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Chemical disinfectants and antiseptics — Quantitative surface test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in the veterinary area on non-porous surfaces without mechanical action — Test method and require

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National foreword

This British Standard is the UK implementation of EN 14349:2012. It supersedes BS EN 14349:2007 which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee CH/216, Chemical disinfectants and antiseptics.

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

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

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

This European Standard was approved by CEN on 22 September 2012.

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Contents

Page

Foreword

This document (EN 14349:2012) 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of an identical text or by endorsement, at the latest by May 2013, and conflicting national standards shall be withdrawn at the latest by May 2013.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

This document supersedes EN 14349:2007.

Data obtained using the former version of EN 14349 may still be used.

It was revised to correct obvious errors and ambiguities, to harmonize the structure and wording with other tests of CEN/TC 216 (existing or in preparation), and to improve the readability of the standard and thereby make it more understandable.

According to the CEN/CENELEC Internal Regulations, the national standards organisations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

Introduction

This European Standard specifies a surface test for establishing whether a chemical disinfectant or antiseptic has bactericidal activity in the area and fields described in the scope.

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

The conditions are intended to cover general purposes and to allow reference between laboratories and product types. Each utilization concentration of the chemical disinfectant or antiseptic, found by this test corresponds to the chosen experimental conditions. However, for some applications, the instructions of use of a product may differ and therefore additional test conditions need to be used.

1 Scope

This European Standard specifies a test method and the minimum requirements for bactericidal activity of chemical disinfectant and antiseptic products that form a homogeneous physically stable preparation when diluted with hard water, or – in the case of ready-to-use-products – with water.

This European Standard applies to products that are used in the veterinary area on non-porous surfaces without mechanical action i.e. in the breeding, husbandry, production, transport and disposal of all animals except when in the food chain following death and entry to the processing industry.

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

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

NOTE 2 This method corresponds to a Phase 2 Step 2 test.

This method cannot be used to evaluate the activity of products against mycobacteria.

2 Normative references

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

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

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

3 Terms and definitions

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

4 Requirements

The product shall demonstrate at least a 4 decimal log (lg) reduction from a water control, when tested in accordance with Table 1 and Clause 5 under simulated low level (3,0 g/l bovine albumin) or high level soiling (10 g/l yeast extract and 10 g/l bovine albumin) on a surface.

Table 1 - Obligatory and additional test conditions

Test conditions	**Bactericidal activity on non-porous surfaces without mechanical action in the veterinary area**
Test organism a) obligatory	*Enterococcus hirae* *Proteus vulgaris* *Pseudomonas aeruginosa* *Staphylococcus aureus*
b) additional	any relevant test organism
Test temperature a) obligatory	10 °C ± 1 °C
b) additional	4 °C ± 1 °C; 20 °C ± 1 °C; 40 °C ± 1 °C
Contact time a) obligatory	30 min ± 10 s
b) additional	1 min ± 5 s; 5 min ± 10 s; 60 min ± 10 s
Interfering substance a) obligatory low level soiling high level soiling	 3,0 g/l bovine albumin 10 g/l yeast extract plus 10 g/l bovine albumin
b) additional	any relevant substance
The obligatory contact time for surface disinfectants stated in Table 1 were chosen to enable comparison of standard conditions. The recommended contact time for the use of the product is within the responsibility of the manufacturer. NOTE For the additional conditions, the concentration defined as a result can be lower than the one obtained under the obligatory test conditions.	

Any additional specific bactericidal activity shall be determined in accordance with 5.2.1 and 5.5.1.1 in order to take into account intended specific use conditions.

5 Test method

5.1 Principle

A test suspension of bacteria mixed with interfering substance is inoculated onto the test surface and dried. After a drying time, 0,1 ml of the product is transferred to the surface, in a manner which covers the dried film. The surface is maintained at a specified temperature for a defined period of time specified in Clause 4 and 5.5.1.1. At the end of that contact time the surface is transferred to a neutralizer so that the action of the disinfectant is immediately neutralized. The numbers of surviving organisms which can be recovered from the surface is determined quantitatively.

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

The test is performed using *Enterococcus hirae*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* as test organisms (Clause 4, Table 1).

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

5.2 Materials and reagents

5.2.1 Test organisms

The bactericidal activity shall be evaluated using the following strains[1)] :

Enterococcus hirae ATCC 10541;

Proteus vulgaris ATCC 13315;

Pseudomonas aeruginosa ATCC 15442;

Staphylococcus aureus ATCC 6538.

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

The required incubation temperature for these test organisms is 36 °C ± 1 °C or 37 °C ± 1 °C (5.3.2.3). The same temperature (either 36 °C or 37 °C) shall be used for all incubations performed during a test and its control and validation.

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

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

5.2.2 Culture media and reagents

5.2.2.1 General

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

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

If additional strains do not grow on the media (5.2.2.3) or cannot be used with diluent (5.2.2.4) additional media shall be used and shall be reported as well as additional incubation conditions.

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

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

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

5.2.2.2 Water

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

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

NOTE The procedure to prepare hard water is described in 5.2.2.6.

5.2.2.3 Tryptone Soya Agar (TSA)

Tryptone soya agar, consisting of:

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 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 ±1) °C.

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

5.2.2.4 Diluent

Tryptone sodium chloride solution, consisting of:

Tryptone, pancreatic digest of casein 1,0 g;

Sodium chloride (NaCl) 8,5 g;

Water (5.2.2.2) to 1 000 ml.

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

5.2.2.5 Neutralizer

The neutralizer shall be validated for the product being tested in accordance with 5.5.1.2, 5.5.1.3 and 5.5.2. 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 B.

5.2.2.6 Hard water for dilution of products

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

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

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

Place 600 ml to 700 ml of water (5.2.2.2) in a 1 000 ml volumetric flask (5.3.2.12) and add 6,0 ml of solution A, then 8,0 ml of solution *B*. Mix and dilute to 1 000 ml with water (5.2.2.2). The pH of the hard water shall be 7,0 ± 0,2, when measured at (20 ±1) °C (5.3.2.4). If necessary, adjust the pH by using a solution of approximately 40 g/l (about 1 mol/l) of sodium hydroxide (NaOH) or approximately 36,5 g/l (about 1 mol/l) of hydrochloric acid (HCl).

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

NOTE When preparing the product test solutions (5.4.2), the addition of the product to the hard water produces different final water hardness in each test tube. In any case the final hardness expressed as calcium carbonate ($CaCO_3$) is in the test tube lower than 375 mg/l.

5.2.2.7 Interfering substances

5.2.2.7.1 General

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

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

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

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

5.2.2.7.2 Low level soiling (bovine albumin solution)

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

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

The final concentration of the bovine albumin in the test procedure (5.5) is 3 g/l.

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

a) Dissolve 10 g yeast extract powder in 150 ml of water (5.2.2.2) in a 250 ml volumetric flask (5.3.2.12) and allow foam to collapse. Make up to the mark with water (5.2.2.2). Transfer to a clean dry bottle and sterilize in the autoclave [5.3.2.1 a)]. Allow to cool to 20 °C ± 5 °C;

b) pipette 25 ml of this solution into a 50 ml volumetric flask (5.3.2.12) and add 10 ml of water (5.2.2.2). Dissolve 1 g of the bovine albumin in the solution in the flask with shaking and allow foam to collapse. Make up to the mark with water (5.2.2.2), sterilize by membrane filtration (5.3.2.7) and keep in 10 ml portions in a refrigerator (5.3.2.8) and use within one month.

The final concentration in the test procedure (5.5) is 10 g/l yeast extract and 10 g/l bovine albumin.

5.2.3 Test surface

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

The surfaces should be used only once.

Prior to use the surfaces should be placed in a beaker (minimum size 50 ml) containing not less than 20 ml of 5 % Decon®[2)] for 60 min. Immediately rinse the discs with running freshly distilled water for 10 s.

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

5.3 Apparatus and glassware

5.3.1 General

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 an autoclave [5.3.2.1 a)]

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

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

5.3.2 Usual microbiological laboratory equipment[3)]

and, in particular, the following:

5.3.2.1 Apparatus for sterilization (moist and dry heat)

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

b) for dry heat sterilization, a hot air oven capable of being maintained at $(180\,^{+5}_{0})$ °C for a minimum holding time of 30 min, at $(170\,^{+5}_{0})$ °C for a minimum holding time of 1 h or at $(160\,^{+5}_{0})$ °C for a minimum holding time of 2 h.

5.3.2.2 Water baths, capable of being controlled at (4 ± 1) °C, (10 ± 1) °C, (20 ± 1) °C, (40 ± 1) °C and (45 ± 1) °C (to maintain melted TSA, 5.2.2.3, 5.5.2.2 and 5.5.2.3).

5.3.2.3 Incubator, capable of being controlled either at (36 ± 1) °C or (37 ± 1) °C (5.2.1). The same temperature shall be used for incubation performed during a test and its control and validation.

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

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

5.3.2.5 Stopwatch.

5.3.2.6 Shakers

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

b) Mechanical shaker.

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

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

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

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

5.3.2.9 Graduated pipettes, of nominal capacities 10 ml, 1 ml, 0,1 ml and 0,05 ml, or calibrated automatic pipettes.

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

5.3.2.11 Glass beads (diameter 3 mm to 4 mm).

5.3.2.12 Volumetric flasks.

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

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

5.4 Preparation of test organism suspensions and product test solutions

5.4.1 Test organism suspensions (test and validation suspension)

5.4.1.1 Introduction

Test and validation suspension are the same in this standard.

5.4.1.2 General

For each test organism, one suspension has to be prepared: this is used as the bacterial “test suspension” to perform the test and the “validation suspension” to perform the controls and method validation.

5.4.1.3 Preservation and stock cultures of test organisms

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

5.4.1.4 Working culture of test organisms

In order to prepare the working culture of the test organisms (5.2.1), prepare a subculture from the stock culture (5.4.1.3) by streaking onto TSA (5.2.2.3) slopes or plates and incubate (5.3.2.3). After 18 h to 24 h prepare a second subculture from the first subculture in the same way and incubate for 18 h to 24 h. From this second subculture, a third subculture may be produced in the same way. The second and (if produced) third subculture are the working cultures.

If it is not possible to prepare the second subculture on a particular day, a 48 h subculture may be used for subsequent subculturing, provided that the subculture has been kept in the incubator (5.3.2.3) during the 48 h period. In these circumstances, prepare a further 24 h subculture before proceeding.

Never produce and use a fourth subculture.

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

5.4.1.5 Test suspension ("*N*") and validation suspension ("N_v")

Test and validation suspension are the same in this standard.

a) Take 10 ml of diluent (5.2.2.4) and place in a 100 ml flask (5.3.2.12) with 5 g of glass beads (5.3.2.11). Take the working culture (5.4.1.4) and transfer loopfuls of the cells into the diluent (5.2.2.4). The cells should be suspended in the diluent by rubbing the loop against the wet wall of the flask to dislodge the cells before immersing in the diluent. Shake the flask for 3 min using a mechanical shaker [5.3.2.6 b)]. Aspirate the suspension from the glass beads and transfer to a tube.

b) Adjust the number of cells in the suspension to 1,5 x 10^9 cfu/ml to 5,0 x 10^9 cfu/ml[5)] using diluent (5.2.2.4), estimating the number of cfu/ml by any suitable means. Maintain this suspension in the water bath at (20 ± 1) °C and use within 2 h.

   Adjust the temperature according to [5.5.1.1 a)] and 5.5.1.4 only immediately before the start of the test.

The use of spectrophotometer for adjusting the number of cells is highly recommended (about 620 nm wavelength – cuvette 10 mm path length). Each laboratory should therefore produce calibration data for each test organism knowing that suitable values of optical density are generally found between 0,150 and 0,460. To achieve reproducible results of this measurement it may be necessary to dilute the test suspension, e.g. 1+9.

NOTE A colorimeter is a suitable alternative.

c) For counting of the bacterial test suspension prepare 10^{-7} and 10^{-8} dilutions of the test suspension using diluent (5.2.2.4).

Mix [5.3.2.6 a)].

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

1) When using the pour plate technique, transfer each 1,0 ml sample into separate Petri dishes and add 15 ml to 20 ml melted TSA (5.2.2.3), cooled to (45 ± 1) °C;

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

For incubation and counting see 5.4.1.6.

5.4.1.6 Incubation and counting of the test and the validation suspensions

a) Incubate (5.3.2.3) the plates for 20 h - 24 h. Discard any plates that are not countable for any reason. Count the plates and determine the total number of cfu. Incubate the plates for a further 20 h - 24 h. Do not recount plates that no longer show well-separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.

b) Note for each plate the exact number of colonies but record >330 for any counts higher than 330 and determine the V_C values according to 5.6.2.2.

c) Calculate the numbers of cfu per 0,025 ml in the test suspension *N* and the validation suspension Nv using the methods given in 5.6.2.3. Verify according to 5.7.

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

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

5.4.2 Product test solutions

Product test solutions shall be prepared in hard water (5.2.2.6) at a minimum three different concentrations to include one concentration in the active range and one concentration in the non-active range (5.8.2). The product as received may be used as one of the product test solutions. Dilutions of ready-to-use products, i.e. products that are not diluted when applied, shall be prepared in water (5.2.2.2) instead of hard water.

For solid products, dissolve the product as received by weighing at least 1,0 g ± 10 mg of the product in a volumetric flask and filling up with hard water (5.2.2.6). Subsequent dilutions (= lower concentrations) shall be prepared in volumetric flasks (5.3.2.12) on a volume/volume basis in hard water (5.2.2.6).

For liquid products, dilutions of the product shall be prepared with hard water (5.2.2.6) in volumetric flasks (5.3.2.12) 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 homogeneous preparation, stable during the whole procedure.

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

5.5 Procedure for assessing the bactericidal activity of the product

5.5.1 General

5.5.1.1 Experimental conditions (obligatory and additional)

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

a) temperature (in °C):

The obligatory and additional temperatures to be tested are specified in Clause 4, Table 1.

The allowed deviation for each chosen temperature is ± 1 °C.

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

The obligatory and additional contact times to be tested are specified in Clause 4, Table 1.

The allowed deviation for each chosen time is ± 10 s, except for 1 min for which it is ± 5 s.

c) interfering substance:

The obligatory interfering substance to be tested is 3,0 g/l bovine albumin (5.2.2.7.2) for low level soiling or 10 g/l bovine albumin plus 10 g/l yeast extract (5.2.2.7.3) for high level soiling according to Clause 4, Table 1 and practical applications. Additional interfering substances may be tested according to specific fields of application.

d) test organisms:

Enterococcus hirae, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Clause 4, Table 1 and 5.2.1).

Additional test organisms may be tested.

5.5.1.2 Neutralization

To determine a suitable neutralizer carry out the validation of the dilution-neutralization method (5.5.2.4 and 5.5.2.5 in connection with 5.5.2.6) 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 taking into account the information given in Annex B.

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

5.5.1.3 General instruction for validation and control procedures

The neutralization and/or removal of the bactericidal and/or bacteriostatic activity of the product shall be controlled and validated - only for the highest product test concentration - for each of the used test organisms and for each experimental condition (interfering substance, temperature, contact time). These procedures (water control, 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 a 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 the inoculated and dried stainless steel discs and all reagents (product test solutions (5.4.2), hard water (5.2.2.6) to the test temperature of θ [5.5.1.1 a)] using the water bath (5.3.2.2) and/or the temperature controlled cabinet (5.3.2.13) controlled at θ. Check that the temperature of the reagents is stabilized at θ.

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

5.5.2 Test procedure (Dilution-neutralization method)[6)]

5.5.2.1 General

The test and the control and validation procedures (5.5.2.2, 5.5.2.3, 5.5.2.4 and 5.5.2.5) shall be carried out at the same time.

5.5.2.2 Test "N_a" – Determination of bactericidal concentrations

The procedure for determining bactericidal concentrations is as follows:

a) To prepare the microbial test suspension pipette 1,0 ml of the interfering substance (5.2.2.7) into a tube. Add 1,0 ml of the test suspension (5.4.1.5). Start the stopwatch (5.3.2.5) immediately, mix [5.3.2.6 a)] and place the tube in a water bath or temperature controlled cabinet at the chosen test temperature θ [5.5.1.1 a)] for 2 min ± 10 s.

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

b) Place the test surfaces (5.2.3) in a sterile Petri dish (5.3.2.10) and ensure that the dish is in a horizontal position. Prepare the test surfaces by inoculating 0,05 ml of the microbial test suspension [5.5.2.2 a)] on to each test surface. The inoculum may not be spread over the surface. Dry the surfaces at 37 °C until they are visibly dry.

---

6) For a graphical representation of this method see C.1

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

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

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

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

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

1) When using the pour plate technique, pipette each 1,0 ml sample into separate Petri dishes and add 15 ml to 20 ml of melted TSA (5.2.2.3), cooled to (45 ± 1) °C.

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

For incubation and counting, see 5.5.2.6.

e) Recover the test surface ("N_a"), let the neutralizer drain off and rinse with 10 ml of water (5.2.2.2). Transfer to a Petri dish (5.3.2.10) containing 10 ml of solidified TSA (5.2.2.3) and place on top of the agar test side uppermost. Add 10 ml of TSA (5.2.2.3) melted and cooled to 45 °C.

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

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

5.5.2.3 Water control "N_w"

The procedure for determining the water control is as follows:

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

a) Place one test surface (5.2.3) in a sterile Petri dish (5.3.2.10) and ensure that the dish is in a horizontal position. Inoculate 0,05 ml of the microbial test suspension [5.5.2.2 a)] on to the test surface. The inoculum may not be spread over the surface. Dry the surface at 37 °C until it is visibly dry [5.5.2.2 b)].

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

Place the surface in a temperature controlled cabinet at the chosen test temperature of (10 ± 1) °C (5.3.2.13).

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

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

For incubation and counting, see 5.5.2.6.

5.5.2.4 Neutralizer control "*B*" - Verification of the absence of toxicity of the neutralizer

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

a) Prepare one inoculated test surface [5.5.2.2 a)].

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

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

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

For incubation and counting, see 5.5.2.6.

5.5.2.5 Method validation "*C*" - Dilution-neutralization validation

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

a) Prepare one test surface [5.5.2.2 a)].

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

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

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

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

For incubation and counting, see 5.5.2.6.

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

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

a) Incubate (5.3.2.3) the plates for 20 h to 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of cfu for each plate. Incubate the plates for a further 20 h to 24 h. 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 V_C values according to 5.6.2.2.

c) Calculate the number of cfu/ml in the test mixture N_a, the water control N_w, and in the validation mixtures *B* and *C* using the method given in 5.6.2.4 and 5.6.2.5. Verify according to 5.7.

5.5.3 Observation of test surface agar

Incubate (5.3.2.3) the agar plates with the test surfaces (5.5.2.2) for 20 h to 24 h. Examine for bacterial colonies. Discard any plates that show colonies and incubate plates for a further 20 h to 24 h. Examine for bacterial colonies and verify according to 5.7.3.

5.6 Experimental data and calculation

5.6.1 Explanation of terms and abbreviations

5.6.1.1 Overview of the different suspensions and test mixtures

N represents the microbial suspension and the validation suspension (N_v), N_a represents the bactericidal test mixture, N_w represents the test mixture in the water control, *B* (neutralizer control) and *C* (method validation) represent the different control test mixtures.

NOTE 1 The control A Experimental conditions control A (Validation of the selected experimental conditions or verification of the absence of any lethal effect in the test conditions) differs from other standard due to the fact it is directly determined in the water control.

N represents the number of cells counted per 0,025 ml of the bacterial test suspension and the validation suspension; N_a, N_w and *B*, *C* represent the number of cells counted per ml in the different test mixtures.

Table 2 - Number of cells counted per 0,025 ml or surface in the different test mixtures

	Number of cells per 0,025 ml in the test suspension	**Number of survivors per surface at the end of the contact time *t***
Test	***N***	***N_a***
Controls	***N, N_v***	***N_w, B, C***

NOTE 2 *N* is calculated per 0,025 ml, *N* and N_v are the same in this standard.

5.6.1.2 V_C values

All experimental data are reported as V_C values:

- in the method (test and controls), a V_C value is the number of cfu counted per 1,0 ml sample.

5.6.2 Calculation

5.6.2.1 General

The first step in the calculation is the determination of the V_C values, the second the calculation of *N*, N_a, N_w,, *B* and *C*. The third step is the calculation of the reduction *R* (5.8).

5.6.2.2 Determination of V_C values

The V_C values are determined as follows.

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

NOTE The lower limit (14) is based on the fact that the variability increases the smaller the number counted in the sample (1,0 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. three plates per 1,0 ml sample with 3 cfu, 8 cfu and 5 cfu give a V_C value of 16. The upper limits (330) reflect the imprecision of counting confluent colonies and growth inhibition due to nutriment depletion. They refer only to the counting on one plate and not necessarily to the sample.

b) For counting the test suspension *N* (5.4.1.4) and for all counts of the method (5.5.2.6), determine and record the V_C values according to the number of plates used per 1,0 ml sample (5.6.2.4 and 5.6.2.5).

If more than one plate per 1,0 ml sample has been used to determine the V_C value, the count per plate should be noted.

If the count on one plate is higher than 330, report the number as “>330”. If more than one plate per 1,0 ml sample has been used and at least one of them shows a number higher than 330, report this V_C value as “more than sum of the counts”, e. g. for “>330, 310, 302”, report “ > 942”.

If a V_C value is lower than 14, report the number (but substitute by “<14” for further calculation in the case of N_a).

c) Only V_C values within the counting limits are taken into account for further calculation, except in the case of N_a (5.6.2.4).

5.6.2.3 Calculation of *N*

N is the number of cells per 0,025 ml in the microbial test suspension (5.5.2.2a). It is less than the number in the bacterial test suspension due to dilution by adding equal parts interfering substance and using 0,05ml for the inocula.

Since two dilutions of the microbial test suspension (5.5.2.2a) are evaluated, calculate the number of cfu/0,025 ml as the weighted mean count using the following formula (1):

$$N = \frac{0{,}025 \times c}{(n_1 + 0{,}1\, n_2) \times d}$$

where

c is the sum of the V_C values taken into account;

n_1 is the number of V_C values taken into account in the lower dilution, i.e. 10^{-7};

n_2 is the number of V_C values taken into account in the higher dilution, i.e. 10^{-8};

d is the dilution factor corresponding to the lower dilution (10^{-7})

Round off the results calculated to two significant figures. For this, if the last figure is below 5, the preceding figure is not modified; if the last figure is more than 5 the preceding figure is increased by one unit; if the last figure is equal to 5, round off the preceding figure to the next nearest even figure. Proceed stepwise until two significant figures are obtained. As a result, the number of cfu/0,025 ml is expressed by a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

EXAMPLE

$$N = \frac{(168+213+20+25)}{2{,}2\times10^{-7}}\times 0{,}025 = \frac{426\times 0{,}025}{2{,}2}\times 10^{7} = 4{,}84\times10^{7} = \lg 7{,}68$$

5.6.2.4 Calculation of N_a and N_w

N_a is the number of survivors per test surface (5.5.2.2) at the end of the contact time and before neutralization. It is tenfold higher than the V_C values due to the addition of neutralizer.

N_w is the number of survivors per water control surface (5.5.2.3) at the end of the contact time and before neutralization. It is tenfold higher than the V_C values due to the addition of neutralizer.

Calculate the mean for each dilution step N_a and N_w using following formula (2):

$$N_a\left(or N_w\right) = \frac{c\times 10}{n\times d}$$

where

c is the sum of V_C values taken into account;

n is the number of V_C values taken into account;

d is the dilution taken into account.

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

EXAMPLES

a) duplicate V_C values N_a: 2, 16 (from the neutralised mixture)

$$N_a = \frac{(<14+16)\times 10}{2} = <150 = <1{,}50\times10^{2} \quad <\lg 2{,}18$$

b) duplicate V_C values (two spread plates per 1,0 ml sample from the neutralised mixture): > 330, 330; 310, 290

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

c) duplicate V_C values N_a^{-1}: 40, 46 (from the 10^{-1} dilution)

$$N_a^{-1} = \frac{(40+46)\times 10}{2\times 10^{1}} = 430\times 10^{1} = 4\,300 = 4{,}3\times 10^{3} \quad \lg 3{,}63$$

Use maximum 2 subsequent dilutions for calculating N_a as a weighted mean.

Exceptions and rules for special cases:

d_1 If one or both duplicate V_C values in three or more subsequent dilutions of N_a are within the counting limits (e. g. N_a^{-2}: 17, 23; N_a^{-1}: 120, 135; N_a: 308, > 330) the whole test is invalid (5.7.1).

d_2 If two subsequent dilutions of N_a show duplicate V_C values within the counting limits calculate N_a as the weighted mean using the following formula (3):

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

where

c is the sum of V_C values taken into account;

z is the dilution factor corresponding to the lower dilution.

5.6.2.5 Calculation of *B* and *C*

B and *C* are the numbers of survivors in the neutralizer control (5.5.2.4) and method validation (5.5.2.5) at the end of the defined times 5 min (*B*) and 30 min (*C*). It is tenfold higher than the V_C values due to the addition of neutralizer.

Calculate *B* and *C* using the following formula (4):

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

where

c is the sum of V_C values taken into account;

n is the number of V_C values taken into account;

d is the dilution taken in to account

If two subsequent dilutions of *B* or *C* show duplicate V_C values within the counting limits calculate N_a as the weighted mean using the following formula (5):

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

where

c is the sum of V_C values taken into account;

z is the dilution factor corresponding to the lower dilution.

5.7 Verification of methodology

5.7.1 General

A test is valid if:

— all results meet the criteria of 5.7.3;

— the requirements of 5.8.2 are fulfilled; and

— it is not invalidated by a result described under 5.6.2.4 c) first special case (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 means of the two results shall be not higher than 15 and not lower than 5. Results below the lower limit are taken as the lower limit number (14). Results above the respective upper limit [5.6.2.2 b)] are taken as the upper limit number.

EXAMPLE

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

= between 5 and 15.

NOTE When the counts obtained on plates are out of the limits fixed for the determination of V_C values [5.6.2.2b)], check for the weighted mean as mentioned above but use only the V_C values within the counting limits for calculation of N.

5.7.3 Basic limits

For each test organism check that:

a) N is between $3{,}75 \times 10^7$ and $1{,}25 \times 10^8$ $(7{,}57 \leq \lg N \leq 8{,}10)$

NOTE N is calculated for 0,025 ml

b) $\lg N_w \geq \lg 6{,}2$

c) $B \geq 0{,}5 \times N_w$

d) $C \geq 0{,}5 \times N_w$;

e) no growth is observed on the treated surface for concentration that pass the test;

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

5.8 Expression of results and precision

5.8.1 Reduction

The reduction ($R = N_w/N_a$) is expressed in logarithm.

For each test organism record the number of cfu in the test procedure for bactericidal activity of the product N_a (5.5.2.2) and in the control procedure N_w (5.5.2.3).

For each product concentration and each experimental condition, calculate and record the decimal log reduction (lg) separately using the following formula:

$\lg R = \lg N_w - \lg N_a$

For validation of the dilution-neutralization method record the number of cfu in the neutralizer toxicity control *B* (5.5.2.4) and the dilution-neutralization control *C* (5.5.2.5).

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

At least one concentration per test (5.5.2.2) shall demonstrate a 4 lg or more reduction and at least one concentration shall demonstrate a lg reduction of less than 4.

5.8.3 Limiting test organism and bactericidal concentration

For each test organism, record the lowest concentration of the product which passes the test ($\lg R > 4$). 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 bactericidal concentration determined according to this European Standard.

5.8.4 Precision, repetitions

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

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

Repetition means the complete test procedure with separately prepared test- and validation suspensions. The repetitions may be restricted to the limiting test organism. The mean of the results of the repetitions - not each single result - shall demonstrate at least a 4 lg reduction 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 bactericidal concentrations determined according to this standard may differ (Clause 4). A product can only pass the test if the requirements of 5.8.2 are fulfilled.

5.9.2 Bactericidal activity for general purposes

The product shall be deemed to have passed the EN 14349 standard if it demonstrates in a valid test at least a 4 lg reduction within 30 min at 10 °C with the chosen interfering substance under the conditions defined by this European Standard when the test organisms are *Enterococcus hirae*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

The bactericidal concentration for general purposes is the concentration active on the limiting strain.

5.9.3 Qualification for certain fields of application

The bactericidal concentration for specific purpose is the concentration of the tested product for which at least a 4 lg reduction is demonstrated in a valid test under the additional chosen test conditions. The product shall be deemed to have passed the EN standard under the obligatory test conditions and the bactericidal concentration for specific purposes may be lower than the one determined for general purposes.

For more details see EN 14885.

5.10 Test report

The test report shall refer to this European Standard (EN 14349).

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

a) identification of the testing laboratory;

b) identification of client;

c) identification of the sample:

   1) name of the product;

   2) batch number – if available – expiry date;

   3) manufacturer – if not known – supplier;

   4) date of delivery;

   5) storage conditions;

   6) product diluent recommended by the manufacturer for use;

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

   8) appearance of the product;

d) test method and its validation:

   full details of the tests for validation of the neutralizer shall be given;

e) experimental conditions:

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

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

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

   4) appearance of product dilutions;

   5) contact time(s);

   6) test temperature(s);

   7) interfering substance(s);

   8) temperature of incubation;

9) neutralizer;

10) identification of the bacterial strains used;

11) drying time of the inoculated test surfaces.

f) test results:

1) controls and validation;

2) evaluation of bactericidal activity;

3) number of repetitions per test organism.

g) special remarks;

h) conclusion;

i) locality, date and identified signature.

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

Annex A
(informative)

Referenced strains in national collections

— *Enterococcus hirae* ATCC 10541

CIP 58.55

DSM 3320

NCIMB 8192

— *Proteus vulgaris* ATCC 13315

CIP 58.60

DSM 30118

NCIMB 4175

NCTC 4175

— *Pseudomonas aeruginosa* ATCC 15442

CIP 103467

DSM 939

NCIMB 10421

— *Staphylococcus aureus* ATCC 6538

CIP 4.83

DSM 799

NCIMB 9518

NCTC 10788

Abreviations:

ATCC American Type Culture Collection

CIP Collection de l'Institut Pasteur

DSM Deutsche Sammlung von Mikrooganismen und Zellkulturen

NCIMB National Collection of Industrial and Marine Bacteria

NCTC National Collection of Type Cultures

Annex B
(informative)

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

IMPORTANT— Neutralizers of the residual antimicrobial activity of chemical disinfectants and antiseptics shall be validated according to the prescriptions of the standard.

Table B.1

Antimicrobial agent	Chemical compounds able to neutralize residual antimicrobial activity	Examples of suitable neutralizers[a]
Quaternary ammonium compounds and fatty amines Amphoteric compounds	Lecithin[b)], Saponin, Polysorbate 80, Sodium dodecyl sulphate, Ethylene oxide condensate of fatty alcohol (non-ionic surfactants) [c)]	- Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. - Polysorbate 80, 30 g/l + sodium dodecyl sulphate, 4 g/l + lecithin, 3 g/l. - Ethylene oxide condensate of fatty alcohol, 3 g/l + lecithin, 20 g/l + polysorbate 80, 5 g/l.
Biguanides and similar compounds	Lecithin [b)], Saponin, Polysorbate 80	- Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l.
Oxidizing compounds (Chlorine, iodine, hydrogen peroxide, peracetic acid, hypochlorites, etc…)	Sodium thiosulphate [d)] Catalase [for hydrogen peroxide or products releasing hydrogen peroxide]	- Sodium thiosulphate, 3 g/l to 20 g/l + polysorbate 80, 30 g/l + lecithin, 3 g/l. - Polysorbate 80, 50 g/l + catalase 0,25 g/l + lecithin 10 g/l.
Aldehydes	L-histidine Glycine	- Polysorbate 80, 30 g/l + lecithin, 3 g/l + L-histidine, 1 g/l (or + glycine, 1 g/l). - Polysorbate 80, 30 g/l + saponin, 30 g/l + L-histidine, 1 g/l (or + glycine, 1 g/l).
Phenolic and related compounds: orthophenylphenol, phenoxyethanol,	Lecithin[b)] Polysorbate 80	- Polysorbate 80, 30 g/l + lecithin, 3 g/l.

triclosan, phenylethanol, etc… Anilides	Ethylene oxide condensate of fatty alcohol[c)]	- Ethylene oxide condensate of fatty alcohol, 7 g/l + lecithin, 20 g/l, + polysorbate 80, 4 g/l.
Alcohols[e)]	Lecithin[b)], Saponin, Polysorbate 80 [e)]	- Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l.

[a)] According to the pH of the tested product, the pH of the neutralizer may be adjusted at a suitable value or prepared in phosphate buffer [ex: phosphate buffer 0,25 mol/l: potassium dihydrogen phosphate (KH_2PO_4) 34 g; distilled water (500 ml); adjusted to pH 7,2 $\pm$ 0,2 with sodium hydroxide (NaOH) 1 mol/l; distilled water up to 1000 ml].

[b)] Egg and soya; egg is preferable.

[c)] The carbon chain-length varies from C_{12} to C_{18} carbon atoms.

[d)] The toxic effect of sodium thiosulphate differs from one test organism to another.

[e)] For the neutralization of short chain alcohols (less than C_5), simple dilution may be appropriate. Care should be taken if the alcohol-based products contain additional antimicrobial agents.

Annex C
(informative)

Graphical representation of the method

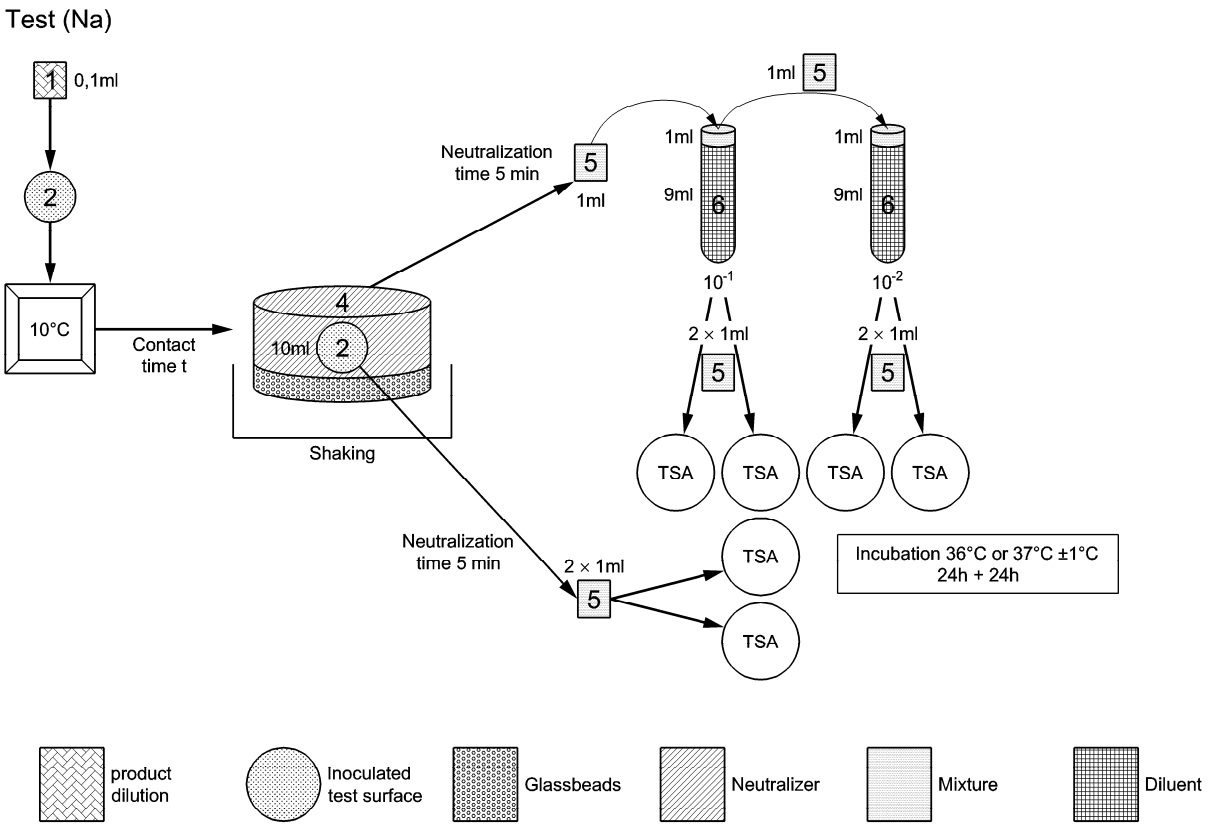

Figure C.1 — Test (N_a)

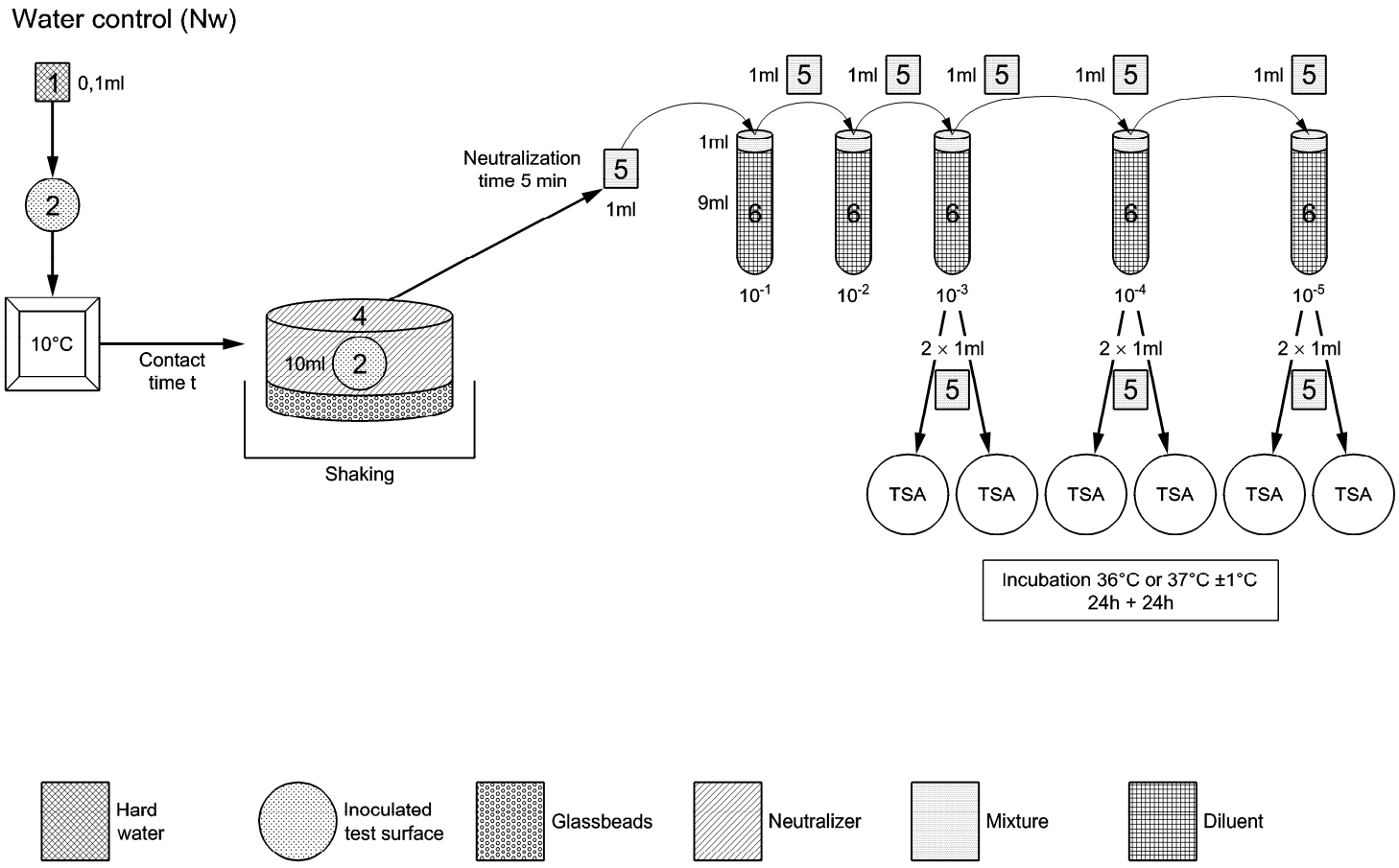

Figure C.2 - Water control (N_w)

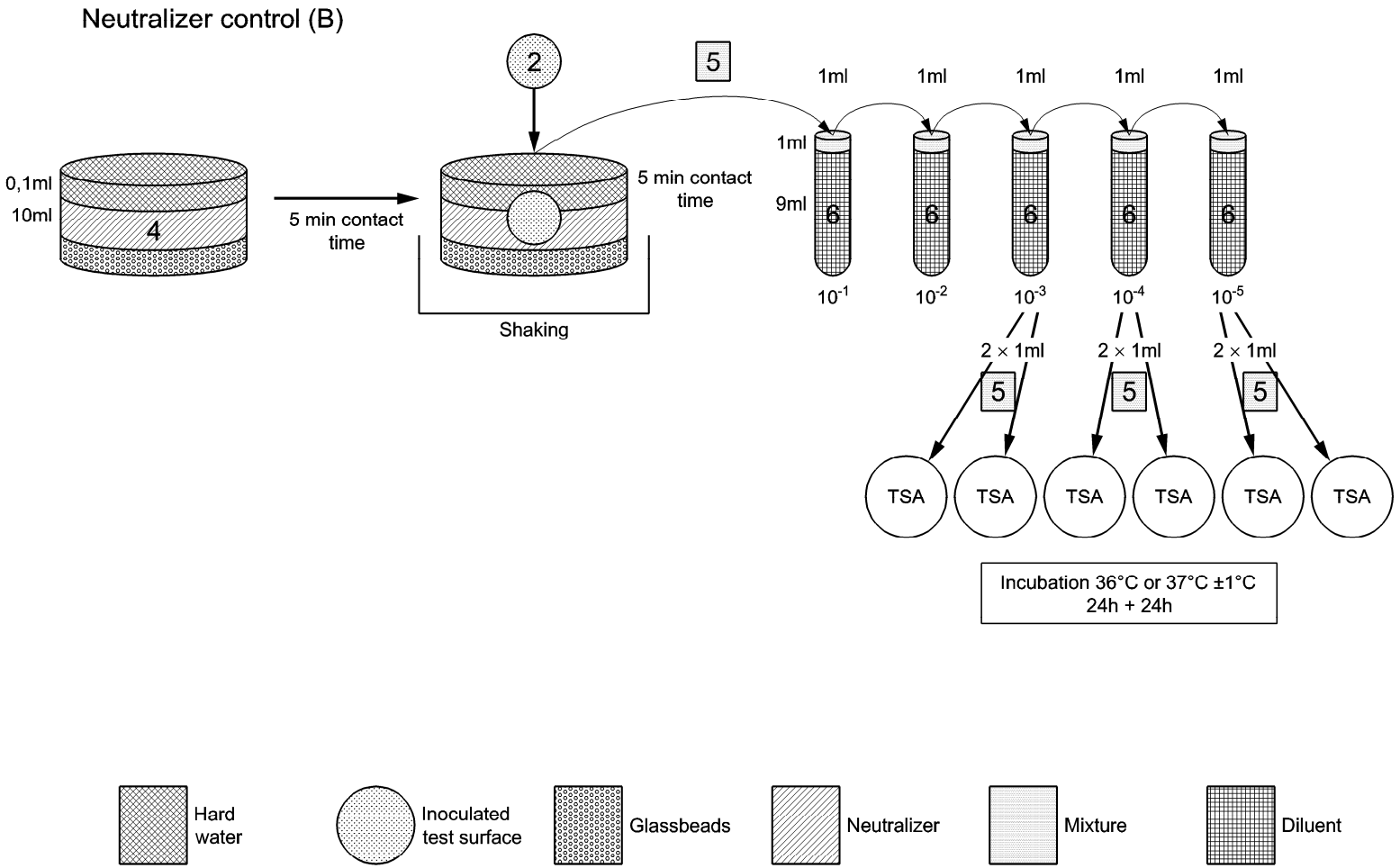

Figure C.3 - Validation – Neutralizer toxicity control (*B*)

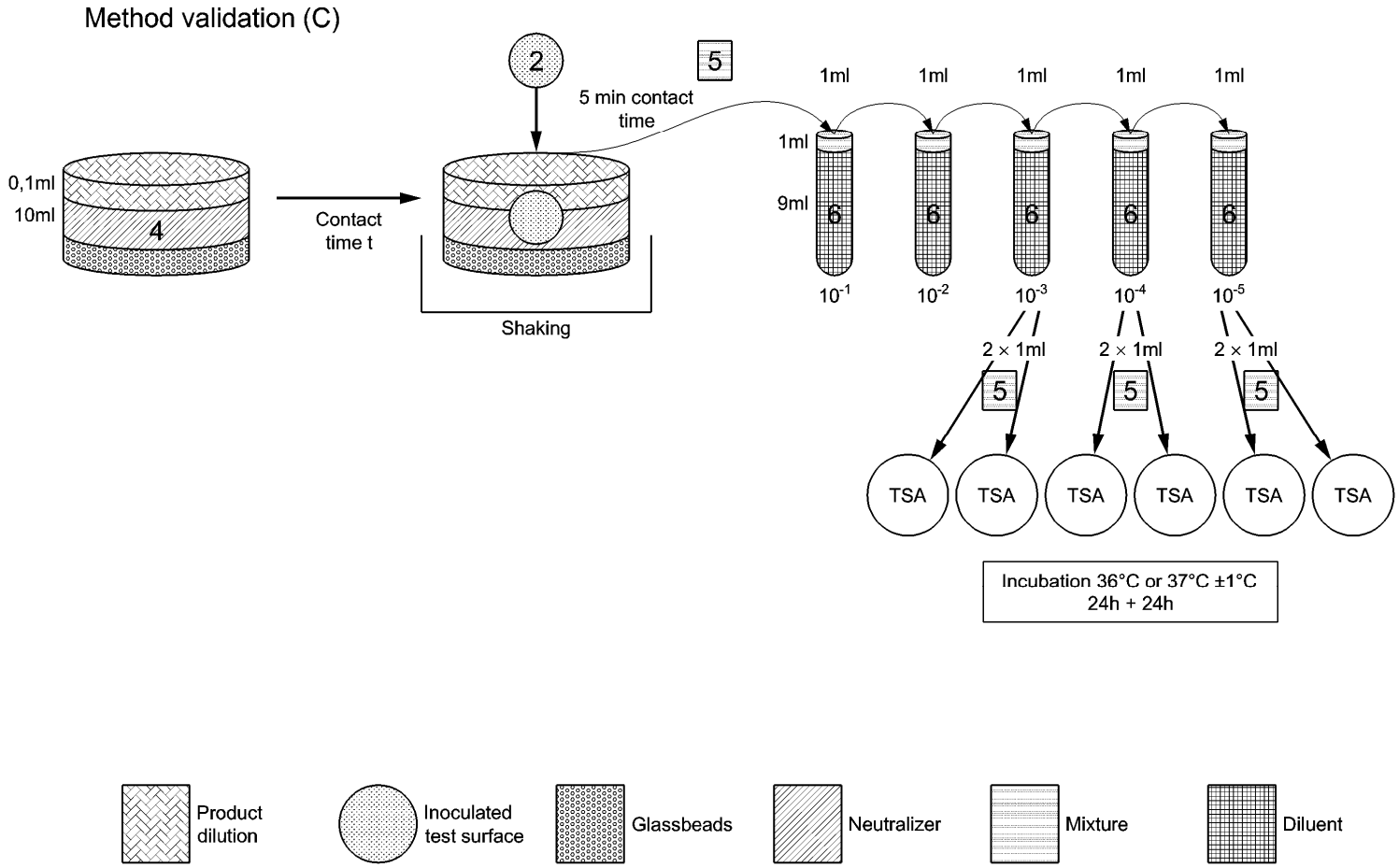

Figure C.4 - Validation – Method Validation (*C*)

Annex D
(informative)

Example of a typical test report

NOTE 1 All names and examples in Annex D are fictitious apart from those used in this European Standard.

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

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TEST REPORT

EN 14349 BACTERICIDAL ACTIVITY

(obligatory and additional conditions)

Client: Centipede Formulations Inc., Mannheim / Euroland

Disinfectant sample

Name of the product: Z **Batch umber**: 11-47-013

Manufacturer or – if not known – **supplier** Centipede Formulations Inc. (manufacturer)

Storage conditions temp and other): Room temperature, darkness

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

Period of testing

Date of delivery of the product: 2011-10-09 **Dates of tests**: See "Test results" (attached)

Experimental conditions

Test method: Dilution-neutralization

Product diluent: hard water **Concentrations of the product tested**: See "Test results" (attached)

Obligatrory conditions:

Test organisms: *Enterococcus hirae* ATCC 10541, *Proteus vulgaris* ATCC 13315, *Pseudomonas aeruginosa* ATCC 15442, Staphylococcus aureus ATCC 6538

Test temperature: 10 °C ± 1 °C **Contact time:** 30 min ± 10 s

Interfering substance: 10 g/l yeast extract + 10 g/l bovine albumin

Incubation temperature: 37 °C ± 1 °C

Neutralizer: 3,0 g/l lecithin in diluent, sterilized in the autoclave

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

EN 14349 (Phase 2. step.2) Product-name: Z Batch No: 11-47-013

Manufacturer: Centipede Formulation Inc. Appearance of the product: liquid, clear, yellowish

Storage conditions (temp. and other): room temperature, darkness

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

Pour plate Spread plate x Number of plates 3/ml Neutralizer: .Lecithin 3,0 g/l in diluent

Test temperature: 10 °C Interfering substances: 10 g/l yeast extract + 10 g/l bovine albumin

Test organism: *P. aeruginosa* ATCC 15442 Drying time on carrier: 40 min (not > 60 min) Incubation temp.: 37 °C

Internal lab. no: QS..58/00 Date of test: 2011-10-19 Responsible person: FangSignature: Fang

Validation and controls

Test and Validation suspension (***N***)			**Neutralizer toxicity control (*B*)**			**Dilution-neutralization control (*C*)** Product conc.: 0,5 %		
	V_{C1}	V_{C2}		V_{C1}	V_{C2}		V_{C1}	V_{C2}
10^{-7}	168	213	10^{-4}	75	76	10^{-4}	80	85
10^{-8}	20	25	10^{-5}	7	8	10^{-5}	9	8
$\bar{x}_{wm}$ = 4,84 x 10^7 = lg 7,68			$\bar{x}$ = 7,55 x 10^6 = lg 6,88			$\bar{x}$ = 8,25 x 10^6 = lg 6,92		
7,57 ≤ lg*N* ≤ 8,10 ☒ yes ☐ no			$\bar{x}$ of *B* ≥ 0,5 x $\bar{x}$ of N_w? ☒ yes ☐ no			$\bar{x}$ of *C* ≥ 0,5 x $\bar{x}$ of N_w? ☒ yes ☐ no		

Water control

Water control (N_w):	N_w	V_{C1}	V_{C2}	$\bar{x}$ = 1,45 x 10^7
	10^{-4}	**150**	**140**	lgNw = 7,16 (lgN_w ≥ lg 6,2) ?
	10^{-5}	**13**	**12**	☒ **yes** ☐ ***no***

Test

Conc. of the product %	Dilution step	Counts per plate		V_{C1}	V_{C2}	lg N_a = lg ($\bar{x}$ or $\bar{x}$ wm)	lg R = (lg N_w =*7,16*)	Contact time (min)
0,125 %	10^{0}	*>330+>330+>330*	*>330+>330+>330*	*>990*	*>990*	*5,04*	*2,12*	*30*
	10^{-1}	*>330+>330+>330*	*324+305+329*	*>990*	*958*			
	10^{-2}	*39+40+45*	*20+33+42*	*124*[a]	*95*[a]			
0,25 %	10^{0}	*>330+>330+>330*	*>330+>330+>330*	*>990*	*>990*	*4,48*	*2,68*	*30*
	10^{-1}	*96+110+92*	*106+111+95*	*298*[a]	*312*[a]			
	10^{-2}	*8+8+11*	*8+12+11*	*27*[a]	*31*[a]			
0,5 %	10^{0}	*3+1+2*	*0+0+0*	*<14*[a]	*< 14*[a]	*<2,15*	*>4,0*	*30*
	10^{-1}	*0+0+o*	*0+0+0*	*< 14*	*< 14*			
	10^{-2}	*0+0+0*	*0+0+0*	*< 14*	*< 14*			
[a] used for calculation								

Remarks:

Explanations:

V_C **= count per ml (one plate or more)**

$\bar{x}$ = average of V_{C1} and V_{C2}

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

R = reduction (lg R = lg N_w – lg N_a)

If N_a <14, lg R = > [lg N_w – <2,15]

See 5.6 for calculation rules.

Bibliography

[1] European Pharmacopoeia (EP), Edition 2005 supplement 2006, Water for injections

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