

# **SLOVENSKI STANDARD**

## **SIST EN 13727:2012+A2:2016**

**01-februar-2016**

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**Kemična razkužila in antiseptiki - Kvantitativni suspenzijski preskus za vrednotenje baktericidnega delovanja kemičnih razkužil in antiseptikov v humani medicini - Preskusna metoda in zahteve (faza 2, stopnja 1)**

Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of bactericidal activity in the medical area - Test method and requirements (phase 2, step 1)

Chemische Desinfektionsmittel und Antiseptika - Quantitativer Suspensionsversuch zur Bestimmung der bakteriziden Wirkung im humanmedizinischen Bereich - Prüfverfahren und Anforderungen (Phase 2, Stufe 1)

Antiseptiques et désinfectants chimiques - Essai quantitatif de suspension pour l'évaluation de l'activité bactéricide en médecine - Méthode d'essai et prescriptions (Phase 2, Étape 1)

**Ta slovenski standard je istoveten z: EN 13727:2012+A2:2015**

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**ICS:**

11.080.20      Dezinfektanti in antiseptiki      Disinfectants and antiseptics

**SIST EN 13727:2012+A2:2016**

**en,fr,de**

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EUROPEAN STANDARD  
NORME EUROPÉENNE  
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**Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of bactericidal activity in the medical area - Test method and requirements (phase 2, step 1)**

Antiseptiques et désinfectants chimiques - Essai quantitatif de suspension pour l'évaluation de l'activité bactéricide en médecine - Méthode d'essai et prescriptions (Phase 2, Étape 1)

Chemische Desinfektionsmittel und Antiseptika - Quantitativer Suspensionsversuch zur Bestimmung der bakteriziden Wirkung im humanmedizinischen Bereich - Prüfverfahren und Anforderungen (Phase 2, Stufe 1)

This European Standard was approved by CEN on 14 October 2013 and includes Amendment 2 approved by CEN on 3 August 2015.

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

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

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

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## European foreword

This document (EN 13727:2012+A2:2015) 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 April 2016, and conflicting national standards shall be withdrawn at the latest by April 2016.

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 includes Amendment 1 approved by CEN on 2013-10-14 and Amendment 2 approved by CEN on 2015-08-03.

This document supersedes <sup>A2</sup> EN 13727:2012+A1:2013 <sup>A2</sup>.

The start and finish of text introduced or altered by amendment is indicated in the text by tags <sup>A1</sup> <sup>A1</sup> and <sup>A2</sup> <sup>A2</sup>.

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association, and supports essential requirements of EU Directive(s).

For relationship with EU Directive(s), see informative Annex ZA, which is an integral part of this document.

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<sup>A2</sup> *deleted text* <sup>A2</sup>

<sup>A1</sup> Data obtained using the former version of EN 13727 may still be used, if a neutralization time of 10 s for all products with contact times of 10 min or shorter has been demonstrated to be sufficient. Data obtained by using the prEN 12054 should not be used as this project was abandoned in 2001. <sup>A1</sup>

According to the CEN/CENELEC Internal Regulations, the national standards organizations 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 suspension test for establishing whether a chemical disinfectant or an antiseptic has a 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. Each utilization concentration of the chemical disinfectant or antiseptic found by this test corresponds to the chosen experimental conditions.

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## 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. Products can only be tested at a concentration of 80 % or less (97 % with a modified method for special cases) as some dilution is always produced by adding the test organisms and interfering substance.

This European Standard applies to products that are used in the medical area in the fields of hygienic handrub, hygienic handwash, surgical handrub, surgical handwash, instrument disinfection by immersion, and surface disinfection by wiping, spraying, flooding or other means.

This European Standard applies to areas and situations where disinfection or antiseptics is medically indicated. Such indications occur in patient care, for example:

- in hospitals, in community medical facilities and in dental institutions;
- in clinics of schools, of kindergartens and of nursing homes;

and may occur in the workplace and in the home. It may also include services such as laundries and kitchens supplying products directly for the patients.

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 1 test.

NOTE 3 This method cannot be used to evaluate the activity of products against *Legionella* in watersystems against mycobacteria and against bacterial spores.

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

## 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 5 decimal log (lg) reduction (for hygienic hand wash at least a 3 lg reduction), when tested in accordance with Table 1 and Clause 5.



Table 1 — Minimum and additional test conditions

Test Conditions	Hygienic handrub and handwash	Surgical handrub and handwash	Instrument disinfection	Surface disinfection
Minimum spectrum of test organisms	<i>P. aeruginosa</i> , <i>S. aureus</i> , <i>E. hirae</i> , <i>E. coli</i> K12	<i>P. aeruginosa</i> , <i>S. aureus</i> , <i>E. hirae</i> , <i>E. coli</i> K12	<i>P. aeruginosa</i> , <i>S. aureus</i> , <i>E. hirae</i> , when temperature is 40 °C or higher: only <i>E. faecium</i>	<i>P. aeruginosa</i> , <i>S. aureus</i> , <i>E. hirae</i>
additional	Any relevant test organism			
Test temperature	according to the manufacturer's recommendation, but between			
	20 °C and 20 °C	20 °C and 20 °C	20 °C and 70 °C	4°C and 30 °C
Contact time	according to the manufacturer's recommendation			
	but between		but no longer than	
	30 s and 60 s	1 min and 5 min	60 min	5 min or 60 min <sup>a</sup>
Interfering substance	<div style="text-align: center;"> <p>ITeH STANDARD PREVIEW</p> <p>(standards.iteh.ai)</p> <p>SIST EN 13727:2012+A2:2016</p> <p><a href="https://standards.iteh.ai/catalog/standards/sist/af43fb4f-da43-4e70-b7b2-f13db7eafa2e/sist-en-13727-2012a2-2016">https://standards.iteh.ai/catalog/standards/sist/af43fb4f-da43-4e70-b7b2-f13db7eafa2e/sist-en-13727-2012a2-2016</a></p> </div>			
clean conditions	0,3 g/l bovine albumin solution (hygienic handrub)	0,3 g/l bovine albumin solution (surgical handrub)	0,3 g/l bovine albumin solution and/or	0,3 g/l bovine albumin solution and/or
dirty conditions	3,0 g/l bovine albumin solution plus 3,0 ml/l erythrocytes (hygienic handwash) <sub>c</sub>	3,0 g/l bovine albumin solution plus 3,0 ml/l erythrocytes (surgical handwash) <sub>c</sub>	3,0 g/l bovine albumin solution plus 3,0 ml/l erythrocytes	3,0 g/l bovine albumin solution plus 3,0 ml/l erythrocytes
additional	clean or dirty; any relevant substance	clean or dirty; any relevant substance	any relevant substance	any relevant substance

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

<sup>a</sup> The contact times for surface disinfectants stated in this table are chosen on the basis of the practical conditions of the product. The recommended contact time for the use of the product is within the responsibility of the manufacturer. Products intended to disinfect surfaces that are likely to come into contact with the patient and / or the medical staff and surfaces, which are frequently touched by different people, leading to the transmission of microorganisms to the patient, shall be tested with a contact time of maximum 5 min. The same applies where the contact time of the product shall be limited for practical reasons. Products for other surfaces than stated above may be tested with a contact time of maximum 60 min.

<sup>b</sup> hygienic and surgical handrub shall be tested as a minimum under clean conditions.

<sup>c</sup> hygienic and surgical handwash shall be tested as a minimum under dirty conditions.

## 5 Test method

### 5.1 Principle

**5.1.1** A sample of the product as delivered and/or diluted with hard water (or water for ready to use products) is added to a test suspension of bacteria in a solution of an interfering substance. The mixture is maintained at one of the temperatures and the contact times specified in Clause 4 and 5.5.1.1. At the end of this contact time, an aliquot is taken; the bactericidal and/or the bacteriostatic action in this portion is immediately neutralized or suppressed by a validated method. The method of choice is dilution-neutralization. If a suitable neutralizer cannot be found, membrane filtration is used. The numbers of surviving bacteria in each sample are determined and the reduction is calculated.

**NOTE** Handwash products are always prediluted with hard water (5.2.2.7). The resulting solution is regarded as a ready-to-use product (5.4.2).

**5.1.2** The test is performed using *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus hirae* and for certain product types *Escherichia coli* K12 as test-organisms (Clause 4, Table 1).

**5.1.3** Other contact times and temperatures within the limits specified in Clause 4, Table 1 may be used. Additional interfering substances and test organisms may be used.

### 5.2 Materials and reagents

#### 5.2.1 Test organisms

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The bactericidal activity shall be evaluated using the following strains as test organisms selected according to Clause 4 (Table 1)<sup>1)</sup>:

- a) *Escherichia coli* K12, NCTC 10538
- b) *Pseudomonas aeruginosa*, ATCC 15442
- c) *Staphylococcus aureus*, ATCC 6538
- d) *Enterococcus hirae*, ATCC 10541
- e) *Enterococcus faecium*, ATCC 6057

**NOTE** See Annex A for strain reference 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.

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<sup>1)</sup> The NCTC and ATCC numbers are the collection numbers of strains supplied by these culture collections. 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.

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

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

All specified pH values are measured at  $20\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ .

### 5.2.2.2 Water

The water shall be freshly glass-distilled water and not demineralized 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 See 5.2.2.7 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
Sodium chloride (NaCl)	5,0 g
Agar	15,0 g
Water (5.2.2.2)	to 1 000,0 ml

Sterilize in the autoclave [5.3.2.1 a)]. After sterilization the pH (5.3.2.4) of the medium shall be equivalent to  $7,2 \pm 0,2$ .

NOTE In case of encountering problems with neutralization (5.5.1.2 and 5.5.1.3) it may be necessary to add neutralizer to 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	8,5 g
Water (5.2.2.2)	to 1 000,0 ml

Sterilize in the autoclave [5.3.2.1 a)]. After sterilization, the pH (5.3.2.4) of the diluent shall be equivalent to  $7,0 \pm 0,2$ .

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**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. It 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 Rinsing liquid (for membrane filtration)**

The rinsing liquid shall be validated for the product being tested in accordance with 5.5.1.2, 5.5.1.3 and 5.5.3. It shall be sterile, compatible with the filter membrane and capable of filtration through the filter membrane under the test conditions described in 5.5.3.

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

**5.2.2.7 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 ( $\text{MgCl}_2$ ) and 46,24 g calcium chloride ( $\text{CaCl}_2$ ) in water (5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration (5.3.2.7) or in the autoclave [5.3.2.1 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 ( $\text{NaHCO}_3$ ) in water (5.2.2.2) and dilute to 1000 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 (5.3.2.9) 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 (5.3.2.4) of the hard water shall be  $7,0 \pm 0,2$ . (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 ( $\text{NaOH}$ ) or approximately 36,5 g/l (about 1 mol/l) of hydrochloric acid ( $\text{HCl}$ ).

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 ( $\text{CaCO}_3$ ) is lower than 375 mg/l in the test tube.

**5.2.2.8 Interfering substance****5.2.2.8.1 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 10 times its final concentration in the test (50 times in the case of the modified method, 5.2.2.8.4).

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.

#### 5.2.2.8.2 Clean conditions (bovine albumin solution – low concentration)

Dissolve 0,30 g of bovine albumin fraction V (suitable for microbiological purposes) in 100 ml of diluent (5.2.2.4).

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) shall be 0,3 g/l ;

#### 5.2.2.8.3 Dirty conditions (Mixture of bovine albumin solution – high concentration with sheep erythrocytes)

Dissolve 3,00 g of bovine albumin fraction V (suitable for microbiological purposes) in 97 ml of diluent (5.2.2.4).

Sterilize by membrane filtration (5.3.2.7).

Prepare at least 8,0 ml fresh defibrinated sheep blood (5.2.2.9). Centrifuge the erythrocytes at 800 *g*<sub>N</sub> for 10 min (5.3.2.13). After discarding the supernatant, resuspend erythrocytes in diluent (5.2.2.4). Repeat this procedure at least 3 times, until the supernatant is colourless.

Resuspend 3 ml of the packed sheep erythrocytes in the 97 ml of sterilized bovine albumin solution (see above). To avoid later contamination this mixture should be split in portions probably needed per day and kept in separate containers for a maximum of 7 days in a refrigerator (5.3.2.8).

The final concentration of bovine albumin and sheep erythrocytes in the test procedure (5.5) shall be 3 g/l and 3 ml/l respectively.

#### 5.2.2.8.4 Clean and dirty conditions for the modified method for ready-to-use products (5.5.4)

Follow the procedures for preparation according to 5.2.2.8.2 and 5.2.2.8.3, but prepare the interfering substance in fivefold higher concentrations, for the dirty conditions maximum 50 ml to avoid problems with the filtration.

- a) Clean conditions (5.2.2.8.2) – dissolve 1,50 g bovine albumin (instead of 0,3 g) in 100 ml of diluent;
- b) Dirty conditions (5.2.2.8.3) – dissolve 7,5 g bovine albumin (instead of 1,5 g) in 42,5 ml of diluent (instead of 48,5 ml). Prepare at least 20 ml (instead of 4,0 ml) sheep blood. Resuspend 7,5 ml (instead of 1,5 ml) of the packed sheep erythrocytes in 42,5 ml of sterilized bovine albumin solution to obtain 50 ml.

#### 5.2.2.9 Defibrinated sheep blood

The defibrinated sheep blood should be sterile (aseptic blood-letting and preparation), pooled from more than one sheep and can be acquired from a commercial supplier.

### 5.3 Apparatus and glassware

#### 5.3.1 General

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

- a) by moist heat, in the autoclave [5.3.2.1 a)];
- b) by dry heat, in the hot air oven [5.3.2.1 b)].

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5.3.2 Usual microbiological laboratory equipment<sup>2)</sup>

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  $20\text{ °C} \pm 1\text{ °C}$ , at  $45\text{ °C} \pm 1\text{ °C}$  (to maintain melted TSA in case of pour plate technique and at additional test temperatures  $\pm 1\text{ °C}$  (5.5.1).

**5.3.2.3** Incubator, capable of being controlled either at  $36\text{ °C} \pm 1\text{ °C}$  or  $37\text{ °C} \pm 1\text{ °C}$  (5.2.1). The same temperature shall be used for incubations performed during a test and its control and validation.

**5.3.2.4** **pH-meter**, having an inaccuracy of calibration of no more than  $\pm 0,1$  pH units at  $20\text{ °C} \pm 1\text{ °C}$ .

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

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**5.3.2.5** **Stopwatch.**

**5.3.2.6** **Shakers**

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- a) Electromechanical agitator, e.g. Vortex<sup>®</sup> mixer<sup>3)</sup>;  
<https://standards.iteh.ai/catalog/standards/sist/af43fb4f-da43-4e70-b7b2-1d5b7eab02c0/sist-en-13727-2012a2-2016>

- 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 of filters of diameter 47 mm to 50 mm and 0,45 µm pore size for sterilization of hard water (5.2.2.7), bovine albumin (5.2.2.8.2, 5.2.2.8.3 and 5.2.2.8.4), and if the membrane filtration method is used (5.5.3).

The vacuum source used shall give an even filtration flow rate. In order to obtain a uniform distribution of the micro-organisms over the membrane and to prevent overlong filtration, the device shall be set so as to obtain the filtration of 100 ml of rinsing liquid in 20 s to 40 s.

**5.3.2.8** **Refrigerator**, capable of being controlled at  $2\text{ °C}$  to  $8\text{ °C}$ .

**5.3.2.9** **Graduated pipettes**, of nominal capacities 10 ml, 1 ml, 100 µl, 1 µl or calibrated automatic pipettes.

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

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<sup>2)</sup> Disposable sterile equipment is an acceptable alternative to reusable glassware.

<sup>3)</sup> Vortex<sup>®</sup> 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.

**5.3.2.11 Glass beads (Diameter 3 mm to 4 mm).**

**5.3.2.12 Volumetric flasks.**

**5.3.2.13 Centrifuge (800  $g_N$ ).**

## **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 General**

For each test organism, two different suspensions have to be prepared: the “test suspension” to perform the test and the “validation suspension” to perform the controls and method validation.

#### **5.4.1.2 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 the 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.

Never produce and use a fourth subculture.

#### **5.4.1.4 Test suspension (M)**

- 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  $1,5 \times 10^8$  cfu/ml<sup>4)</sup> to  $5,0 \times 10^8$  cfu/ml using diluent (5.2.2.4) ( $1,5 \times 10^9$  cfu/ml to  $5,0 \times 10^9$  cfu/ml in the case of the modified method – 5.5.4), estimating the number of cfu by any suitable means. Maintain this test suspension in the water bath at 20 °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.

**NOTE** 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 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. A colorimeter is a suitable alternative.

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<sup>4)</sup> cfu/ml = colony forming unit(s) per millilitre.