the spores are washed off and resuspended with the help of the glass beads once again. Aspirate the suspension from the glass beads and transfer into the 100 ml culture flask.

The collected suspension is purified by filtration through 2 layers of sterile gauze (5.3.2.17) in a sterile funnel (5.3.2.18). The filtrate is filled in screw capped tubes (5.3.2.14) and centrifuged for 30 min at 3 000 g at 10 °C to 15 °C in a centrifuge (5.3.2.13). The liquid is removed and the sediment is suspended in 65 % 2-propanol (not more than 50 % of the use volume of the tube!). To inactivate the remaining vegetative cells leave for 3 h at 20 °C.

Dilute the 2-propanol by adding the same amount of water (5.2.2.2) and centrifuge for 30 min at 3 000 g at 10 °C to 15 °C in a centrifuge (5.3.2.13). The liquid is removed and the sediment is suspended in water (5.2.2.2). Repeat the centrifugation and washing for 5 times.

For purity control smears (per 100 ml -10 smears) on microscopical slides of the suspension are stained with a spore stain and examined under the microscope (5.3.2.16). The amount of remaining vegetative cells should not exceed 20 % per field of view, otherwise the suspension shall be filtered and washed again as described above or discarded.

Adjust the bacterial spore suspension to $> 10^9$ cfu/ml using water (5.2.2.2), estimating the number of cfu by any suitable means. Store the bacterial spore suspension for 4 weeks in a refrigerator (2 °C to 8 °C) before use.

For counting prepare a 10^{-6}, 10^{-7} and 10^{-8} dilution using water (5.2.2.2). Mix (5.3.2.6).

Take a sample of 1,0 ml of each dilution in duplicate and spread each 1,0 ml sample on an appropriate number of surface dried plates containing blood agar (5.2.2.4).

Contaminants can be easily detected by this method.

For incubation and counting see 5.4.1.5.

Bacterial spore suspensions delivered by a central commercial or institutional source can also be used if they have been prepared according to the method given. In this case the spore count of the stock suspension and the resistance of spores against reference substances (5.5.2.2) shall be determined and documented within one week after being received.

Bacterial spores shall be stored in water (5.2.2.2) at 2 °C to 8 °C (5.3.2.19). Bacterial spore suspensions shall be controlled for susceptibility against a glutaraldehyde-standard and a peracetic acid-standard (5.5.2.2) before starting a test series with a disinfectant and if it is used continously at least once a month. Stored bacterial spore suspensions should not be opened between uses.

NOTE 1 Bacterial spore suspensions can be stored in the refrigerator at 2 °C to 8 °C for years. It is recommended to replace them every 2 years by a new prepared bacterial spore suspension.

NOTE 2 If the bacterial spore suspensions are stored in separate tubes, control for susceptibility against a glutaraldehyde-standard and a peracetic acid-standard (5.4.3) when opening a new tube.

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

5.4.1.3 Test suspensions ("N1", "N2")

a) To prepare the test suspension “N1”, dilute the stock spore suspension (5.4.1.2) using water (5.2.2.2) to obtain 3 x 10^8 cfu/ml to 1 x 10^9 cfu/ml.

   For counting prepare 10^{-6}, 10^{-7} and 10^{-8} dilutions of the test suspension using water (5.2.2.2). Mix (5.3.2.6). Take a sample of 1,0 ml of each dilution in duplicate and inoculate using the spread plate or the pour plate technique;

b) To prepare the suspension “N2”, dilute the test suspension “N1” using water (5.2.2.2) to obtain 3 x 10^2 cfu/ml to 1 x 10^3 cfu/ml.

   For counting prepare a 10^{-1} and 10^{-2} dilution using water (5.2.2.2). Mix (5.3.2.6).

   Take a sample of 1,0 ml in duplicate out of dilution10^0, 10^{-1} and 10^{-2} and inoculate using the spread plate or the pour plate technique.

When using:

c) the spread plate technique, spread each 1,0 ml sample on an appropriate number of surface dried plates containing TSA (5.2.2.3);

d) the pour plate technique, pipette each 1,0 ml sample in separate Petri dishes and add 12 ml to 15 ml melted TSA (5.2.2.3), cooled to 45 °C $\pm$ 1 °C.

Check the purity in parallel for bacterial spore suspension “N1” by spreading 1 ml out of dilution 10^{-7} and for bacterial spore suspension “N2” out of dilution 10^{-1} on an appropriate number of surface dried blood agar plates (5.2.2.4).

For incubation and counting see 5.4.1.5.

Check size, shape, colour and surface properties of colonies grown on blood agar in order to assure purity of the bacterial spore suspensions by visual control.

5.4.1.4 Validation suspension (“Nv”)

To prepare the validation suspension, dilute the test suspension “N1” (5.4.1.3) using water (5.2.2.2) to obtain 3 x 10^4 cfu/ml to 1 x 10^5 cfu/ml.

For counting prepare a 10^{-2}, 10^{-3} and 10^{-4} dilution using water (5.2.2.2). Mix (5.3.2.6).

Take a sample of 1,0 ml in duplicate out of each dilution (10^{-2}, 10^{-3} and 10^{-4}) and inoculate using the spread plate or the pour plate technique (5.4.1.3).

For incubation and counting see 5.4.1.5.

5.4.1.5 Incubation and counting of the test and the validation suspensions

a) Incubate (5.3.2.3) the plates for minimum 4 and maximum 7 days at 36 °C $\pm$ 1 °C (or 37 °C $\pm$ 1 °C). Control growth every day. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units (cfu) of the sporeforming bacteria. Do not recount

plates which no longer show well separated colonies. Recount the remaining plates. If the number has increased use only the higher number for further evaluation.

b) Note for each plate the exact number of colonies but record > 330 for any counts higher than 330 and determine the Vc values according to 5.6.2.1.

c) Calculate the numbers of cfu/ml in the test suspension “N1”, “N2” and in the validation suspension “Nv” using the method given in 5.6.2. Verify according to 5.7.

5.4.2 Product test solutions

The concentration of a product test solution shall be 1,25 times the desired test concentration because it is diluted to 80 % during the test and the method validation (5.5.2).

Product test solutions shall be prepared in water (5.2.2.2) at minimum three different concentrations to include one concentration in the active range and one concentration in the non-active range. The concentrations shall decrease in a geometrical progression with a factor of at least 2 (i.e. at least doubling the dilutions). The product as received may be used as one of the product test solutions; in this case the highest tested concentration is 80 %.

For solid products, dissolve the product as received by weighing at least 1,0 g ± 10 mg of the product in a volumetric flask (5.3.2.11) and filling up with water (5.2.2.2). Subsequent dilutions (i.e. lower concentrations) shall be prepared in volumetric flasks (5.3.2.11) on a volume/volume basis in water (5.2.2.2).

For liquid products, dilutions of the product shall be prepared with water (5.2.2.2) in volumetric flasks (5.3.2.11) on a volume/volume basis.

The product test solutions shall be prepared freshly and used in the test within 2 h. They shall give a physically homogenous preparation, stable during the whole procedure. If during the procedure a visible inhomogeneity appears due to the formation of a precipitate or flocculant, it shall be recorded in the test report.

NOTE Counting micro-organisms embedded in a precipitate or flocculant is difficult and unreliable.

The concentration of the product stated in the test report shall be the (desired) test concentration. Record the test concentration in terms of mass per volume or volume per volume and details of the product sample as received.

5.4.3 Preparation of reference test solutions

The concentration of the reference test solutions shall be 1,25 times the required test concentration because it is diluted to 80 % during the test and the method validation (Annex F).

a) To prepare the 1 % glutaraldehyde (GA)[5)] standard dissolve 2,5 g 50 % GA and 0,25 g $NaHCO_3$ in 97,5 g water (5.2.2.2).

The pH shall be equivalent to 7,6 ± 0,2 when measured at 20 °C.

b) To prepare a 3 % glutaraldehyde standard (GA) dissolve 7,5 g 50 % GA and 0,75 g $NaHCO_3$ in 92,5 g water (5.2.2.2).

The pH shall be equivalent to 7,6 ± 0,2 when measured at 20 °C.

5) Glutaraldehyde: 50% Glutaraldehyde.

c) To prepare the 0,05 % peracetic acid (PA[6)]) standard dissolve 0,25 ml 15 % PA in 59,75 ml water (5.2.2.2).

The pH shall be equivalent to 3,2 ± 0,2 when measured at 20 °C.

d) To prepare the 0,1 % peracetic acid (PA) standard dissolve 0,5 ml 15 % PA in 59,5 ml water (5.2.2.2).

The pH shall be equivalent to 3,1 ± 0,2 when measured at 20 °C.

5.5 Procedure for assessing the sporicidal activity of the product

5.5.1 General

5.5.1.1 Experimental conditions (obligatory and additional)

Besides the obligatory temperature, contact times and test organisms additional experimental conditions may be selected according to the practical use considered for the product (4):

a) temperature (θ in °C):

— obligatory temperature to be tested is θ = 20 °C;

allowed deviation for each chosen temperature is □ 1 °C ;

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

— obligatory contact times to be tested are t = 30 min, 60 min or 120 min;

allowed deviation for each chosen contact time is □ 10 s, for 120 min □ 60 s;

c) test organisms:

— obligatory test organisms are: *Bacillus cereus* and *Bacillus subtilis* (5.2.1);

If required for specific applications, additional test organisms may be chosen.

5.5.1.2 Selection of neutralizer

The method of choice is dilution-neutralization method. To determine a suitable neutralizer carry out the validation of the dilution neutralization method (5.5.2.3 and 5.5.2.4 in connection with 5.5.2.5) using a suitable neutralizer, chosen according to laboratory experience and published data.

The neutralizer has to form a homogenous, clear and not cloudy preparation when added to TSB.

If this neutralizer is not valid, repeat the validation test using an alternative neutralizer (Annex B) containing a combination of polysorbate 80 (30 g/l), saponin (30 g/l), L-histidine (1 g/l), lecithin (3 g/l), sodiumthiosulphate (5 g/l).

NOTE In special circumstances it may be necessary to add neutralizer to TSA (5.2.2.3).

If neutralizer is added to TSA the same amount shall be added to TSA used in the test procedure.

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6) Peracetic acid 15 %: aqueous solution of peracetic acid (min. 15 % acetic acid + max. 24 % Hydrogene peroxide + min. 16 % peracetic acid + min. 40 % H_2O). Stored at 4 °C to 8 °C.

5.5.1.3 Validation and control procedures - General instruction

The neutralization and/or removal of the sporicidal and/or sporistatic activity of the product shall be controlled and validated - only for the highest product test concentration - for each of the used test organisms and for each of the chosen experimental conditions (temperature, contact time). These procedures (neutralizer control and method validation) shall be performed at the same time with the test and with the same neutralizer used in the test.

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

5.5.1.4 Equilibration of temperature

Prior to testing, equilibrate all reagents (product test solutions (5.4.2), bacterial spore test suspension (5.4.1.3), validation suspension (5.4.1.4), neutralizer (5.2.2.5), water (5.2.2.2) to the test temperature $\theta = 20$ °C ± 1 °C using the water bath (5.3.2.2) controlled at 20 °C ± 1 °C.

5.5.1.5 Precautions for manipulation of test organisms

Do not touch the upper part of the test tube sides when adding the test - or the validation suspensions.

5.5.2 Method (Dilution-neutralization)

The test and the control and validation procedures (5.5.2.1 to 5.5.2.4) shall be carried out in parallel.

5.5.2.1 Test “Na” (Determination of sporicidal concentrations), Water control “Nw”

a) Pipette 8,0 ml of one of the product test solutions (5.4.2) into a tube and add 1,0 ml of water (5.2.2.2). Add 1,0 ml of the bacterial spore suspension N1 (5.4.1.3). Start the stopwatch immediately, mix (5.3.2.6) and place the tube in a water bath controlled at 20 °C ± 1 °C [5.5.1.1 a)].

   The activity of the product shall be determined for a contact time chosen from one of the following: 30, 60 or 120 min $\pm$ 10 s. Just before the end of the chosen contact time, mix (5.3.2.6) again;

b) At the end of the chosen contact time take a 0,1 ml sample of the test mixture “Na” and transfer into a tube containing 10,0 ml TSB with neutralizer (ONT) [5.2.2.6 b)]. Mix (5.3.2.6) and place in a water bath controlled at 20 °C ± 1 °C.

   After a neutralization time of 30 min ± 10 s, immediately make a serial of ten-fold dilutions out of the neutralized test mixture “Na” (containing neutralizer, product test solution, test suspension) in 9 ml TSB with neutralizer and determine the spore count of the original neutralization tube (ONT) and every dilution 10^{-1}, 10^{-2} and 10^{-3} by the spread plate or by the pour plate technique on TSA (5.4.1.3) in order to determine Na.

   For incubation and counting see 5.5.2.5.

c) Perform the procedure a) – b) using the other product test solutions at the same;

d) For the water control “Nw” perform the procedure a) - b), but pipette 9 ml of water (5.2.2.2) instead of the product test solution into a tube and add 1,0 ml bacterial spore suspension “N1”. Start the stopwatch immediately, mix (5.3.2.6) and place the tube in a water bath controlled at 20 °C ± 1 °C [5.5.1.1 a)] for the longest contact time chosen in the test.

   At the end of t mix and take a 0,1 ml sample of this mixture “Nw” and transfer into a tube containing 10 ml of water. Mix (5.3.2.6) and place in a water bath controlled at 20 °C ± 1 °C. After 30 min ± 10 s mix and make a serial of ten-fold dilutions out of the mixture “Nw” in 9 ml water (5.2.2.2) and determine the spore

count out of dilution 10^{-2}, 10^{-3} and 10^{-4} by the spread plate or by the pour plate technique on TSA (5.4.1.3) in order to determine Nw.

For incubation and counting see 5.5.2.5.

e) Perform the procedure a) – c) applying the other obligatory and - if appropriate - other additional experimental conditions (5.5.1.1).

The principle is given in Figure C.1.

5.5.2.2 Control of bacterial spore suspension with reference substances

Perform the test procedure described in 5.5.2.1 a) – b) using the glutaraldehyde (GA) and peracetic acid (PA) standard (5.4.3) instead of the product test solution. Suitable neutralizers are 20 g/l histidine for glutaraldehyde and 10 g/l sodium thiosulfate for peracetic acid.

The results obtained shall meet the requirements fixed in Annex F and Annex G.

5.5.2.3 Neutralizer control "B" (Verification of the absence of toxicity of the neutralizer)

a) Pipette 1,0 ml of the spore test suspension “N1” (5.4.1.3) into a tube. Add 9,0 ml of water (5.2.2.2).

Start the stopwatch at the beginning of the addition, mix (5.3.2.6), and place the tube in a water bath controlled at 20 °C ± 1 °C for the longest contact time in the test;

b) At the end of this time mix (5.3.2.6) and transfer a sample of 0,1 ml of this mixture “B” in 10 ml TSB with neutralizer (ONT) [5.2.2.6 b)]. Mix (5.3.2.6), and place the tube in a water bath controlled at 20 °C ± 1 °C. After 30 min ± 10 s mix and prepare 10^{-2}, 10^{-3} and 10^{-4} dilutions out of the mixture “B” in 9 ml TSB with neutralizer and determine the spore count by the spread plate or by the pour plate technique on TSA (5.4.1.3) in order to determine B.

For incubation and counting see 5.5.2.5.

5.5.2.4 Method validation “C” (Dilution-neutralization validation) and long time toxicity control

For **validation of dilution-neutralization** prepare 3 different dilutions of the disinfectant. One in the concentration, one with double concentration and one with half the concentration to be tested in the actual test.

Prepare twice as much tubes with 10 ml of TSB with neutralizer [5.2.2.6 b], for dilution-neutralization-validation) and TSB without neutralizer [5.2.2.6 a)], for determination of sporistatic activity) as product-dilution steps to be tested.

For **long time toxicity control** prepare one tube with 9 ml TSB with neutralizer [5.2.2.6 b] and add 1,0 ml water (5.2.2.2). Pipette 0,1 ml of the validation suspension “Nv” (5.4.1.4) into the tube and mix.

a) For validation of dilution-neutralization pipette 0,1 ml of the validation suspension “Nv” (5.4.1.4) into each tube and mix. Add 0,1 ml of the corresponding product-dilution to each tube. Start a stopwatch at the beginning of the addition, mix (5.3.2.6) and place the tubes in a water bath controlled at 20 °C ± 1 °C;

b) After 30 min ± 1 min neutralization time mix (5.3.2.6) again and make a serial of ten-fold dilutions out of each tube in 9 ml TSB with neutralizer from the corresponding tubes and in TSB alone from the tubes without neutralizer and determine the spore count by the spread plate or by the pour plate technique on TSA (5.4.1.3) in order to determine C.

Incubate (5.3.2.3) the plates, all tubes (after 30 min neutralization) and the long time toxicity control for minimum 4 and maximum 7 days at 36 °C ± 1 °C (or 37 °C ± 1 °C). Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units. Control growth every day. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. If the number has increased use only the higher number for further evaluation.

Repeat the test at least one time on another day.

The principle of the validation of neutralization is given in Figure C.2.

5.5.2.5 Incubation and counting of the test mixture and the control and validation mixtures

a) Incubate (5.3.2.3) the plates and original neutralization tubes (ONT) (5.5.2.1, 5.5.2.3 and 5.5.2.4) for minimum 4 and maximum 7 days at 36 °C ± 1 °C (or 37 °C ± 1 °C). Control growth every day. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units. (cfu). Do not recount plates which no longer show well separated colonies. Recount the remaining plates. If the number has increased use only the higher number for further evaluation.

   Add 0,1 ml of spore-suspension “N2” (5.4.1.3) to every original neutralization tube (ONT) which shows no visible growth after 4 day incubation and incubate for 4 days at 36 °C ± 1 °C (or 37 °C ± 1 °C). Check ONT for visible growth after 4 day incubation.

NOTE If there is no visible growth in ONT the test is invalid, because neutralization has to be regarded not effective in the test;

b) Note for each plate the exact number of colonies but record > 330 for any counts higher than 330 and determine the viable count (Vc) values according to 5.6.2.1.

c) Calculate the numbers of cfu/ml using the method given in 5.6.2.

   Verify according to 5.7.

5.6 Experimental data and calculation

5.6.1 Explanation of terms and abbreviations

a) Overview of the different suspensions/test mixtures:

   — “N1”, “N2” and “Nv” represent the bacterial spore suspensions, “Na” represents the sporicidal test mixture, “Nw” (water control), “B” (neutralizer control), “C” (method validation) represent the different control test mixtures;

   — N1, Nv, Na, and Nw, B, C represent the number of cells counted **per ml** in the different test mixtures according to the Table 1:

Table 1 – Numbers of cells and survivors per ml

	Number of cells **per ml** in the bacterial spore suspensions	Number of survivors **per ml** in the test mixtures at the end of the contact-time t of 30 min (C)
Test	N1 Test suspension	Na, Nw, B (before neutralisation)
Controls	Nv Validation suspension	C

b) Viable counts = Vc values.

All experimental data are reported as Vc values:

Vc value is the number of cfu counted per 1,0 ml sample

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

5.6.2 Calculation

The first step is the determination of the Vc values, the second the calculation of N1, N2, Nv, Na, Nw, B and C. The third step is the calculation of the reduction R (5.8).

5.6.2.1 Determination of Vc values

a) The usual limits for counting bacteria on agar plates are between 15 and 300 colonies. In this standard a deviation of 10 % is accepted, so the limits are 14 and 330.

NOTE 1 The lower limit (14) is based on the fact that the variability is increasing the smaller the number counted in the sample (1 ml or 0,1 ml) is and therefore subsequent calculations may lead to wrong results. The lower limit refers only to the sample (and not necessarily to the counting on one plate), e.g. two plates per 1 ml sample with 11 and 5 cfu give a Vc value of 16.

The upper limits (330) reflect the imprecision of counting confluent colonies and growth inhibition due to nutriment depletion. It refers only to the counting on one plate, and not necessarily to the sample;

b) According to the number of plates used per 1 ml sample [see 5.6.1 b], determine and record the Vc values.

NOTE 2 If more than one plate per 1 ml sample has been used to determine the Vc value the counting per plate should be noted.

If the count on one plate is higher than 330 report the number “> 330”. If at least one of the plates per 1 ml sample shows a number higher than 330 report the number of this Vc value as “> sum of the counts”, e.g. for “> 330, 308”, report “> 638”.

If a Vc value is lower than 14 report the number but substitute by “< 14” for further calculations (Na);

c) Only Vc values within the respective counting limits are taken into account for further calculation, except in the case of Na (5.6.2.3).

5.6.2.2 Calculation of N1 and N2

N1 is the number of cells per ml in the test suspension "N1" (5.4.1.3), N2 is the number of cells per ml in the test suspension "N2" (5.4.1.3).

Since two dilutions of the test suspension (5.4.1.3 in connection with 5.4.1.5) are evaluated, calculate the number of cfu/ml as the weighted mean count using the following formula:

$$N = \frac{c}{\left(n_1 + 0{,}1\, n_2 \right) d}$$

where

c is the sum of Vc-values taken into account;

n_1 is the number of Vc-values taken into account in the lower dilution, i.e. (10^{-6});

n_2 is the number of Vc-values taken into account in the higher dilution, i.e. (10^{-7});

d is the dilution factor corresponding to the lower dilution.

Round off the results calculated to two significant figures. For this, if the last figure is below 5, the preceding figure is not modified; if the last figure is more than 5 the preceding figure is increased by one unit; if the last figure is equal to 5, round off the preceding figure to the next nearest even figure. Proceed stepwise until two significant figures are obtained. As a result the number of cfu/ml is expressed by a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

EXAMPLE

$$N = \frac{168 + 213 + 20 + 25}{(2 + 0{,}1 \times 2)\ 10^{-6}} = \frac{426}{2{,}2 \times 10^{-6}} = 1{,}9363 \times 10^{8} = 1{,}9 \times 10^{8} \text{ (in cfu/ml)}$$

5.6.2.3 Calculation of Na and Nw

a) Na is the number of survivors per ml in the test mixture [5.5.2.1 a)] at the end of the contact time and before neutralization. It is hundredfold higher than the Vc values due to the sample volume of 0,1 ml transferred into ONT (dilution 1:100) [5.5.2.1 b)];

b) Nw is the number of cells per ml in the test mixture [5.5.2.1 d)] at the end of the contact time and before neutralization. It is hundredfold higher than the Vc values due to the sample volume of 0,1 ml transferred into ONT (dilution 1:100) [5.5.2.1 d)].

Calculate Na and Nw using the following formula:

$$\text{Na, Nw} = \frac{c \times 100}{\mathrm{n} \times d}$$

where

c is the sum of Vc-values taken into account;

n is the number of Vc-values taken into account;

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

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

EXAMPLES

a) duplicate Vc values in dilution step Na^0: 2, 16

$$Na = \frac{(<14 + 16) \times 100}{2 \times 10^{0}} = \frac{<3000}{2x10^{0}} = <1\ 500 \times 10^{0} = <1{,}5 \times 10^{3} \text{ (in cfu/ml)}$$

b) duplicate Vc values in dilution step Na^{-3}: > 330, > 330

$$Na = \frac{(>330 + >330)\ x100}{2\ x\ 10^{-4}} = \frac{>66000}{2\ x\ 10^{-4}} = >33000 \times 10^{4} = >3{,}3\ x\ 10^{8} \text{ (in cfu/ml)}$$

c) duplicate Vc values in dilution step Na^{-3}: >330, 300

$$Na = \frac{(>330 + 300)\ x\ 100}{2\ x\ 10^{-4}} = \frac{>63000}{2\ x\ 10^{-4}} = >31500 \times 10^{4} = >3{,}15\ x\ 10^{8}\ (\text{in cfu/ml})$$

d) Use maximum two subsequent dilutions for calculating Na as weighted mean. Exceptions and rules for special cases:

d1) If one or both duplicate Vc values in three or more subsequent dilutions of Na (including Na^{0}) are within the counting limits (e.g. Na^{-2}: 17, 23; Na^{-1}: 120, 135; Na^{0}: 308, > 330) the whole test is invalid (5.7);

d2) If two subsequent dilutions of Na or Nw show duplicate Vc values within the counting limits calculate Na as the weighted mean using the formula:

$$Na,\ Nw = \frac{c \times 100}{2{,}2 \times d}$$

c is the sum of Vc-values taken into account;

d is the dilution factor corresponding to the lower dilution, e.g. Na^{-2} is the lower dilution in comparison with Na^{-3};

d3) If in two subsequent dilutions of Na both Vc values of the higher dilution are within the counting limits and one Vc value of the lower dilution is “more than”, calculate Na as weighted mean, using the formula as in d2.

EXAMPLE

$$Na = \frac{(>330 + 320 + 46 + 38)\ x\ 100}{2{,}2\ x\ 10^{0}} = \frac{>73400}{2{,}2\ x\ 10^{0}} = >33363{,}64 \times 10^{0} = >3{,}34\ x\ 10^{4}\ (\text{in cfu/ml})$$

d4) If in two subsequent dilutions of Na one of the higher dilution duplicate Vc values shows “< 14”, take only the lower dilution as result for Na.

5.6.2.4 Calculation of Nv

Nv is the number of cfu/ml in the validation suspension (5.4.1.4).

Calculate Nv using the following formula:

$$N_{V} = \frac{c}{n \times d}$$

where

c is the sum of Vc-values taken into account;

n is the number of Vc-values taken into account;

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

5.6.2.5 Calculation of B and C (Controls and method validation)

B and C are the numbers of survivors in the neutralizer control (5.5.2.3) and method validation (5.5.2.4) at the end of the longest contact time in the actual test (B) or the defined time 30 min (C). They are hundredfold

higher than the Vc values due to the sample volume of 0,1 ml transferred into ONT (dilution 1:100) (5.5.2.3 and 5.5.2.4).

Calculate B and C using the following formula:

$$B, C = \frac{c \times 100}{n \times d}$$

where

c is the sum of Vc-values taken into account;

n is the number of Vc-values taken into account;

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

5.7 Verification of methodology

5.7.1 General

A test is valid if:

— all results meet the criteria of 5.7.2 and 5.7.3;

— the requirements of 5.8.2 are fulfilled;

— it is not invalidated by a result described under 5.6.2.3 d1).

5.7.2 Control of weighted mean counts

For results calculated by weighted mean of two subsequent dilutions (e.g. “N”) the quotient of the mean of the two results shall not be higher than 15 and not lower than 5. Results below the lower limit are taken as the lower limit number (14). Results above the respective upper limit (5.6.2.1) are taken as the upper limit number (330). (Example for N: 10^{-6} dilution: 168 + 215 cfu/ml, 10^{-7} dilution: 20 < 14 cfu/ml; $(168 + 215)/(20 + 14)$ = 383/34 = 11, 26 = between 5 and 15).

5.7.3 Basic limits

For each test organism check that:

a) N1 is between 3 x 10^8 cfu/ml and 1 x 10^9 cfu/ml ($8{,}48 \leq \lg N1 \leq 9{,}00$);

b) N2 is between 3 x 10^2 cfu/ml and 1 x 10^3 cfu/ml ($2{,}48 \leq \lg N2 \leq 3{,}00$);

c) Nw is between 3 x 10^7 cfu/ml and 1 x 10^8 cfu/ml ($7{,}48 \leq \lg Nw \leq 8{,}00$);

d) Nv is between 3 x 10^4 cfu/ml and 1 x 10^5 cfu/ml ($4{,}48 \leq \lg Nv \leq 5{,}00$);

e) B is equal or greater than Nw ($8{,}00 \geq \lg Nw \geq 7{,}48$);

f) C is between 3 x 10^4 cfu/ml and 1 x 10^5 cfu/ml (equal Nv) ($4{,}48 \leq \lg Nv \leq 5{,}00$);

g) ONT shows growth after inoculation;

h) Control of weighted mean counts (5.7.2): Quotient is not lower than 5 and not higher than 15.

5.8 Expression of results and precision

5.8.1 Reduction in viable counts

The reduction (R = Nw/Na) is expressed in logarithm.

For each test organism record the number of cfu/ml in the water control Nw [5.5.2.1d] and of the results of the test Na [5.5.2.1 a)].

For each product concentration and each experimental condition calculate and record the decimal log reduction separately using the formula:

$$R = \frac{\text{Nw}}{\text{Na}} \text{ or lg } R = \text{lg Nw} - \text{lg Na}$$

For the controls and validation of dilution-neutralization method record the results of B and C (5.6.2.5) and their comparison with Nw and Nv (5.7.3).

5.8.2 Control of active and non-active product test solution (5.4.2)

For the product to pass the test at least one concentration shall demonstrate at least a 4 lg reduction in viable counts and at least one concentration shall demonstrate a lg reduction of less than 4.

5.8.3 Limiting test organism and sporicidal concentration

For each test organism, record the lowest concentration of the product which passes the test (lg $R \geq 4$). Record as the limiting test organism the test organism requiring the highest of these concentrations (it is the least susceptible to the product in the chosen experimental conditions).

The lowest concentration of the product active on the limiting test organism is the sporicidal concentration determined according to this document.

5.8.4 Precision, replicates

Taking into account the precision of the methodology determined by a statistical analysis based on data provided by a collaborative study for bacteria and fungi, replication of the test (6 replicates for a precision of $\pm$ 1 lg in reduction) is recommended (Annex F). Replication means the complete test procedure with separately prepared test - and validation suspensions. The replicate of the test may be restricted to the limiting test organism. The mean of the results of the replicates - not each single result - shall demonstrate at least a 4 lg reduction in viable counts and shall also be calculated and recorded.

5.9 Interpretation of results – conclusion

5.9.1 General

According to the chosen experimental conditions (obligatory or obligatory and additional) the sporicidal concentrations determined according to this document may differ (4).

5.9.2 Sporicidal activity

The product shall be deemed to have passed the EN 14347 standard if it demonstrates in a valid test at least a 4 lg reduction in viable counts within 30 min, 60 min or 120 min or less at 20 °C under the conditions defined by this document when the test organisms are dormant spores of *Bacillus cereus* and *Bacillus subtilis*.

A product which passes the test is characterized as possessing sporicidal activity. In order to qualify the product as a chemical disinfectant and/or antiseptic for a defined purpose, it shall be evaluated using additional standard tests which are appropriate to its intended use.

Information regarding the additional standard tests which shall be used to qualify a product as chemical disinfectant and/or antiseptic for a defined purpose is given in Annex E.

5.9.3 Interpretation of the validation of dilution-neutralization method

The dilution-neutralization is considered valid when after neutralization the mean value of the spore count in the tube with disinfectant in double the concentration used in the actual test (see Figure C.2) is in the range of the control ± standard deviation (see Annex G).

If the sporistatic concentration was hit in the range of the tested disinfectant concentrations, there should be at least one dilution step between the sporistatic concentration (dilution in TSB, cultivation on TSA) and the lowest concentration neutralized in the procedure (see Figure C.2).

NOTE: It is not always possible to determine the exact sporistatic (MIC) concentration in the test, because it may lay outside the range of the concentrations of the product or between two dilutions tested here. In such cases no exact determination of the MIC is necessary in order to limit the work load if it may be seen from the actual results that the MIC is below the active concentration.

Visible growth of the test strain (reconfirmed by subcultivation) should be seen in the incubated neutralizer test mixture (TSB + neutralizer + spores + disinfectant) at the dilution step representing the highest concentration in the actual test.

Visible growth of the test strain should be seen in the incubated mixture (TSB + neutralizer + spores and long time toxicity of the neutralizer).

5.10 Test report

The test report shall refer to this standard (EN 14347).

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

a) identification of the testing laboratory;

b) identification of the sample:

   1) name of the product;

   2) batch number and – if available – expiry date;

   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);

   8) appearance of the product;

c) test method and its validation:

   Full details of the test for validation of the neutralizer shall be given;

d) experimental conditions:

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

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

   3) product test concentrations;

   4) appearance of the product dilutions;

   5) contact time(s);

   6) test temperature(s);

   7) stability and appearance of the mixture during the procedure (note the formation of any precipitate or flocculant);

   8) temperature of incubation;

   9) neutralizer;

   10) identification of the bacterial strains used;

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

e) test results:

   1) controls and validation;

   2) evaluation of sporicidal activity;

   3) number of replicates per test-organism;

f) special remarks;

g) conclusion;

h) locality, date and identified signature.

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

Annex A
(informative)

Referenced strains in national collections

- Bacillus cereus: ATCC 12826
  CIP 105151
- Bacillus subtilis: ATCC 6633
  DSM 347
  NCTC 10400
  CCM 19999
  IAM 1069
  NCIB 8054
  CIP 52-62

Annex B
(informative)

Suitable neutralizers

B.1 General

Neutralizers given under B.2 are generally used neutralizers and maybe too low in concentration for sporicidal testing. Preferred neutralizers from B.2.1, B.2.2 and B.3 should be used in this context

The lists in B.2 and B.3 are not exhaustive and other reagents may be used.

B.2 Neutralizers

Any of the following neutralizers may be used:

— lecithin 3 g/l; polysorbate 80[7)] 30 g/l; sodium thiosulphate[8)] 5 g/l; L-histidine 1 g/l; saponin 30 g/l in diluent (5.2.2.4) or in phosphate buffer 0,0025 mol/l;

— phosphate buffer 0,25 mol/l:

  — potassium-dihydrogen phosphate ($K H_2 PO_4$) 34 g;

  — water (5.2.2.2) 500 ml;

  — adjusted to pH 7,2 ± 0,2 with sodium hydroxide (NaOH) 1 mol/l;

  — water (5.2.2.2) up to 1 000 ml;

  — sterilized in an autoclave (5.3.1);

— fresh egg yolk diluted to 5 % (V/V) or 0,5 % (V/V);

— 30 g/l polysorbate 80; 4 g/l sodium dodecyl sulphate ($C_{12}H_{25}NaO_4S$); lecithin 3 g/l;

— 7 % (V/V) ethylene oxide condensate of fatty alcohol; 20 g/l lecithin; 4 % (V/V) polysorbate 80;

— 4 % (V/V) ethylene oxide condensate of fatty alcohol; 4 g/l lecithin;

— 30 g/l polysorbate 80; lecithin 3 g/l; L-histidine 1 g/l;

— glycine as a function of the product concentration;

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7) Analytical quality, non-hydrolysed in accordance with EP. 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.

8) Sodium thiosulfate waterfree (without cristal water). Sodium thiosulfate containing cristal water: the adaequate concentration should be used.

— 30 g/l polysorbate 80; lecithin 3 g/l;

— phospholipid emulsion (commercial) at 50 mg/ml (diluted 1 to 10);

— sodium thioglycollate at 0,5 g/l or 5 g/l;

— L-cysteine at 0,8 gl or 1,5 g/l;

— thiomalic acid at 0,075 % (V/V) (adjusted to pH 7,0 with sodium hydroxide (NaOH));

— sodium thiosulphate at 3 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 7,0;

— polysorbate 80 30 g/l; saponin 30 g/l; L-histidine 1 g/l; L-cysteine 1 g/l.

B.2.1 For Aldehydes

— histidine 20 g/l, polysorbate 80[9)], 30 g/l, lecithin 3 g/l saponine 30 g/l;

— histidine 10 g/l and sterile inactivated horse serum 100 ml/l;

— histidine 20 g/l.

B.2.2 For oxidizing agents

— sodium thioglycolate 5 g/l, polysorbate 80[9)] 30g/l, lecithin 3 g/l and saponine 30 g/l;

— cysteine 1,5 g/l, sodium thinosulfate 3 g/l, polysorbate 80[9)] 30 g/l, lecithin 3 g/l and saponine 30 g/l;

— catalase or peroxidase: One unit of these enzymes catalyzes the decomposition of 1,4 moles of hydrogene peroxide per minute at 25 °C and at pH 7;

— sodium thiosulfate 5 g/l or 10 g/l.

B.3 Neutralizer added to the agar for counting:

— 10 % (V/V) of a solution containing 0,7 g/l lecithin and 5 % (V/V) polysorbate 80;

— 10 % (V/V) of a solution containing 10 g/l lecithin and 5 % (V/V) polysorbate 80;

— 10 % (V/V) of a solution containing fresh egg yolk 1,5 % (V/V) and 5 % (V/V) polysorbate 80.

NOTE Sodium thiosulfate should not be added to TSA.

9) Analytical quality, non-hydrolized (TWEEN 80 in accordance with European Pharmacopaeia volume 1).

Annex C
(informative)

Graphical representations of test method

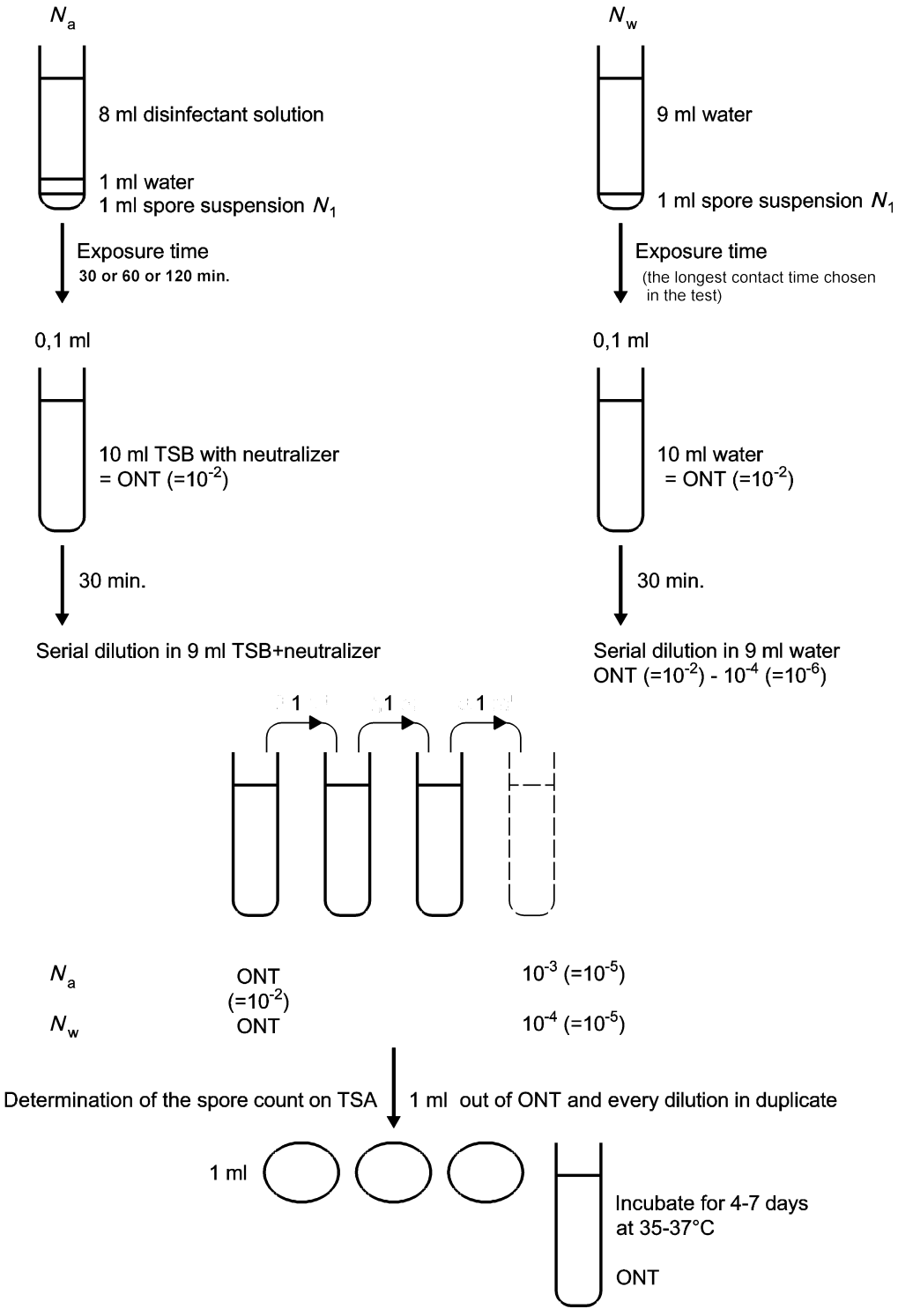

Figure C.1 — Test procedure

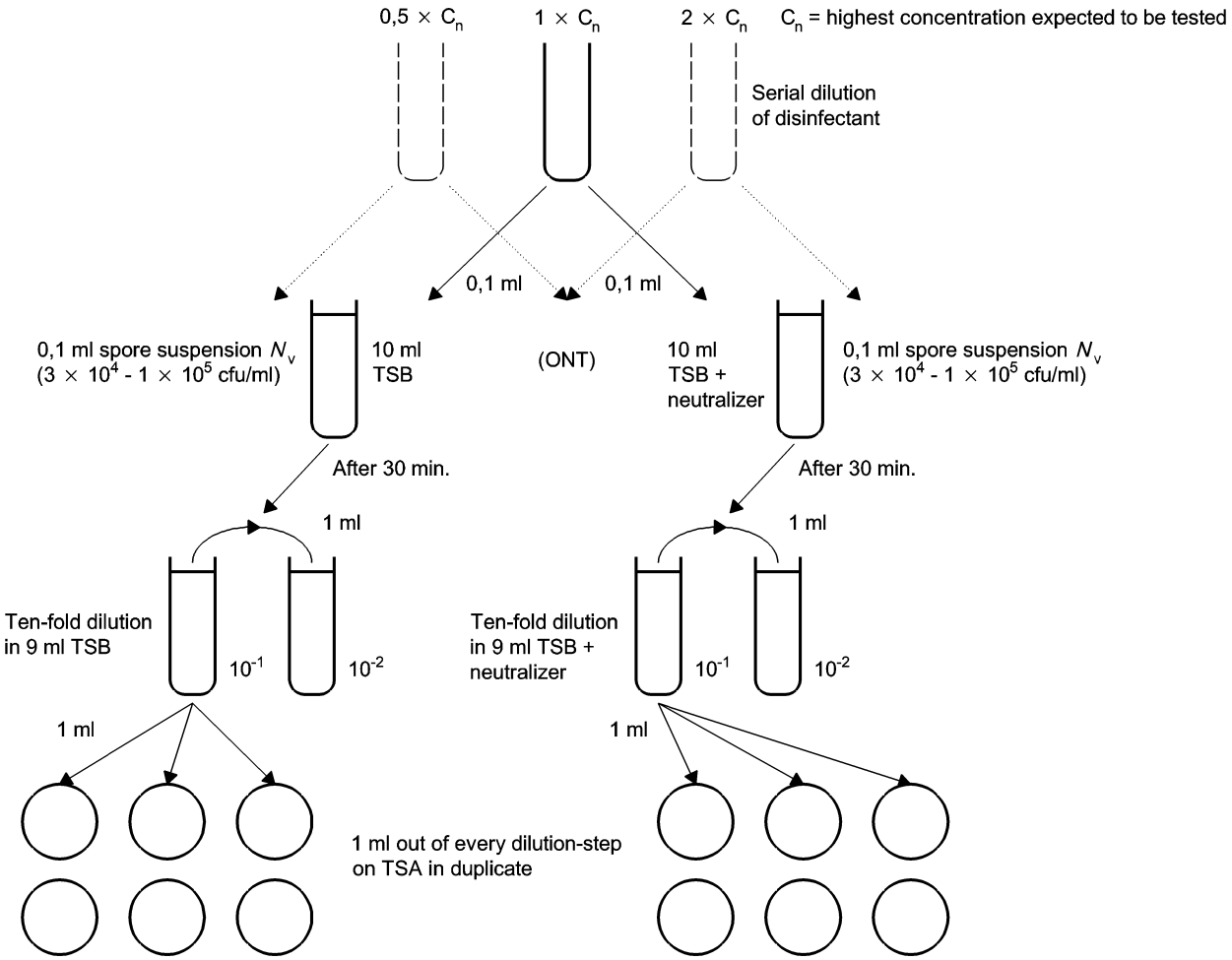

Figure C.2 — Test procedure

Annex D
(informative)

Example of a typical test report

H.H.Q. Laboratories
Antiseptville/Euroland
Tel. ++011.57 83 62-0
Fax ++011-57 83 62-19
e-mail:h.h.Q.lab.@net.com

TEST REPORT

TEST: EN 14347

Identification of the disinfectant-sample:

Name of the product: Z

Batch number: 91-71-51

Manufacturer: Centipede Formulations Inc

Storage conditions: Room temperature and darkness

Product diluent recommended by the manufacturer for use: Potable water

Active substance(s) and its (their) concentration(s): Not indicated

Period of testing - date of delivery of the product: .2003-10-23..; dates of test: 2003-10-24, 2003-10-25, 2003-10-26, 2003-10-27, 2003-10-28, 2003-11-05.....;

Experimental conditions:

Temperature: 20 °C Exposure times: 30 min, 60 min, 120 minutes

Results see Annex

Conclusion:

According to EN 14347, the batch 91-71-51 of product Z, when diluted at 1 % (V/V) in water, possesses sporicidal activity in 120 minutes at 20 °C for referenced strains Bacillus cereus and Bacillus subtilis. The mean reduction of six replicates with the limiting test organism was $1,2 \times 10^4$. In order to qualify the product as an antiseptic and/or chemical disinfectant for a defined purpose, it will be evaluated using additional standard tests which are appropriate to its intended use.

Antiseptville, 2003-11-10.

Alexandra May, M.D., Ph.D., Scientific Director.

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

NOTE 2 In Annex D page 2 a table of test results for Bacillus cereus is given as an example.

Test results

EN.14347...(Phase 1) **Product name**:…Z…….. **Batch No**.:..91-71-51……
Manufacturer: Centipede Formulations Inc… **Storage conditions** (temp. and other):…….
Room temperature:..22 °C… **Remarks**: appearance of the product: liquid, clear, yellowish.

Dilution neutralization method Pour plate **X** Spread plate Number of plates…3../ml
Neutralizer in TSB: ..Lecithin 3,0 g/l in diluent… Neutralizer in TSA:……..

Test temperature:….20 °C…. **Test organism**:…Bacillus cereus ATCC 12826…..
Incubation temperature:…37 °C…… Last purity and sensitivity control of spores: 2003-11-01
Internal lab. No.:…..QS 58/00 **Date** of test: 2003-11-05 Responsible person:……..…
Signature:…….

Diluent used for product test solutions:…..water (freshly glass distilled)…….

Appearance of the product test solutions:…clear……...

Validation and controls

Determination of spore count of suspension N1: ξ = ….4,41 x 10^8….. **s** = … 1,31 (x 10^8)…

Test suspension (N1)	N1	Vc1	Vc2
	10^{-6}	329	316
	10^{-7}	39	34
	10^{-8}		
	x_{wm}= 3,26 x 10^8 lg_{xwm} = 8,51 8,48 ≤ lg N1 ≤ 9,00 ? **X** yes no		

Test suspension (N2)	N2	Vc1	Vc2
	10^0	319	304
	10^{-1}	33	36
	10^{-2}		
	x_{wm} = 3,15 x 10^2 lg_{xwm} = 2,5 2,48 ≤ lg N2 ≤ 3,00 ? **X** yes no		

Validation suspension (Nv)	Nv	Vc1	Vc2
	10^{-2}		
	10^{-3}	54	61
	10^{-4}	4	7
	x = 5,75 x 10^4 lg_x = 4,76 4,48 ≤ lg Nv ≤ 5,00 ? **X** yes no		

Method validation (C)

with neutralizer without neutralizer

Neutralizer control (B)	B	Vc1	Vc2
	10^{-3}		
	10^{-4}	72	86
	10^{-5}	7	8
	$x = 7,9 \times 10^{7}$ $lg_x = 7,9$ $7,48 \leq lg\ Nw \leq 8,00$? **X** yes no		

Concentration of the product: ..2.. %	C1	Vc1	Vc2
	ONT		
	10^{-1}	39	48
	10^{-2}	2	4
	$x = 4,35 \times 10^{4}$ $lg_x = 4,63$ $4,48 \leq lg\ Nv \leq 5,00$? **X** yes no		

Concentration of the product: ..2.. %	C4	Vc1	Vc2
	ONT	0	0
	10^{-1}	-	-
	10^{-2}	-	-
	$x = < 1,5 \times 10^{3}$		

Long time toxicity control

Visible growth **X** yes no

Concentration of the product: ..1.. %	C2	Vc1	Vc2
	ONT		
	10^{-1}	43	49
	10^{-2}	4	5
	$x = 4,6 \times 10^{4}$ $lg_x = 4,66$ $4,48 \leq lg\ Nv \leq 5,00$? **X** yes no		

Concentration of the product: ..1.. %	C5	Vc1	Vc2
	ONT	0	0
	10^{-1}	-	-
	10^{-2}	-	-
	$x = < 1,5 \times 10^{3}$		

Concentration of the product: ..0,5.. %	C3	Vc1	Vc2
	ONT		
	10^{-1}	58	64
	10^{-2}	4	7
	$x = 6,1 \times 10^{4}$ $lg_x = 4,78$ $4,48 \leq lg\ Nv \leq 5,00$? **X** yes no		

Concentration of the product: ..0,5.. %	C6	Vc1	Vc2
	ONT		
	10^{-1}	28	30
	10^{-2}	4	5
	$x = 2,9 \times 10^{4}$ $lg_x = 4,46$		

Sporistatic concentration (if determined): …1…(0,01)..%

Test

Conc. of the product %	Exposure time min									Water control (Nw)			Log reduction lgNw-lgNa
	30			60			120						
…1…		Vc1	Vc2		Vc1	Vc2		Vc1	Vc2		Vc1	Vc2	30 min: 2,76
	ONT			ONT	21	17	ONT	-	-	10^{-2}			60 min: 4,6
	10^{-1}	124	139	10^{-1}	3	1	10^{-1}	-	-	10^{-3}	> 976	> 990	
	10^{-2}	9	13	10^{-2}	-	-	10^{-2}	-	-	10^{-4}	69	84	
	10^{-3}	-	-	10^{-3}	-	-	10^{-3}	-	-	$x = 7,65 \times 10^7$			
	$x = 1,32 \times 10^5$			$x = 1,9 \times 10^3$			$x = < 1,5 \times 10^3$			$lg_x = 7,88$			120 min: >4,7
	$lg_x = 5,12$			$lg_x = 3,28$			$lg_x = < 3,18$			$7,48 \leq lg\ Nw \leq 8,00$?			
										X yes no			
..0,5...		Vc1	Vc2		Vc1	Vc2		Vc1	Vc2				30 min: 2,38
	ONT			ONT	228	246	ONT	4	2				60 min: 3,51
	10^{-1}	329	311	10^{-1}	25	21	10^{-1}	-	-				
	10^{-2}	33	28	10^{-2}	4	2	10^{-2}	-	-				
	10^{-3}	5	3	10^{-3}	-	-	10^{-3}	-	-				
	$x_{wm} = 3,19 \times 10^5$			$x_{wm} = 2,36 \times 10^4$			$x = <1,5 \times 10^3$						120 min: >4,7
	$lg_{xwm}lg = 5,5$			$lg_{xwm} = 4,37$			$lg_x = <3,18$						
..0,25.		Vc1	Vc2		Vc1	Vc2		Vc1	Vc2				30 min: 1,39
	ONT			ONT			ONT	269	288				60 min: 2,88
	10^{-1}			10^{-1}	99	103	10^{-1}	32	29				
	10^{-2}	312	302	10^{-2}	11	10	10^{-2}	2	4				
	10^{-3}	33	31	10^{-3}	-	1	10^{-3}	-	-				
	$x_{wm} = 3,08 \times 10^6$			$x = 1,01 \times 10^5$			$x_{wm} = 2,81 \times 10^4$						120 min: 3,43
	$lg_{xwm} = 6,49$			$lg_x = 5,00$			$lg_{xwm} = 4,45$						
……		Vc1	Vc2		Vc1	Vc2		Vc1	Vc2				30 min:
	ONT			ONT			ONT						60 min:
	10^{-1}			10^{-1}			10^{-1}						
	10^{-2}			10^{-2}			10^{-2}						120 min:
	10^{-3}			10^{-3}			10^{-3}						

ONT shows visible growth after inoculation X yes no

Remarks:

Explanations:

Vc = count per ml (one plate or more)

$\bar{x}$ = average of Vc_1 and Vc_2

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

R = reduction (lg R = lg N_W – lg Na)

ξ = mean value

s = standard deviation

Annex E
(informative)

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

E.1 Introduction

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

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

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

For applications where phase 2/step 2 tests without phase 2/step 1 tests are used to support product claims, the justification for omitting phase 2/step 1 tests should be given. Such applications will be indicated either in the 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.

E.3 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 document will be to specify in detail the relationship of the various tests to one another and to use recommendations.

Annex F
(normative)

Precision of the Test Result

Control of spore suspension with reference substances

Log reductions of bacterial spore suspensions shall be as follows:

GA-standard	Exposure time	30 min
B. cereus (1 % GA)		3,45 ± 0,7
B. subtilis (3 % GA)		1,55 ± 1,15
PA-standard	Exposure time	15 min
B. cereus (0,1 % PA)		1,25 ± 0,5
B. subtilis (0,05 % PA)		2,4 ± 0,4

Annex G
(normative)

Determination of spore count - mean value and standard deviation

Take 5 test-tubes, each containing 10 ml of TSB, add 0.1 ml of bacterial spore suspension N1 to each tube and mix thoroughly. For counting prepare 10^{-3} and 10^{-4} dilutions of the test suspension using TSB (9 + 1) ml. Take a sample of 1,0 ml of each dilution in duplicate and spread each 1,0 ml sample on an appropriate number of surface dried plates containing TSA to determine the spore count by the method of the weightened mean value using the following formula.

It is hundredfold higher due to the volume of 0,1 ml of bacterial spore suspension N1 transferred to 10 ml TSB.

$$N = \frac{c \times 100}{\left(n_1 + 0{,}1 n_2\right) \cdot d}$$

where

c is the sum of Vc-values taken into account;

n_1 is the number of Vc-values taken into account in the lower dilution, i.e. (10^{-3});

n_2 is the number of Vc-values taken into account in the higher dilution, i.e. (10^{-4});

d is the dilution factor corresponding to the lower dilution.

Repeat this procedure at least on another day. Calculate the mean value (ξ) and the standard deviation (s) from the results.

$$\xi = \frac{\sum x}{n} \qquad s = \frac{\sum \left(x_i - \bar{x}\right)^2}{n-1} = \frac{\left(x_1 - \bar{x}\right)^2 + \left(x_2 - \bar{x}\right)^2 + \left(x_3 - \bar{x}\right)^2 + \ldots}{n-1}$$

where

x is the colony count in each tube;

n is the number of colony counts;

x_i single colony count out of each tube;

$\bar{x}$ average number of colonies.

The results have to be noted in the test report.

Bibliography

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

[2] European Pharmacopoeia (EP) - edition 1997 suppl. 2000, *Water for injections*

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