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# Chemical disinfectants and antiseptics — Quantitative surface test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in veterinary area on porous surfaces without mechanical action — Test method and requirements (phase 2, step 2)

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# Chemical disinfectants and antiseptics - Quantitative surface test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in veterinary area on porous surfaces without mechanical action - Test method and requirements (phase 2, step 2)

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 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 porösen Oberflächen ohne mechanische Wirkung - Prüfverfahren und Anforderungen (Phase 2, Stufe 2)

This European Standard was approved by CEN on 30 November 2013.

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**CEN-CENELEC Management Centre: Avenue Marnix 17, B-1000 Brussels**

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# Foreword

This document (EN 16437:2014) has been prepared by Technical Committee CEN/TC 216 “Chemical disinfectants and antiseptics”, the secretariat of which is held by AFNOR.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by August 2014 and conflicting national standards shall be withdrawn at the latest by August 2014.

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# Introduction

This European Standard specifies a surface test for establishing whether a chemical disinfectant or antiseptic, for use on porous surfaces without mechanical action, in the veterinary area. has or does not have bactericidal activity under the laboratory conditions defined by this European Standard, which influence the action of disinfectants in practical use.

The laboratory test takes into account practical conditions of application of the product including pre-drying test organisms and interfering substances on a surface, contact time and temperature, 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 utilisation 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 can 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 disinfectants 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 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.

NOTE 3 This method cannot be used to evaluate the activity of products against mycobacteria or bacterial spores.

## 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 (including Legionella), mycobactericidal, sporicidal, fungicidal and virucidal (including bacteriophages) 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 soiling (3,0 g/l bovine albumin).

**Table 1 — Obligatory and additional test conditions**

| Test Conditions | Bactericidal activity on porous surfaces without mechanical action in the veterinary area |
|---|---|
| Test organism<br>a) obligatory | *Enterococcus hirae*<br>*Proteus vulgaris*<br>*Pseudomonas aeruginosa*<br>*Staphylococcus aureus* |
| b) additional | any relevant test organism |
| Test temperature<br>a) obligatory | 10 °C ± 1 °C |
| b) additional | 4 °C ± 1 °C; 20 °C ± 1 °C; 40 °C ± 1 °C |
| Contact time<br>a) obligatory | 60 min ± 10 s |
| b) additional | 1 min ± 5 s, 5 min ± 10 s, 15 min + 10 s, 30 min ± 10 s; 120 min ± 10 s |
| Interfering substance<br>a) obligatory | 3,0 g/l bovine albumin |
| b) additional | any relevant substance |
| NOTE 1 The obligatory contact times 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.<br>NOTE 2 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 the drying time the test surface is immersed into a sample of the product as delivered and/or diluted with hard water (for ready to use products: water) ensuring that the test surface is totally covered for one minute. The test surface is removed from the product solution and 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 test surface is transferred to a neutraliser so that the action of the disinfectant is immediately neutralised. The bacteria are removed from the surface by ultrasound treatment. The numbers of surviving bacteria 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 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.

### 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 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.

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.

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

#### 5.2.2.2 Water

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

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

NOTE See 5.2.2.6 for the procedure to prepare hard water.

#### 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 |
| D(+)-Glucose | 2,5 g |
| Sodium chloride (NaCl) | 5,0 g |
| Agar | 15,0 g |
| Water (5.2.2.2) | to 1 000 ml |

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

In case of encountering problems with neutralisation (5.5.1.2 and 5.5.1.3), it may be necessary to add neutraliser to the TSA. Annex B gives guidance on the neutralisers that may be used. It is recommended not to use a neutraliser 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 |

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

#### 5.2.2.5 Neutraliser

The neutraliser shall be validated for the product being tested in accordance with 5.5.1.2, 5.5.1.3 and 5.5.2. The neutraliser shall be sterile. The neutraliser is added to diluent (5.2.2.4) and TSB (5.2.2.8).

NOTE Information on neutralisers 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. Sterilise by membrane filtration (5.3.2.7) or in the autoclave [5.3.2.1 a)]. Autoclaving – if used - may cause a loss of liquid. In this case make up to 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. Sterilise 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 °C ± 1 °C. 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 a different final water hardness in each test tube. In any case the final hardness expressed as calcium carbonate (CaC03) 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, detergents) shall be defined.

NOTE The term “interfering substance” is used even if it contains more than one substance.

##### 5.2.2.7.2 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).

Sterilise 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.

#### 5.2.2.8 Tryptone Soya Broth (TSB) with neutraliser

Tryptone Soya broth, consisting of:

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

Sterilise in the autoclave [5.3.2.1 a)]. After sterilisation the pH of the medium shall be equivalent to 7,3 ± 0,2 when measured at 20 °C ± 1 °C.

An adequate neutraliser shall be added according to its chemical properties before or after autoclaving (5.2.2.5). TSB with neutraliser should be filled into glass tubes in portions of 10 ml.

#### 5.2.3 Test surface[2)]

Poplar wood: Size: 10mm wide, 20 mm long and 0,6 mm −1,0 mm thick with visually smooth cut edges. Cut from sliced veneer, stored at least one year before use, from untreated wood of the European poplar tree.

Prior to use put the surfaces into a glass Petri dish in a single layer and sterilise in the autoclave [5.3.2.1 a)] for 15 min.

Test surfaces should be kept in a sterile vessel until use. The surfaces should be used only once.

### 5.3 Apparatus and glassware

#### 5.3.1 General

Sterilise 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)].

#### 5.3.2 Usual microbiological laboratory equipment[3)]

and in particular, the following:

##### 5.3.2.1 Apparatus for sterilisation (moist and dry heat)

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

b) for dry heat sterilisation, 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 bath,** capable of being controlled at 4 °C ± 1 °C, 10 °C ± 1 °C, 20 °C ± 1 °C, 40 °C ± 1 °C (5.5.1) and 45 °C ± 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 at 36 °C ± 1 °C or 37 °C ± 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 °C ± 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

2) DES-IN-TEST Supply Walbrunnenstrasse D-70599 Stuttgart Tel. ++49 (0) 711 45 54 06. This information is given for the convenience of the users of this European Standard and does not constitute an endorsement by CEN of this product.

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

#### 5.3.2.6 Shakers

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

b) Mechanical shaker.

**5.3.2.7 Membrane filtration apparatus,** constructed of a material compatible with the substances to be filtered with a filter holder of at least 50 ml volume and suitable for use with filters of diameter 47 mm to 50 mm and 0,45 µm pore size for sterilisation of hard water (5.2.2.6) and bovine albumin (5.2.2.7).

**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 °C ± 1 °C.

**5.3.2.14 Ultrasonic bath,** capable of operating at a frequency 30 kHz to 55 kHz, maximal output 1 000 W.

## 5.4 Preparation of test organism suspension and product test solutions

### 5.4.1 Test organism suspension (test and validation suspension)

NOTE Test and validation suspension are the same in this European Standard.

#### 5.4.1.1 General

For each test organism, one suspension shall 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.

#### 5.4.1.2 Preservation and stock cultures of test organisms

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

#### 5.4.1.3 Working culture of test organisms

In order to prepare the working culture of test organisms (5.2.1), prepare a subculture from the stock culture (5.4.1.2) 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.

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.

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.

#### 5.4.1.4 Test suspension ("N")

a) Take 10 ml of diluent (5.2.2.4) and place in a 100 ml flask with 5 g of glass beads (5.3.2.11). Take the working culture (5.4.1.3) 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 $1{,}5 \times 10^9$ cfu/ml[5)] to $5{,}0 \times 10^9$ cfu/ml using diluent (5.2.2.4), estimating the number of cfu/ml by any suitable means. Maintain this test suspension in the water bath at 20 °C ± 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 a spectrophotometer for adjusting the number of cells is highly recommended (about 620 nm wavelength – cuvette 10 mm path length). Each laboratory should therefore produce calibration data for each test organism. To achieve reproducible results of this measurement it may be necessary to dilute the test suspension.

NOTE A colourimeter is a suitable alternative.

c) For counting 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 the spread plate technique.

1) When using the pour plate technique, transfer each 1,0 ml sample into separate Petri dishes and add 15 ml to 20 ml melted TSA (5.2.2.3), cooled to 45 °C ± 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 5.4.1.5.

#### 5.4.1.5 Incubation and counting of the test suspension

a) Incubate (5.3.2.3) the plates for 20 h to 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 to 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 Vc values according to 5.6.2.2.

c) Calculate the numbers of cfu per 0,05 ml in the test suspension N using the methods given in 5.6.2.3. Verify according to 5.7.

NOTE 0,1 ml of an equal parts mixture of the test suspension and interfering substance is added to the surface therefore 0,05 ml of the suspension is added.

5) cfu/ml = colony forming unit per milliliter

#### 5.4.2 Product test solution

Product test solutions shall be prepared in hard water (5.2.2.6) at 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 (i.e. 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 homogenous 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 substance 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 contact time is ± 10 s.

c) interfering substance:

The obligatory interfering substance to be tested is 3,0 g/l bovine albumin (5.2.2.7.2) for 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 Neutralisation

To determine a suitable neutraliser carry out the validation of the dilution-neutralisation method (5.5.2.4 and 5.5.2.5 in connection with 5.5.2.6) using a suitable neutraliser, chosen according to laboratory experience and/or published data.

If this neutraliser is not valid, repeat the validation test using an alternative neutraliser taking into account the information given in Annex B.

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

#### 5.5.1.3 General instructions for the validation and control procedures

The neutralisation shall be controlled and validated - only for the highest product test concentration - for each of the test organisms and for each experimental condition (interfering substance, temperature, contact time). These procedures (water control, neutraliser control and method validation) shall be performed at the same time with the test and with the same neutraliser used in the test.

If, because of problems with neutralisation a neutraliser 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 neutraliser as well.

#### 5.5.1.4 Equilibration of temperature

Prior to testing, equilibrate the inoculated and dried test surfaces and all reagents (product test solutions (5.4.2) and 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 stabilised at θ.

The neutraliser (5.2.2.5), TSB with neutraliser (5.2.2.8) and diluent (5.2.2.4) shall be equilibrated at a temperature of 20 °C ± 1 °C.

### 5.5.2 Test procedure (Dilution-neutralisation 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 “Na” - 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 (5.4.1.4). Start the stopwatch (5.3.2.5) immediately, mix [5.3.2.6 a)] and place the tube in a water bath controlled 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 sterile Petri dishes (5.3.2.10) and ensure that the dishes are in a horizontal position. Prepare the test surfaces by inoculating 0,1 ml of the microbial test suspension [5.5.2.2 a)] on to each test surface. Dry the surfaces at 37 °C until they are visibly dry.

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

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 fan). For this reason, no time duration is given and the minimum required time for 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 (Na) pipette 20 ml of each product test solution (5.4.2) into separate sterile Petri dishes. Immerse the inoculated and dried test surfaces. Ensure that the test surfaces are completely covered by the product test solution. Start the stopwatch immediately and after 1 min ± 5 s transfer the surfaces into sterile Petri dish and place them on their edge against the side of the dish. See Annex C for diagrammatic representation.

Place the surfaces in a temperature controlled cabinet (5.3.2.13) at the chosen test temperature θ.

d) At the end of t, transfer each of the surfaces (Na) to a separate vessel containing 10 ml of TSB with neutraliser (5.2.2.8): Mix for 15 s [5.3.2.6 a)]. Place the vessels in a water bath controlled at 20 °C ± 1 °C. After 25 min ± 10 s mix [5.3.2.6 a)] again and place the vessels in an ultrasonic bath (5.3.2.14) with ice to maintain the temperature between at 0 °C and 4 °C. Treat the vessels with ultrasound for 5 min ± 10 s to remove bacterial cells from the test surface.

Prepare a series of 10-fold dilutions from $10^{-1}$ to $10^{-6}$ of the neutralised mixture in diluent (5.2.2.4). Take a 1,0 ml sample of the neutralised mixture and each of the dilutions in duplicate and inoculate using the 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 – 20 ml melted TSA (5.2.2.3), cooled to 45 °C ± 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) Perform the procedure a) to d) using the other product test solutions at the same time.

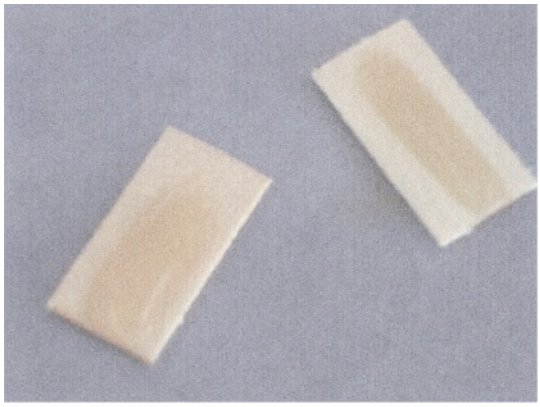
f) Perform the procedures a) to e) applying the other obligatory and - if appropriate - other additional experimental conditions (5.5.1.1).

#### 5.5.2.3 Water control “Nw”

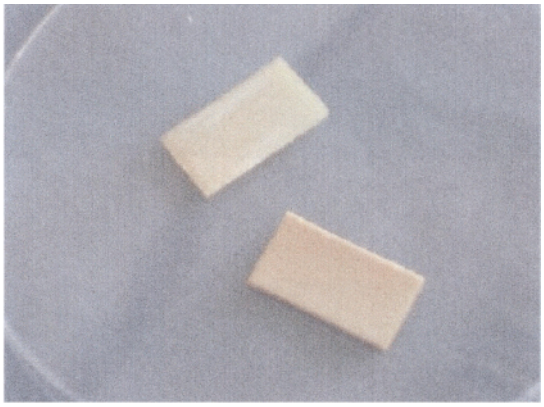
The procedure for determining the water control is as follows:

NOTE The control A - Experimental conditions control (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”.

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 0,1 ml of the microbial test suspension [5.5.2.2 a)] on to the test surface. Dry the surface at 37 °C until it is visibly dry [5.5.2.2 b)]. See Figure 1.



Inoculated test surfaces before drying



Inoculated test surfaces after drying

**Figure 1**

b) For the water control (Nw) pipette 20 ml of hard water (5.2.2.6) into a sterile Petri dish. Immerse the inoculated and dried test surface. Ensure that the test surface is completely covered by the hard water. Start the stopwatch immediately and after 1 min ± 5 s transfer the surface into a sterile Petri dish and place it on its edge against the side of the dish.

Place the surface in a temperature controlled cabinet (5.3.2.13) at the chosen test temperature θ.

c) At the end of the longest chosen t in the test, transfer the surface (Nw) into a vessel containing 10 ml of TSB with neutraliser (5.2.2.8): Mix for 15 s [5.3.2.6 a)]. Place the vessel in a water bath controlled at 20 °C ± 1 °C. After 25 min ± 10 s mix [5.3.2.6 a)] again and place the vessel in an ultrasonic bath (5.3.2.14) with ice to maintain the temperature between at 0 and 4 °C. Treat the vessel with ultrasound for 5 min ± 10 s.

Prepare a series of 10-fold dilutions from $10^{-1}$ to $10^{-6}$ of the neutralised mixture in diluent (5.2.2.4). Take 1,0 ml samples of the $10^{-4}$ to $10^{-6}$ dilutions in duplicate and inoculate using the pour plate or spread plate technique [5.5.2.2 d)].

For Incubation and counting see 5.5.2.6.

#### 5.5.2.4 Neutraliser control “B” - verification of the absence of toxicity of the neutraliser

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

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

b) Pipette 10 ml of TSB with neutraliser (5.2.2.8) into a vessel, add an inoculated and dried test surface, mix [5.3.2.6 a)] and place the tube in a water bath controlled at 20 °C ± 1 °C for 25 min ± 10 s.

Just before the end of this time, mix [5.3.2.6 a)] again and place the tube in an ultrasonic bath (5.3.2.14) (maintaining the temperature in the ultrasonic bath at 0 °C – 4 °C by addition of ice) and treat it with ultrasound for 5 min ± 10 s.

At the end of this time prepare a series of 10-fold dilutions from $10^{-1}$ to $10^{-6}$ of this mixture “B” in 9,0 ml diluent (5.2.2.4). Take a sample of 1,0 ml of the dilutions $10^{-4}$ to $10^{-6}$ 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-neutralisation validation

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

a) Prepare one inoculated test surface as in [5.5.2.2 a) and b)] and also one uninoculated surface.

b) Pipette 20 ml of the highest concentration of the product test solution used in the test (5.5.2.2) into a sterile Petri dish. Immerse the one sterile uninoculated test surface. Ensure that the test surface is completely covered by the product test solution. After 1 min ± 10 s transfer the surface into a sterile Petri dish and place it on its edge against the side of the dish.

   Place the surface in a temperature controlled cabinet (5.3.2.13) at the chosen test temperature θ.

c) At the end of t, transfer the surface into a tube containing 10 ml TSB with neutraliser (5.2.2.8) mix [5.3.2.6 a)] and place the tube in a water bath controlled at 20 °C ± 1 °C for 25 min ± 10 s. Just before the end of this time add the inoculated test surface [5.5.2.2 a)], mix [5.3.2.6 a)] and place the tube in an ultrasonic bath (maintaining the temperature in the ultrasonic bath at 0 °C – 4 °C by addition of ice) and treat it with ultrasound for 5 min ± 10 s.

At the end of this time prepare a series of 10-fold dilutions of the mixture in diluent (5.2.2.4) to produce $10^{-1}$ to $10^{-6}$ dilutions. Take a 1,0 ml sample of the $10^{-4}$ to $10^{-6}$ 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.

#### 5.5.2.6 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 at 37 °C ± 1 °C. Discard any plates which are not countable (for any reason). Count the plates and determine the total 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 Vc values according to 5.6.2.2.

c) Calculate the numbers of cfu/surface in the test mixture Na, in the water control Nw 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.

## 5.6 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, Na represents the bactericidal test mixture, Nw represents the test mixture in the water control, B (neutraliser control) and C (method validation) represent the different control test mixtures.

NOTE 1 The control A - Experimental conditions control (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 0,05 ml of the bacterial test suspension and the validation suspension Nv; Na, Nw and B, C represent the number of cells counted per surface in the different test mixtures.

**Table 2 — Number of cells per 0,05 ml or per surface**

| | Number of cells per 0,05 ml in the test suspension | Number of survivors per surface at the end of the contact time t |
|---|---|---|
| Test | *N* | *Na* |
| Controls | *N, Nv* | *Nw, B, C* |

NOTE 2 N is calculated per 0,05 ml, N and Nv are the same in this European Standard.

#### 5.6.1.2 Vc values

All experimental data are reported as Vc values:

— in the method (test and controls), a Vc 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 Vc values, the second the calculation of N, Na, Nw, B and C. The third step is the calculation of the reduction R (5.8).

#### 5.6.2.2 Determination of Vc values

a) The usual limits for counting bacteria on agar plates are between 15 and 300 colonies. 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 Vc value of 16. The upper limit (330) reflects 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 Vc 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 Vc 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 Vc value as “more than sum of the counts”, e.g. for “>330, 280, 305” report “>915”.

If a Vc value is lower than 14, report the number (but substitute by ” < 14” for further calculations in the case of Na).

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

#### 5.6.2.3 Calculation of N

N is the number of cells per 0,05 ml in the microbial test suspension [5.5.2.2 a)]. It is less than the number in the bacterial test suspension due to dilution by adding equal parts interfering substance and using 0,1 ml for the inocula.

Since two dilutions of the microbial test suspension (5.5.2.2) are evaluated, calculate the number of cfu/0,05 ml as the weighted mean count using the following formula:

$$N = \frac{0,05 \times c}{(n1 + 0,1 \times n2) \times 10^7}$$

where

- $c$ is the sum of the Vc values taken into account;
- $n1$ is the number of Vc values taken into account in the lower dilution, i.e. $10^{-7}$;
- $n2$ is the number of Vc values taken into account in the higher dilution, i.e. $10^{-8}$;
- $10^7$ 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/0,05 ml is expressed by a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

EXAMPLE

$$N = \frac{(168 + 213 + 20 + 25) \times 0,05}{2,2 \times 10^{-7}} = \frac{426 \times 0,05 \times 10^7}{2,2} = 9,68 \times 10^7$$

#### 5.6.2.4 Calculation of Na and Nw

Na is the lg number of survivors per test surface (5.5.2.2) at the end of the contact time and before neutralisation. It is tenfold higher than the Vc values due to the addition of neutraliser.

Nw is the lg number of survivors per water control surface (5.5.2.3) at the end of the contact time and before neutralisation. It is tenfold higher than the Vc values due to the addition of neutraliser.

Calculate the mean for each dilution step Na and Nw using following formula:

$$Na\,(or Nw) = \frac{c \times 10}{n \times d}$$

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 taken into account.

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

Examples:

a) duplicate Vc values Na: 0, 0 (from the neutralised mixture, $10^0$)

$$Na = \frac{(<14 + <14)}{2} \times 10 = <140 = <1,40 \times 10^2; \quad \lg(<1,40 \times 10^2) = <2,15$$

b) duplicate Vc values Na: 2, 16 (from the neutralised mixture, $10^0$)

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

c) duplicate Vc values (two spread plates per 1,0 ml sample from the neutralised mixture, $10^0$): > 330, 330; 310, 290

$$Na = \frac{(>660 + 600)}{2} \times 10 = >6\,300 = >6,3 \times 10^3; \quad \lg(>6,3 \times 10^3) = >3,80$$

d) duplicate Vc values Na-1: 40, 46 (from the $10^{-1}$ dilution)

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

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

Exceptions and rules for special cases:

d1 If one or both duplicate Vc values in three or more subsequent dilutions of Na are within the counting limits (e. g. $Na^{-2}$: 17, 23; $Na^{-1}$: 120, 135; $Na^0$: 308, > 330) the whole test is invalid (5.7.1).

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

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

where

*c* is the sum of Vc 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 neutraliser control (5.5.2.4) and method validation (5.5.2.5). It is tenfold higher than the Vc values due to the addition of neutraliser.

Calculate B and C using the following formula:

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

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 taken in to account.

If two subsequent dilutions of B or C show duplicate Vc values within the counting limits calculate B or C as the weighted mean using the following formula:

$$B\left(orC\right)=\frac{c\times 10}{2{,}2\times 10^{z}}$$

where

*c* is the sum of Vc 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;

— it is not invalidated by a result described under 5.6.2.4 d) first special case (d1).

### 5.7.2 Control of weighted mean counts

For results calculated by weighted mean of two subsequent dilutions 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 (330) are taken as the upper limit number.

Example for N:

$10^{-7}$ dilution: 168 + 215 cfu, $10^{-8}$ dilution: 20 + < 14 cfu; (168 + 215) / (20 + 14) = 383/34 = 11,26 = between 5 and 15.

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

### 5.7.3 Basic limits

For each test organism check that:

a) N is between $7{,}5 \times 10^{7}$ cfu/ml and $2{,}5 \times 10^{8}$ cfu/ml ($7{,}88 \leq \lg N \leq 8{,}4$);

NOTE N is calculated for 0,05 ml.

b) $\lg Nw \geq 6{,}5$;

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

d) $C \geq 0,5 \times Nw$;

e) 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) is the ratio between Nw and Na.

The reduction is usually expressed as a logarithm.

$lg\ R = lg\ Nw - lg\ Na$

For each test organism record the number of cfu/surface in the test procedure for bactericidal activity of the product Na (5.5.2.2) and in the control procedure Nw (5.5.2.3).

For each product concentration and each experimental condition, calculate and record the decimal log reduction (lg) separately.

For validation of the dilution-neutralisation method record the number of cfu/ml in the neutraliser toxicity control B (5.5.2.4) and the dilution-neutralisation 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 in 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 1 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.

NOTE 2 To calculate the mean all lg values are converted to real numbers, e.g. 5,2 lg to about 158,000. The mean is the arithmetic mean of these converted numbers.

## 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 16437 standard if it demonstrates in a valid test at least a 4 lg reduction within 60 min at 10 °C with 3,0 g/l bovine albumin 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 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 16437).

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

a) identification of the testing laboratory;

b) identification of the client;

c) identification of the sample:

   1) name of the product;

   2) batch number and – 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 neutraliser 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) neutraliser;

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 |

Abbreviations:

| | |
|---|---|
| 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)

# Neutralisers - Examples of neutralisers of the residual antimicrobial activity of chemical disinfectants and antiseptics

**Important** Neutralisers of the residual antimicrobial activity of chemical disinfectants and antiseptics shall be validated according to the prescriptions of the standard

| **Antimicrobial agent** | **Chemical compounds able to neutralise residual antimicrobial activity** | **Examples of suitable neutralisers** [a] |
|---|---|---|
| Quaternary ammonium compounds and fatty amines<br>Amphoteric compounds | Lecithin, Saponin, Polysorbate 80, Sodium dodecyl sulphate, Ethylene oxide condensate of fatty alcohol (non-ionic surfactants) [b] | −30 g/l Polysorbate 80 + 30 g/l saponin + 3g/l.lecithin.<br>−30 g/l Polysorbate 80 + 4 g/l sodium dodecyl sulphate + 3g/l lecithin.<br>−3 g/l Ethylene oxide condensate of fatty alcohol + 20g/l lecithin + 5g/l polysorbate 80. |
| Biguanides and similar compounds | Lecithin [c], Saponin, Polysorbate 80 | −30 g/l Polysorbate 80 + 30 g/l saponin + 3g/l lecithin. |
| Oxidising compounds<br>(Chlorine, iodine, hydrogen peroxide, peracetic acid, hypochlorites, etc…) | Sodium thiosulphate [d]<br>Catalase [for hydrogen peroxide or products releasing hydrogen peroxide] | −3 g/l to 20 g/l Sodium thiosulphate + 30 g/l polysorbate 80 + 3 g/l lecithin.<br>−50 g/l Polysorbate 80 + 0,25g/l catalase + 10 g/l lecithin. |
| Aldehydes | L-histidine<br>Glycine | −30 g/l Polysorbate 80 + 3g/l lecithin + 1g/l L-histidine (or + 1 g/l glycine).<br>−30 g/l Polysorbate 80 + 30 g/l saponin + 1 g/l L-histidine (or + 1 g/l glycine). |
| Phenolic and related compounds: orthophenylphenol, phenoxyethanol, triclosan, phenylethanol, etc…<br>Anilides | Lecithin<br>Polysorbate 80<br>Ethylene oxide condensate of fatty alcohol[b] | −30 g/l Polysorbate 80 + 3g/l lecithin.<br>−7 g/l Ethylene oxide condensate of fatty alcohol + 20 g/l lecithin + 4 g/l polysorbate 80. |
| Alcohols | Lecithin, Saponin, Polysorbate 80 [e] | −30 g/l Polysorbate 80 + 30 g/l saponin +3 g/l lecithin. |

[a] According to the pH of the tested product, the pH of the neutraliser 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 ± 0,2 with sodium hydroxide (NaOH) 1 mol/l; distilled water up to 1 000 ml].

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

[c] Egg and soya; egg is preferable.

[d] The toxic effect of sodium thiosulphate differs from one microorganism to another.

[e] For the neutralisation 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

NOTE 1 Other neutraliser mixtures may be required for products containing more than one antimicrobial agent.

NOTE 2 The concentration of the various neutralising compounds or of the neutraliser as such may not be adequate to neutralise high concentrations of the product.

# Annex C
(informative)

## Graphical representations of dilution-neutralisation method

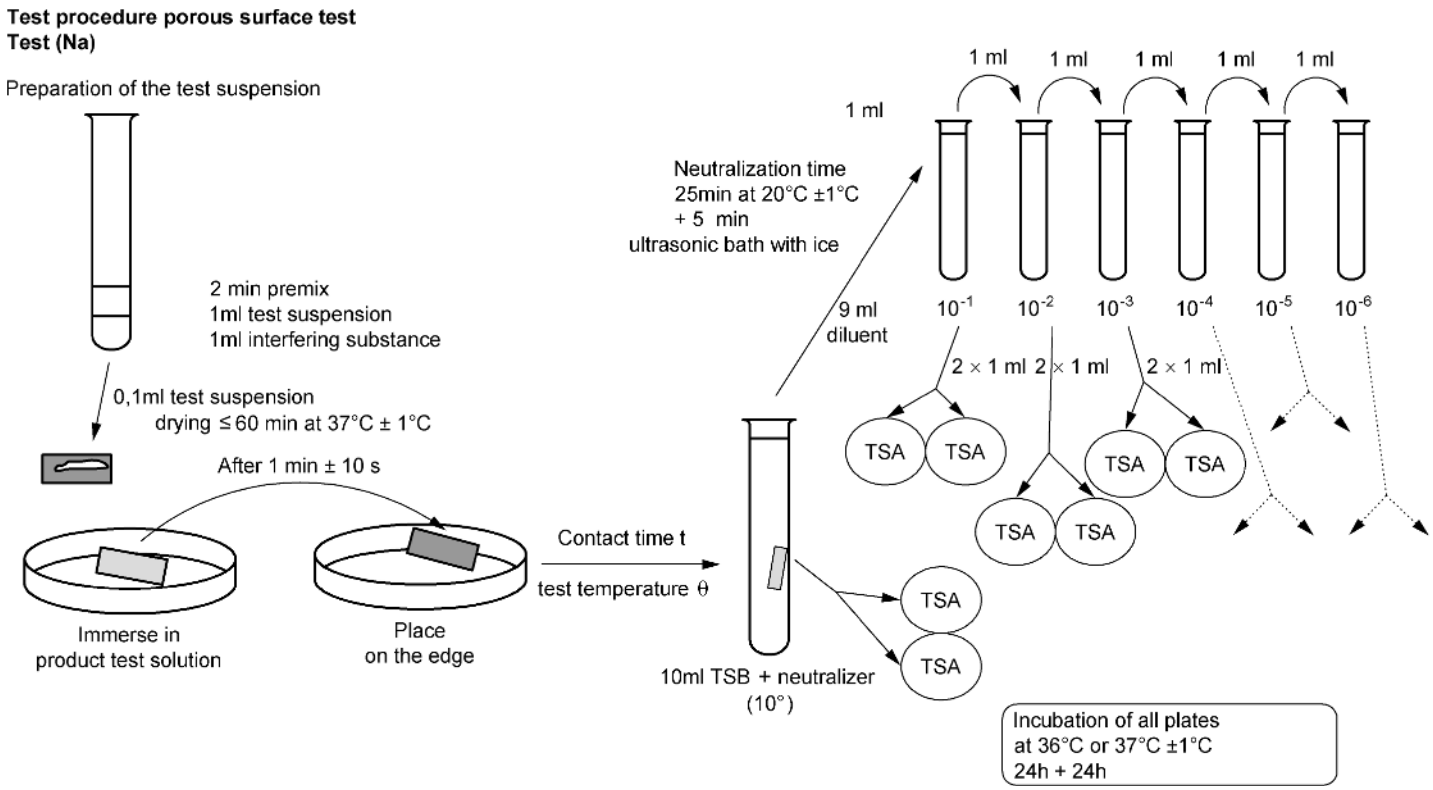



**Figure C.1 —Test (Na)**

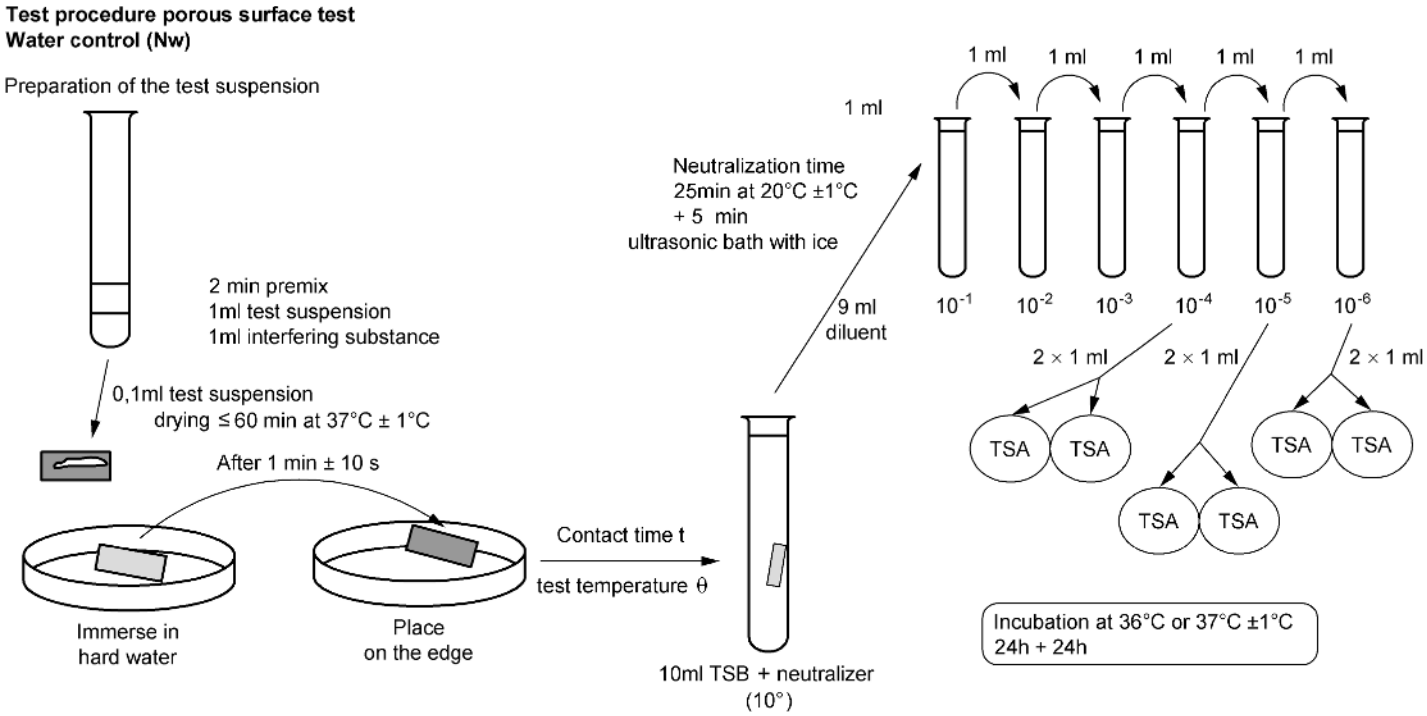



**Figure C.2 —Water control Nw**

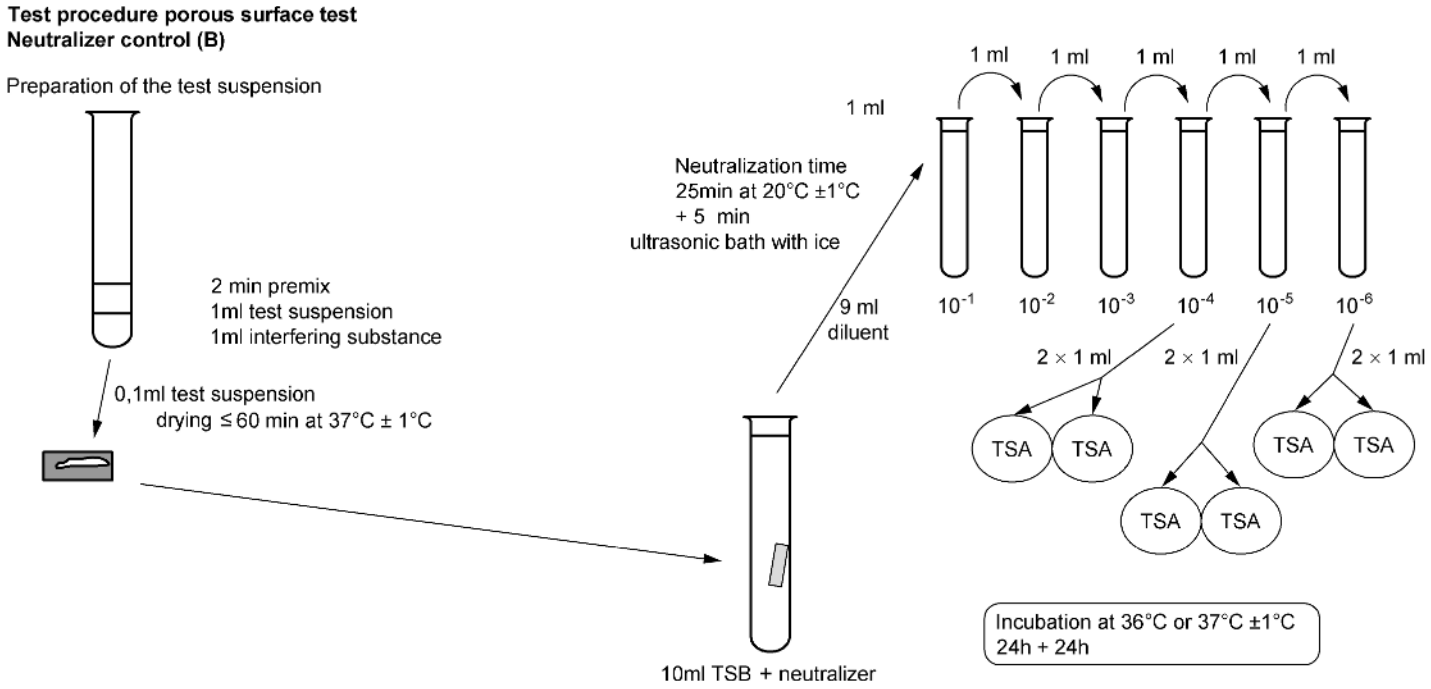



**Figure C.3 — Neutralizer control (B)**

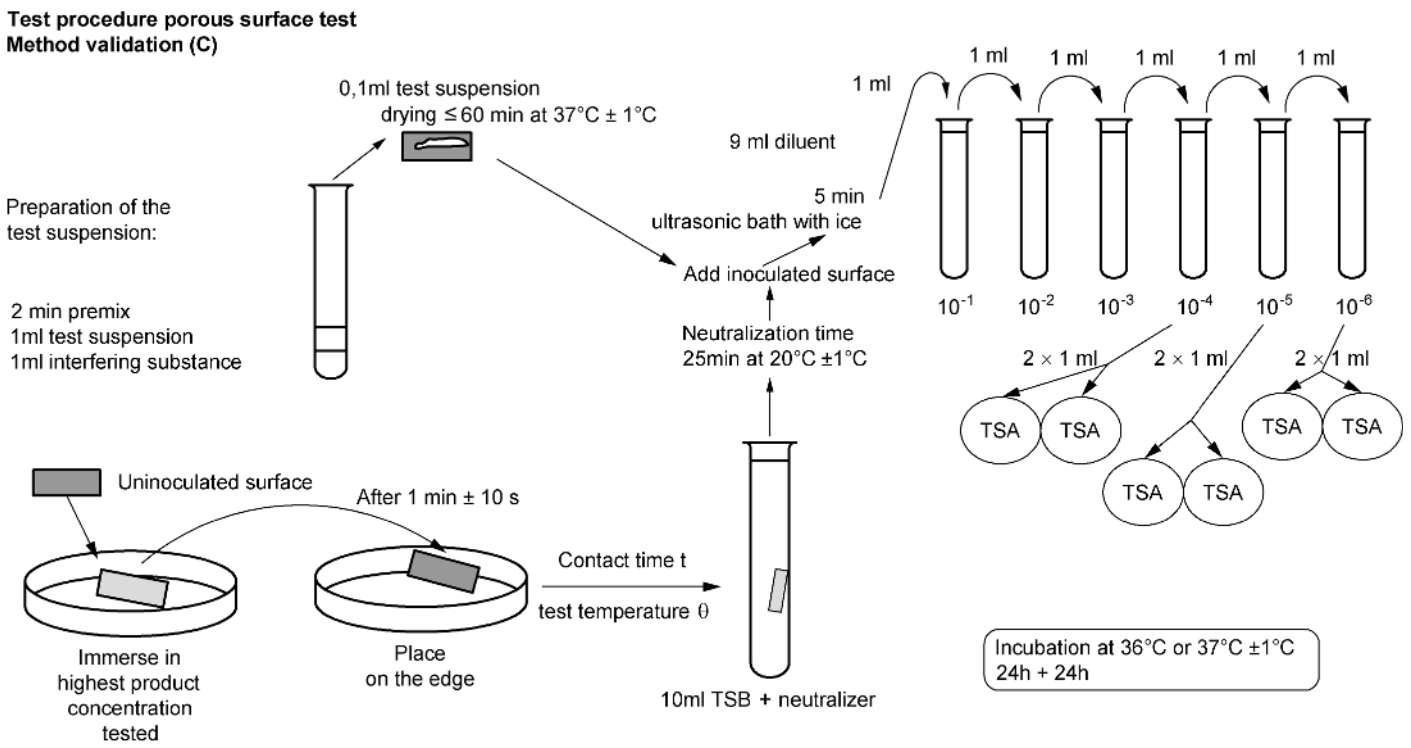



**Figure C.4 — Method validation (C)**

# 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 16437**, BACTERICIDAL ACTIVITY**

**(obligatory and additional conditions)**

**Client:** Centipede Formulations Inc., Markkleeberg / Euroland

Determination of veterinary bactericidal activity in low soiling conditions:

a) Identification of the test Laboratory;

b) Identification of the sample:

| | |
|---|---|
| Name of the product ..... | Z |
| Batch number ..... | 10–47–013 |
| Manufacturer | |
| Date of delivery ..... | 2010–10–06 |
| Expiry date …… | 2013–01–18 |
| Storage conditions ..... | Laboratory storage conditions |
| Active substance(s) and its/their concentrations..... | Not indicated |

c) Test method and its validation:

| | |
|---|---|
| Method..... | Dilution neutralisation |
| Neutraliser..... | 30 g/l lecithin, sterilised in the autoclave |

d) Experimental conditions:

| | |
|---|---|
| Period of analysis ..... | 2010–10–06 to 2010–11–06 |
| Appearance of the product and its dilutions ..... | Colourless clear liquid, soluble in hard water |

| | |
|---|---|
| Product test concentrations | 5 ml/l (0,5 %), 7,5 ml/l (0,75 %), 10 ml/l (1 %) |
| Test temperature ..... | 10 °C ± 1 °C |
| Contact time ..... | 30 min ± 10 s |
| Interfering substance ..... | 3,0 g/l bovine albumin |
| Product diluent ..... | Hard water |
| Temperature of incubation ..... | 37 °C ± 1 °C |
| Counting procedure ..... | Pour plate |
| Bacterial strains used ..... | *Enterococcus hirae* ATCC 10541 |
| | *Proteus vulgaris* ATCC 13315 |
| | *Pseudomonas aeruginosa* ATCC 15442 |
| | *Staphylococcus aureus* ATCC 6538 |
| Drying time of inoculated test surfaces | 45 min |

e) Test results;

f) Conclusion:

According to EN 16437 the batch 10–47–013 of the product Z possesses bactericidal activity for the referenced strains of *Enterococcus hirae, Proteus vulgaris, Pseudomonas aeruginosa* and *Staphylococcus aureus* at a concentration of X % (v/v) at 10 °C with a 60 min contact time and under simulated soiling conditions.

g) Locality, date and identified signature.

**Test results (bactericidal quantitative carrier test)**

EN 16437 (Phase 2. step.2**)** Product-name**:** .***Z***.. Batch No: ..***91–71–61***..

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 ☒ Number of plates **3 / ml** Neutraliser: .***Lecithin 3,0 g/l in diluent***.

**……..**

Test temperature: ***10°C*** **Interfering substances**: .*bovine albumin 3 g /l……*

Test organism: ***P. aeruginosa ATCC 10231***. Drying time on carrier: ***40*** **min** Incubation temp.: ***36°C***
**……**(not > 60 min)

Internal lab. no: **QS..*58/00***.... Date of test:***2010–11–06.*** Responsible person: ***Fang***... Signature:.. ***Fang***....

**Validation and controls**

| Test suspension **(N)** | | | | | Neutraliser control (**B**) | | | | | Method validation (**C**) Product conc.: *1,0 ml/l* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Counts per plate | | $Vc_1$ | $Vc_2$ | | Counts per plate | | $Vc_1$ | $Vc_2$ | | Counts per plate | | $Vc_1$ | $Vc_2$ |
| $10^{-7}$ | *50+49+46* | *51+48+50* | *145* | *149* | $10^{-4}$ | *69+75+73* | *84+62+76* | *217* | *222* | $10^{-4}$ | *59+78+63* | *65+68+70* | *200* | *203* |
| $10^{-8}$ | *5+8+6* | *8+7+6* | *19* | *21* | $10^{-5}$ | *11+7+8* | *9+6+7* | *26* | *22* | $10^{-5}$ | *5+7+6* | *7+8+5* | *18* | *20* |
| $\bar{x}$ wm = $1,5 \times 10^9 \times 0,05 = 7,6 \times 10^7$<br>lg $6,6 \times 10^7 = 7,88$ | | | | | $\bar{x}$ wm = $2,2 \times 10^7$<br>lg $2,2 \times 10^7 = 7,34$ | | | | | $\bar{x}$ wm = $2,0 \times 10^7$<br>lg $2,0 \times 10^7 = 7,30$ | | | | |
| 7,88 ≤ lgN ≤ 8,40 ?<br>☒ yes ☐ no | | | | | lgB ≥ 0,5 x Nw?<br>☒ yes ☐ no | | | | | lgC ≥ 0,5 x Nw?<br>☒ yes ☐ no | | | | |

**Water control**

| Water control **(Nw):** | | Counts per plate | | $Vc_1$ | $Vc_2$ | $\bar{x}$ wm = *1,4 × 10⁷ lg 1,4 × 10⁷ = 7,15*<br>lg Nw ≥ 6,5 ?<br>☒ yes ☐ *no* |
|---|---|---|---|---|---|---|
| | $10^{-4}$ | *42+35+62* | *55+46+40* | *139* | *141* | |
| | $10^{-5}$ | *4+7+5* | *4+4+6* | *16* | *14* | |

**Test**

| Conc. of the product % | Dilution step | Counts per plate | | $Vc_1$ | $Vc_2$ | **lg Na** = lg ($\bar{x}$ or $\bar{x}$ wm) | **lg R** (lg Nw = *7,15*) | Contact time (min) |
|---|---|---|---|---|---|---|---|---|
| *0,5 %* | $10^{0}$ | *> 330+ > 330+ > 330* | *> 330+ > 330+ > 330* | *> 990* | *> 990* | *5,04* | *2,11* | *60* |
| | $10^{-1}$ | *> 330+ > 330+ > 330* | *324+305+329* | *> 990* | *958* | | | |
| | $10^{-2}$ | *39+40+45* | *20+33+42* | *124** | *95** | | | |
| *0,75 %* | $10^{0}$ | *> 330+ > 330+ > 330* | *> 330+ > 330+ > 330* | *> 990* | *> 990* | *4,48* | *2,67* | *60* |
| | $10^{-1}$ | *96+110+92* | *106+111+95* | *298** | *312** | | | |
| | $10^{-2}$ | *8+8+11* | *8+12+11* | *27** | *31** | | | |
| *1,0 %* | $10^{0}$ | *3+0+2* | *1+1+3* | *< 14** | *< 14** | *< 2,15* | *> 5,0* | *60* |
| | $10^{-1}$ | *0+0+0* | *0+0+0* | *< 14* | *< 14* | | | |
| | $10^{-2}$ | *0+0+0* | *0+0+0* | *< 14* | *< 14* | | | |

**Remarks**: *used for calculation

Explanations:

Vc = count per ml (one plate or more)

R = reduction (lg R = lg Nw – lg Na)

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

If Na < 140, lg R = > [lg Nw – 2,15]

$\bar{x}$wm = weighted mean of $\bar{x}$ (see 5.6.2 - 5.7 for calculation rules)

# Bibliography

[1] EUROPEAN PHARMACOPOEIA (EP). Edition 1997 supplement 2000, Water for injections

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