SLOVENSKI STANDARD
SIST EN 14347:2005

01-junij-2005

Kemična razkužila in antiseptiki - Osnovno sporocidno delovanje - Preskusna metoda in zahteve (faza 1, stopnja 1)

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

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

Désinfectants et antiseptiques chimiques - Activité sporicide de base - Méthode d'essai et prescriptions (phase 1)

Ta slovenski standard je istoveten z: **EN 14347:2005**

ICS:

71.100.35	Kemikalije za dezinfekcijo v industriji in doma	Chemicals for industrial and domestic disinfection purposes

SIST EN 14347:2005 **en,fr,de**

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)

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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of an identical text or by endorsement, at the latest by September 2005, and conflicting national standards shall be withdrawn at the latest by September 2005.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 ± 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 ± 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.

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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 ± 1 °C (or 37 °C ± 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 ± 1 °C (or 37 °C ± 1 °C) and then for 21 days at 30 °C ± 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.