BRITISH STANDARD

BS EN 14563:2008

Chemical disinfectants and antiseptics — Quantitative carrier test for the evaluation of mycobactericidal or tuberculocidal activity of chemical disinfectants used for instruments in the medical area — Test method and requirements (phase 2, step 2)

ICS 11.080.20

BSI British Standards

National foreword

This British Standard is the UK implementation of EN 14563:2008.

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

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Chemical disinfectants and antiseptics - Quantitative carrier test for the evaluation of mycobactericidal or tuberculocidal activity of chemical disinfectants used for instruments in the medical area - Test method and requirements (phase 2, step 2)

Désinfectants et antiseptiques chimiques - Essai quantitatif de porte-germe pour l'évaluation de l'activité mycobactéricide ou tuberculocide des désinfectants chimiques utilisés pour instruments en médecine humaine - Méthode d'essai et prescriptions (phase 2, étape 2)

Chemische Desinfektionsmittel und Antiseptika - Quantitativer Keimträgerversuch zur Prüfung der mykobakteriziden oder tuberkuloziden Wirkung chemischer Desinfektionsmittel für Instrumente im humanmedizinischen Bereich - Prüfverfahren und Anforderungen (Phase 2, Stufe 2)

This European Standard was approved by CEN on 18 October 2008.

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Foreword

This document (EN 14563:2008) 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of an identical text or by endorsement, at the latest by May 2009, and conflicting national standards shall be withdrawn at the latest by May 2009.

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

For relationship with EU Directive 93/42/EEC, see informative annex ZA, which is an integral part of this document.

Other methods to evaluate the efficacy of chemical disinfectants and antiseptics for different applications in the medical field are in preparation.

A collaborative trial will be undertaken to provide a precision annex to this standard.

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, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland and the United Kingdom.

Introduction

This European Standard specifies a carrier test for establishing whether a chemical disinfectant for use on instruments (surgical instruments, anaesthesia material, endoscopes etc.) has a mycobactericidal or tuberculocidal activity in the area described in the scope.

The laboratory test closely simulates practical conditions of application including pre-drying mycobacteria on a carrier, contact time, temperature, test organisms and interfering substances, i.e. conditions which may influence the action of chemical disinfectants in practical situations.

The obligatory conditions are intended to cover general purposes and to allow reference between laboratories and product types. Each utilization concentration of the chemical disinfectant found by this test corresponds to defined experimental conditions. However, for some applications the recommendations 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 mycobactericidal or tuberculocidal activity of chemical disinfectant 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 medical area for disinfecting instruments by immersion – even if they are not covered by the EEC/93/42 Directive on Medical Devices.

This European Standard applies to areas and situations where disinfection 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, kindergartens and 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.

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

NOTE This method corresponds to a phase 2, step 2 test.

2 Normative references

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

EN 12353, *Chemical disinfectants and antiseptics – Preservation of 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 European Standard, the terms and definitions given in EN 14885 apply.

4 Requirements

The product, when tested in accordance with Clause 5 under simulated clean conditions (0,3 g/l bovine albumin solution) or simulated dirty conditions (3,0 g/l bovine albumin solution, plus 3,0 ml/l washed sheep erythrocytes) according to its practical applications and under the obligatory test conditions, (one or two selected test organisms, 20 °C, 60 min), shall demonstrate at least a decimal log (lg) reduction in counts of 4.

The mycobactericidal activity shall be evaluated using the following two test organisms: *Mycobacterium avium* and *Mycobacterium terrae*.

The tuberculocidal activity shall be evaluated using the following test organism: *Mycobacterium terrae*.

Where indicated, additional specific mycobactericidal or tuberculocidal activity shall be determined applying other contact times, temperatures and interfering substances in accordance with 5.5.1.1, in order to take into account intended specific use conditions.

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

5 Test method

5.1 Principle

5.1.1 A test suspension of mycobacteria in a solution of interfering substances is spread on a glass carrier. After drying the carrier is immersed into a sample of the product as delivered and/or diluted with hard water (for ready to use products: water). The carrier is maintained at 20 °C ± 1 °C for 60 min ± 10 s (obligatory test conditions). At the end of this contact time, the carrier is transferred into a neutralizer containing glass beads. The mycobacteria are to be severed from the surface by shaking. The numbers of surviving mycobacteria in each sample are determined and the reduction is calculated.

5.1.2 The test is performed using *Mycobacterium avium* and *Mycobacterium terrae* or only *Mycobacterium terrae* as test organisms (obligatory test conditions).

5.1.3 Additional and optional contact times and temperatures are specified. Additional interfering substances may be used.

5.2 Materials and reagents

5.2.1 Test organisms

The mycobactericidal activity shall be evaluated using the following two test-organisms[1)]:

— *Mycobacterium avium* ATCC 15769

— *Mycobacterium terrae* ATCC 15755

The tuberculocidal activity shall be evaluated using only *Mycobacterium terrae*.

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

5.2.2 Culture media and reagents

5.2.2.1 General

All weights of chemical substances given in this 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.

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 the product named.

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.

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 (5.3.1). 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 Middlebrook and Cohn 7H10 medium enriched by 10 % OADC (MCO)

Middlebrook 7H10 agar – powder	19,0 g
Glycerol ($C_3H_8O_3$) (see bibliographic reference [2])	5,0 ml
Water (5.2.2.2)	to 900,0 ml

Heat to boiling to dissolve completely. Sterilize in the autoclave (5.3.1) and cool to 50 °C to 55 °C. Add 100 ml Middlebrook OADC enrichment under aseptic conditions. Fill 18 – 20 ml per plate (5.3.2.10). The pH of the medium shall be equivalent to 6,6 ± 0,2 when measured at 25 °C.

NOTE In special circumstances (problems with neutralization – see 5.5.1.2 and 5.5.1.3) it may be necessary to add neutralizer to MCO (see Annex B). It is not recommended to use neutralizer that causes opalescence in the agar.

5.2.2.4 Diluent

Tryptone Sodium Chloride Solution:

Tryptone, pancreatic digest of casein	1,0 g
Sodium chloride (NaCl)	8,5 g
Water (5.2.2.2)	to 1 000,0 ml

Sterilize in the autoclave (5.3.1). 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 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 Sterile defibrinated sheep blood

The sterile defibrinated sheep blood can be acquired from a commercial supplier or prepared according to EN 14820.

5.2.2.7 Hard water for dilution of products

Prepare:

— Solution A: Dissolve 19,84 g anhydrous magnesium chloride ($MgCl_2$) or an equivalent of hydrated magnesium chloride and 46,24 g anhydrous calcium chloride ($CaCl_2$) or an equivalent of hydrated calcium chloride in water (5.2.2.2) and dilute to 1 000 ml. Sterilize in the autoclave (5.3.1). Store the solution in a refrigerator (5.3.2.8) for no longer than one month.

— 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 a refrigerator (5.3.2.8) for no longer than one week.

— Hard water: For the preparation of 1 l hard water, place 600 ml – 700 ml 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. 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 (see 5.4.2) the addition of the product to this hard water produces a different final water hardness in each test tube. In any case the final hardness is lower than 300 mg/l of calcium carbonate ($CaCO_3$) 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.

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 In the following, 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 1 month.

— The final concentration of the bovine albumin in the test procedure (see 5.5) is 0,3 g/l.

5.2.2.8.3 Dirty conditions (Mixture of bovine albumin solutions – high concentration with sheep erythrocytes (see 5.2.2.6))

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 sterile defibrinated sheep blood (5.2.2.6). Centrifuge the sheep blood at 800 g_N for 10 min. 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 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 at 2 °C to 8 °C.

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

5.3 Apparatus and glassware

5.3.1 General

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

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

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

5.3.2 Usual microbiological laboratory equipment [2)] and in particular the following:

5.3.2.1 Apparatus for sterilization:

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

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 °C ± 1 °C, at 50 °C to 55 °C (to prepare the MCO – see 5.2.2.3) and at additional test temperatures ± 1 °C (5.5.1).

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

NOTE 1 The same temperature should be used for all incubations performed during a test and its controls and validation.

NOTE 2 A CO_2 incubator and a temperature of 36 °C ± 1 °C are better suited for the test organisms. If a CO_2 incubator is not used, the inoculated plates should be protected from drying by sealing with insulating tape or packing them into polyethylene bags.

5.3.2.4 pH-meter, having an inaccuracy of calibration of not more than ± 0,1 pH units at (20 ± 1) °C.

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

5.3.2.5 Stopwatch.

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

5.3.2.6 Shakers

a) Electromechanical agitator, e.g. Vortex® mixer[3)].

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) and bovine albumin (5.2.2.8).

The vacuum source used shall give an even filtration flow rate.

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 and 1,0 ml and 0,1 ml. Calibrated automatic pipettes may be used.

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 Glass beads (Diameter: 0,25 mm to 0,5 mm).

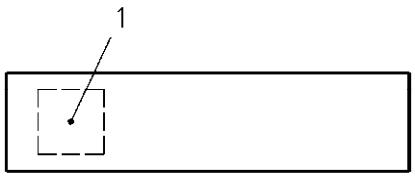
5.3.2.14 Centrifuge (800 g_N and 2 000 g_N).

5.3.2.15 Cylindrical plastic screw cap tubes, contents of about 15 ml, diameter about 18 mm (for the carrier).

5.3.2.16 Coned bottom screw cap tubes, contents of 50 ml (diameter about 28 mm).

5.3.2.17 Frosted glass carriers, 15 mm x 60 mm x 1 mm, one surface sandblasted.

Glass carriers shall be used once only. For preparation the glass carrier is boiled 10 min in a suitable detergent, cleaned minimum 3 times with water (5.2.2.2) and at the end once with ethanol (70 Vol.%). Mark a 10 mm square at one end of the dried carrier on its sandblasted surface, about 2 mm off the three edges. Sterilize in the heat oven [5.3.1b)]. After the sterilization process the markings of the “inoculation square” shall be clearly visible.



Key

1 Inoculation square

Figure 1 — Frosted glass carrier with markings

3) Vortex® in 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.

5.4 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 shall 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 of test organisms

The test organisms 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), subculture directly from the defrosted cryovials (5.4.1.2) by streaking onto at least two plates containing MCO (5.2.2.3) and incubate (5.3.2.3). After 21 days prepare a second subculture from the first subculture in the same way and incubate for 21 days. The first and/or the second subculture is/are the working culture(s).

Never produce and use a third subculture.

5.4.1.4 Test suspension (*N*)

a) Prepare a suitable homogeneous suspension from the working cultures (5.4.1.3) using

- either the homogenization by glass beads: with the aid of a plastic disposable loop transfer the test organisms from at least two plates of the working culture (5.4.1.3) into a coned bottom screw cap tube (5.3.2.16) containing 6-7 g of dry glass beads (5.3.2.11). The test organisms are homogenized by mixing [5.3.2.6a)] for at least 5 min to distribute them homogeneously on the beads and on most of the parts of the screw cap tube’s internal surface. Add 10 ml water (5.2.2.2) drop by drop and resuspend them by mixing [5.3.2.6a)]. After 20 min sedimentation time the supernatant is transferred to a tube.

- or the homogenization by Potter S 1 apparatus: pipette 5,0 ml water (5.2.2.2) on each of the plates (at least two) of the working culture (5.4.1.3) and recover with a glass spatula the test organisms. Pipette all of the liquid from the plates into a 25 ml centrifugation tube. Add up to 18 ml water (5.2.2.2). Wash with water (5.2.2.2), centrifuging 3 times at approximately 2 000 g_N for 15 min. After each centrifuging, discard the supernatant, resuspend by mixing [5.3.2.6a)] and fill up to the original volume with water (5.2.2.2).

  After the last centrifuging discard the supernatant and transfer the sediment into a 15 ml glass vessel of the Potter S 1 apparatus. Fill up to 15 ml with water (5.2.2.2). Mix, cool with ice and homogenize for 15 min.

  After 20 min sedimentation time, during which enough ice for cooling should be present, the supernatant is transferred to a tube.

NOTE 1 Other methods of homogenization are allowed provided that the number of colony forming unit(s) per millilitre (cfu/ml) obtained is appropriate and stable during the time of the test and microscopic examination shows that the suspension is as homogeneous as after the two procedures described above.

NOTE 2 Do not use tensio-active substances (surfactants).

b) Adjust the number of cells in the (supernatant) suspension to 1,5 x 10^9 cfu/ml[4] to 5,0 x 10^9 cfu/ml using water (5.2.2.2). Maintain this test suspension in the water bath at 20 °C ± 1 °C and use at the day of preparation. Adjust the temperature according to 5.5.1.1a) 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 a calibration curve for each test organism knowing that suitable values of optical density are generally found between 0,200 and 0,400. 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.

c) For counting prepare 10^{-7} and 10^{-8} dilutions of the test suspension using water (5.2.2.2). Mix [(5.3.2.6a)].

d) Take a sample of 1,0 ml of each dilution in duplicate. Divide each sample in two nearly equal amounts and spread onto separate surface dried plates (5.3.2.10) containing MCO (5.2.2.3), i.e. four plates per duplicate 1,0 ml samples.

For incubation and counting see 5.4.1.6.

5.4.1.5 Validation suspension (N_V)

a) To prepare the validation suspension, dilute the test suspension (5.4.1.4) with water (5.2.2.2) to obtain 3,0 x 10^2 cfu/ml to 1,6 x 10^3 cfu/ml [about one fourth (1 + 3) of the 10^{-6} dilution].

b) For counting prepare a 10^{-1} dilution with water (5.2.2.2). Mix [5.3.2.6a)]. Take a sample of 1,0 ml in duplicate. Divide each sample in two nearly equal amounts and spread onto separate surface dried plates (5.3.2.10) containing MCO (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 21 days. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units (cfu) for each plate.

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/ml in the test suspension N and in the validation suspension N_V using the method given in 5.6.2.3 or 5.6.2.5. Verify according to 5.7.

5.4.2 Product test solution

Product test solutions shall be prepared in hard water (5.2.2.7) at minimum three different concentrations to include one concentration in the active range and one concentration in the non-active range. The product as received may be used as one of the product test solutions.

Dilutions of ready-to-use products, i.e. products which 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 g ± 10 mg of the product in a volumetric flask and filling up with hard water (5.2.2.7). Subsequent dilutions (i.e. lower concentrations) shall be prepared in volumetric flasks (5.3.2.12) on a volume/volume basis in hard water (5.2.2.7).

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

For liquid products, dilutions of the product shall be prepared with hard water on a volume/volume basis using volumetric flasks (5.3.2.12).

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

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

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 mycobactericidal / tuberculocidal 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 may be selected according to the practical use considered for the product (Clause 4):

a) temperature θ (in °C):

- obligatory temperature to be tested is θ = 20 °C;
- additional temperatures (Clause 4) may be chosen according to the manufacturer's recommendation, but not higher than 60 °C;
- allowed deviation for each chosen temperature is ± 1 °C.

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

- obligatory contact time to be tested is *t* = 60 min;
- additional contact times (Clause 4) may be chosen from 5 min, 15 min and 30 min;
- allowed deviation for each chosen contact time is ± 10 s.

c) interfering substance:

- obligatory interfering substance to be tested is 0,30 g/l bovine albumin (5.2.2.8.2) under clean or a mixture of 3 ml/l sheep erythrocytes and 3,0 g/l bovine albumin (5.2.2.8.3) under dirty conditions – according to practical applications.
- Additional interfering substances may be tested according to specific fields of application.

d) test organisms:

- obligatory test organisms for testing mycobactericidal activity are:
  - *Mycobacterium avium* and *Mycobacterium terrae* (5.2.1).
- obligatory test organism for testing tuberculocidal activity is: *Mycobacterium terrae.*

5.5.1.2 Selection of neutralizer

To determine a suitable neutralizer carry out the validation of the dilution neutralization method (see 5.5.2.3, 5.5.2.4 and 5.5.2.5 in connection with 5.5.2.6) using a 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.

NOTE In special circumstances it may be necessary to add neutralizer to MCO (5.2.2.3). If neutralizer is added to MCO the same amount should be added to MCO used in the test procedure.

5.5.1.3 Validation and control procedures – General instructions

The neutralization and/or removal of the mycobactericidal and/or mycobacteriostatic 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 (experimental condition control, neutralizer control and method validation) shall be performed always at the same time with the test and with the same neutralizer used in the test.

In the case of ready-to-use-products use water (5.2.2.2) instead of hard water.

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

5.5.1.4 Equilibration of temperature

Prior to testing, equilibrate all reagents (product test solutions (5.4.2), test suspension (5.4.1.4), validation suspension (5.4.1.5), hard water (5.2.2.7) and interfering substance (5.2.2.8)) to the test temperature of θ using the water bath (5.3.2.2) controlled at θ. Observe the provisions laid down in 5.4.1.4.b). Check that the temperature of the reagents is stabilized at θ.

The neutralizer (5.2.2.5) and water (5.2.2.2) shall be equilibrated at a temperature of 20 °C ± 1 °C.

In the case of ready-to-use-products, water (5.2.2.2) shall be additionally equilibrated to the test temperature of θ.

5.5.1.5 Precautions for manipulation of test organisms

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

5.5.1.6 Inoculation of the carriers

Pipette 1,0 ml of interfering substances (5.2.2.8) into a tube. Add 9,0 ml of the test suspension (5.4.1.4). Mix [5.3.2.6a)] and pipette 0,05 ml of this mixture on the “inoculation square” of a carrier (5.3.2.17) and distribute equally inside the square, e.g. with the tip of the pipette. Let the inoculum dry at 36 °C ± 1 °C until visible dryness, maximum 60 min. Use the carrier immediately after the end of the drying time.

Note the drying time in the test report.

5.5.2 Method[5)]

5.5.2.1 General

The test and the control and validation procedures (see 5.5.2.2 until 5.5.2.6) shall be carried out in parallel – separately for each experimental condition (see 5.5.1.1).

5.5.2.2 Test N_a (Determination of bactericidal concentrations), water control N_W

a) Pipette 10 ml of one of the product test solutions (5.4.2) into a cylindrical screw cap tube (5.3.2.15) placed in a water bath controlled at the chosen test temperature of θ [5.5.1.1a)]. Immerse an inoculated carrier (5.5.1.6) immediately after the drying process has been finished. Secure that the inoculation square is completely covered by the product test solution (5.4.2). Start the stopwatch and leave for the chosen contact time *t* [5.5.1.1b)].

b) At the end of *t* transfer the carrier into a second cylindrical screw cap tube (5.3.2.15), placed in a water bath controlled at 20 ºC and filled with 10 ml of neutralizer (5.2.2.5) and approximately 1 ml of glass beads (5.3.2.13). Restart the stopwatch and mix [5.3.2.6a)] for 15 s. After a neutralization time of 5 min ± 10 s, mix [5.3.2.6a)] and immediately take a sample of 1,0 ml of the neutralized test mixture N_a (containing neutralizer, product test solution, interfering substance, test suspension) in duplicate. Divide each sample in two portions of approximately equal size and spread onto separate surface dried plates containing MCO (5.2.2.3). Additionally transfer 0,5 ml of the test mixture N_a into a tube containing 4,5 ml of neutralizer (10^{-1} dilution of N_a), mix [5.3.2.6a)] and dilute accordingly to produce 10^{-2} and 10^{-3} dilutions of N_a with neutralizer. Take samples of 1,0 ml from each dilution tube in duplicate. Divide and spread as described above. The number of 1,0 ml samples of N_a shall be altogether 8.

For incubation and counting see 5.5.2.6.

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

d) Water control N_W: Perform the procedure a) - b), but instead of the product test solution pipette 10 ml of hard water (5.2.2.7) or – in the case of ready-to-use products – water (5.2.2.2). Deviating from b) produce 10^{-4} and 10^{-5} dilutions from the neutralized test mixture N_W for incubation and counting (total number of 1,0 ml samples: 4 = duplicate of 10^{-4} + 10^{-5}).

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

5.5.2.3 Experimental condition control "*A*" (Validation of the selected experimental conditions or verification of the absence of any lethal effect in the test conditions)

a) Pipette 1,0 ml of the interfering substance used in the test (5.5.2.2) into a tube. Add 1,0 ml of the validation suspension (5.4.1.5). Start the stopwatch immediately, mix [5.3.2.6a)] and place the tube in a water bath controlled at θ for 2 min ± 10 s. At the end of this time add 8,0 ml of hard water (5.2.2.7). (In the case of ready-to-use products: water (5.2.2.2) instead of hard water). Restart the stopwatch at the beginning of the addition. Mix [5.3.2.6a)] and place the tube in a water bath controlled at θ for *t*. Just before the end of *t*, mix [5.3.2.6a)] again.

b) At the end of *t*, take a sample of 1,0 ml of this mixture *A* in duplicate. Divide and spread as described in 5.5.2.2 b).

For incubation and counting see 5.5.2.6.

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

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

a) Pipette 8,0 ml of the neutralizer – used in the test (5.5.2.2) – and 1,0 ml of water (5.2.2.2) into a tube. Add 1,0 ml of the validation suspension (see 5.4.1.5). Start the stopwatch at the beginning of the addition, mix [5.3.2.6a)], and place the tube in a water bath controlled at 20 °C ± 1 °C for 5 min ± 10 s. Just before the end of this time, mix [5.3.2.6a)].

b) At the end of this time take a sample of 1,0 ml of this mixture *B* in duplicate. Divide and spread as described in 5.5.2.2.b).

For incubation and counting see 5.5.2.6.

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

a) Pipette 1,0 ml of the interfering substance used in the test (5.5.2.2) into a tube. Add 1,0 ml of water (5.2.2.2) and then, starting a stopwatch, 8,0 ml of the product test solution only of the highest concentration used in the test (5.5.2.2). Mix [5.3.2.6a)] and place the tube in a water bath controlled at θ for *t*. Just before the end of *t*, mix [5.3.2.6a)] again.

NOTE It is not necessary to prepare the highest concentration of the product test solution 1,25 times higher than the derived (actually tested) concentration though it is diluted during the method validation by interfering substance and water (8+1+1). In the test N_a the amount of neutralizer in relation to the product test solution is much higher.

b) At the end of *t* transfer 1,0 ml of the mixture into a tube containing 8,0 ml of neutralizer (used in 5.5.2.2). Restart the stopwatch at the beginning of the addition, mix [5.3.2.6a)] and place the tube in a water bath controlled at 20 °C ± 1 °C for 5 min ± 10 s. Add 1,0 ml of the validation suspension (5.4.1.5). Start a stopwatch at the beginning of the addition and mix [5.3.2.6a)]. Place the tube in a water bath controlled at 20 °C ± 1 °C for 30 min ± 1 min. Just before the end of this time, mix [5.3.2.6a)] again. At the end of this time take a sample of 1,0 ml of the mixture *C* in duplicate. Divide and spread as described in 5.5.2.2. b).

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

a) Incubate (5.3.2.3) the plates for 21 days. Discard any plates which are not countable (for any reason). Count the plates and determine the number of cfu for each plate.

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/ml in the test mixtures N_a and N_W and in the validation mixtures *A*, *B* and *C* using the methods given in 5.6.2.3b), 5.6.2.4 and 5.6.2.6. 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 / test mixtures:

— *N* and N_V represent the mycobacterial suspensions, N_a represents the mycobactericidal test mixture, N_W represents the test mixture in the water control, *A* (experimental conditions control), *B* (neutralizer control), *C* (method validation) represent the different control test mixtures.

— *N*, N_V, N_{V0}, N_a, and *A*, *B*, *C* represent the number of cells counted ***per ml*** in the different test mixtures according to Table 1:

Table 1 — Number of cells counted per ml in the different test mixtures

	Number of cells ***per ml*** in the mycobacterial suspensions	Number of cells ***per ml*** in the test mixtures at the beginning of the contact time (time 0)	Number of survivors ***per ml*** in the test mixtures at the end of the contact-time *t* (*A*) or of 5 min (*B*) or of 30 min (*C*)
Test	*N* Test suspension	*N*/20 (= theoretical number on the carrier)	N_a, N_w (before neutralization)
Controls	N_v Validation suspension	N_{v0} ($N_{v0} = N_v/10$)	*A*, *B*, *C*

5.6.1.2 V_C-values

All experimental data are reported as V_C-values:

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 is the determination of the V_C-values, the second the calculation of N, N_a, N_W, N_V, N_{V0}, A, B and C. The third step is the calculation of the reduction R (5.8).

5.6.2.2 Determination of V_C-values

a) The usual limits for counting mycobacteria 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 1 The lower limit (14) is based on the fact that the variability increases the smaller the number counted in the sample (1 ml) is and therefore subsequent calculations may lead to wrong results. The lower limit refers only to the sample (and not necessarily to the counting on one plate), e.g. two plates per 1 ml sample with 11 cfu and 5 cfu give a V_C-value of 16. The upper limit (330) reflects the imprecision of counting confluent colonies and growth inhibition due to nutriment depletion. It refers only to the counting on one plate, and not necessarily to the sample.

b) According to the number of plates used per 1 ml sample (5.6.1.2), determine and record the V_C-values.

NOTE 2 The countings per plate should be noted.

If the count on one plate is higher than 330 the number should be reported "> 330". If at least one of the plates per 1 ml sample shows a number higher than 330 the number of this V_C-value should be reported as "more than sum of the counts", e.g. for ">330, 308", "> 638" should be reported. If a V_C-value is lower than 14 report the number but substitute by "< 14" for further calculations in the case of N_a .

c) Only V_C-values within the respective 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 and N_W

a) *N* is the number of cells per ml in the test suspension (5.4.1.4). Since two dilutions of the test suspension (5.4.1.4 in connection with 5.4.1.6) are evaluated, calculate the number of cfu/ml as the weighted mean count using the formula (1):

$$N = \frac{c}{(n_1 + 0{,}1 n_2)10^{-7}} \qquad (1)$$

where

c sum of V_C-values taken into account;

n_1 number of V_C-values taken into account at the first dilution (10^{-7});

n_2 number of V_C-values taken into account at the second dilution (10^{-8});

10^{-7} dilution factor corresponding to the lowest dilution.

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

EXAMPLE $N = \frac{168 + 213 + 20 + 25}{(2 + 0{,}1 \times 2)10^{-7}} = \frac{426}{2{,}2 \times 10^{-7}} = 1{,}9363 \times 10^{9} = 1{,}9 \times 10^{9}$ (cfu/ml)

b) N_W is the number of cells per ml in the test mixture [5.5.2.2d)] at the end of the contact time and before neutralization. It is tenfold higher than the V_C-values (5.5.2.6) due to the addition of neutralizer [5.5.2.2b) and d)]. Calculate the number of cfu/ml as the weighted mean count of the 10^{-4} and 10^{-5} dilutions using the formula (2):

$$N_W = \frac{c \times 10}{(n_1 + 0{,}1 n_2)10^{-4}} \qquad (2)$$

For explanation of c, n_1, n_2 , the calculation rules and example see a).

5.6.2.4 Calculation of N_a

N_a is the number of survivors per ml in the test mixture [5.5.2.2a)] at the end of the contact time and before neutralization. It is tenfold higher than the V_C-values (5.5.2.6) due to the addition of neutralizer [5.5.2.2b)].

a) Calculate the mean for each dilution step N_a^0, N_a^{-1}, N_a^{-2}, N_a^{-3} using the formula (3):

$$N_a^0, N_a^{-1}, N_a^{-2}, N_a^{-3} = \frac{c \times 10}{n} \qquad (3)$$

where

c sum of V_C-values taken into account;

n number of V_C-values taken into account.

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

EXAMPLE 1

duplicate V_C-values N_a^{-1}: 2, 16

$$N_a^{-1} = \frac{(<14+16)\times 10}{2} = <150\times 10^1 = <1500 = <1{,}5\times 10^3$$

duplicate V_C-values N_a^{-2} : > 660, > 660

$$N_a^{-2} = \frac{(>660+>660)\times 10}{2} = >6600\times 10^2 = >660.000 = >6{,}6\times 10^5$$

duplicate V_C-values N_a^{0}, > 660, 600

$$N_a^{0} = \frac{(>660+600)\times 10}{2} = >6300\times 10^0 = >6300 = >6{,}3\times 10^3$$

b) For calculation of N_a use only N_a^0, N_a^{-1}, N_a^{-2}, N_a^{-3} results where one or both V_C-values are within the counting limits. Exceptions and rules for special cases:

If all subsequent dilutions of N_a show mean values of "more than", take only the highest dilution (10^{-3}) as result for N_a.

EXAMPLE 2

	Vc_1	Vc_2		mean x 10
N_a^0	> 660	> 660	=	> 6 600
N_a^{-1}	> 660	> 660	=	> 6 600
N_a^{-2}	> 660	> 660	=	> 6 600
N_a^{-3}	> 660	> 660	=	> 6 600

N_a = > 6 600 x 10^3 = > 6,6 x 10^6

If all subsequent dilutions of N_a show mean values of "less than", take only the lowest dilution (10^0) as result for N_a.

EXAMPLE 3

	Vc_1	Vc_2		mean x 10
N_a^0	< 14	18	=	< 160
N_a^{-1}	< 14	< 14	=	< 140
N_a^{-2}	< 14	< 14	=	< 140
N_a^{-3}	< 14	< 14	=	< 140

N_a = < 160 x 10^0 = < 1,6 x 10^2

If one or both duplicate V_C-values in only one dilution of N_a are within the counting limits, use this result as N_a.

EXAMPLE 4

	Vc_1	Vc_2		mean x 10
N_a^0	> 660	> 660	=	> 6 600
N_a^{-1}	96	107	=	1 015
N_a^{-2}	< 14	< 14	=	< 140
N_a^{-3}	< 14	< 14	=	< 140

N_a = 1 015 x 10^1 = 1,0 x 10^4

If the higher dilution in two subsequent dilutions of N_a shows a mean value of "less than" and the lower dilution shows a mean value of „more than", take only the lower dilution as N_a value.

EXAMPLE 5

	Vc_1	Vc_2		mean x 10
N_a^0	> 660	> 660	=	> 6 600
N_a^{-1}	> 660	> 660	=	> 6 600
N_a^{-2}	< 14	29	=	< 215
N_a^{-3}	< 14	< 14	=	< 140

N_a = > 6 600 x 10^1 = > 6,6 x 10^4

c) Use maximum 2 subsequent dilutions for calculating N_a as a weighted mean. Rules for special cases:

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

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 formula (4):

$$N_a = \frac{c \times 10}{2,2 \times 10^{z}} \qquad (4)$$

where

- c sum of V_C-values taken into account;
- z dilution factor corresponding to the lower dilution, e.g. N_a^{-2} is the lower dilution in comparison with N_a^{-3}.

If in two subsequent dilutions of N_a both V_C-values of the higher dilution are within the counting limits and one V_C-value of the lower dilution is „more than", calculate N_a as the weighted mean, using the formula (4).

EXAMPLE 6

	Vc_1	Vc_2	mean x 10
N_a^0	> 538	320	> 4 290
N_a^{-1}	46	57	515
N_a^{-2}	< 14	< 14	< 140
N_a^{-3}	< 14	< 14	< 140

$$N_a = \frac{(> 538 + 320 + 46 + 57) \text{ x } 10}{2,2 \text{ x } 10^0} =$$

> 4 368,18 = > 4,4 x 10^3

If in two subsequent dilutions of N_a one of the higher dilution duplicate values shows "< 14", take only the lower dilution as result for N_a.

EXAMPLE 7

	Vc_1	Vc_2		mean x 10
N_a^0	> 660	> 660	=	> 6 600
N_a^{-1}	> 660	> 660	=	> 6 600
N_a^{-2}	569	> 660	=	> 12 290
N_a^{-3}	< 14	26	=	< 200

N_a = > 12 290 x 10^2 = > 1,2 x 10^6

5.6.2.5 Calculation of N_V and N_{V0}

N_V is the number of cells per ml in the validation suspension (5.4.1.5). It is tenfold higher than the counts in terms of V_C-values due to the dilution step of 10^{-1} [5.4.1.5b)].

N_{V0} is the number of cells per ml in the mixtures *A*, *B* or *C* at the beginning of the contact time (time 0). It is one tenth of the mean of the V_C-values of N_V [5.4.1.6c)] – taken into account.

Calculate N_V and N_{V0} using the formula (5) and (6):

$$N_V = \frac{c \times 10}{n} \quad (5)$$

$$N_{V0} = \frac{c}{n} \quad (6)$$

where

c sum of V_C-values taken into account;

n number of V_C-values taken into account.

5.6.2.6 Calculation of *A*, *B*, *C* (Controls and method validation)

A, *B* and *C* are the numbers of survivors in the experimental conditions control (5.5.2.3), neutralizer control (5.5.2.4) and method validation (5.5.2.5) at the end of the contact time *t* (*A*) or of the defined times 5 min (*B*) and 30 min (*C*). They correspond to the mean of V_C-values of the mixtures *A*, *B* and *C* – taken into account.

Calculate *A*, *B* and *C* using the formula (7):

$$A, B, C = \frac{c}{n} \quad (7)$$

where

c sum of V_C-values taken into account;

n number of V_C-values taken into account.

5.7 Verification of methodology

5.7.1 General

A test is valid if

— all results meet the criteria of 5.7.3 and,

— 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).

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 two means shall not be higher than 15 and not lower than 5. Results below the lower limit are taken as the lower limit number (14). Results above the respective upper limit [5.6.2.2b)] are taken as the upper limit number.

EXAMPLE For *N*: 10^{-7} dilution: > 330 + 302 cfu, 10^{-8} dilution: 20 + 25 cfu; (330 + 302) / (20 + 25) = 632/45 = 14,04 = between 5 and 15.

NOTE When the counts obtained on plates are out of the limits fixed for the determination of *Vc* values (5.6.2.2), 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 :

a1) N is between 1,5 x 10^9 cfu/ml and 5,0 x 10^9 cfu/ml ($9{,}17 \leq \lg N \leq 9{,}70$)

a2) N_W is not less than 1,4 x 10^6 cfu/ml ($\lg N_W \geq 6{,}15$) and not more than 0,05 x N ($\lg N_W \leq (\lg N - 1{,}3)$)

NOTE The dilution caused by the addition of neutralizer is taken into account. For the limits of N_W the lower limit (6,15) is the minimum value of N_W to enable the demonstration of the required *R* (5.8.1). The upper limit reflects the dilution of *N* before dried on the carrier (5.5.1.6).

b) N_{V0} is between 30 and 160 cfu/ml (3,0 x 10^1 and 1,6 x 10^2) (N_V is between 3,0 x 10^2 and 1,6 x 10^3 cfu/ml)

c) *A*,*B*,*C* are equal to or greater than 0,5 x N_{V0}

d) 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/ml in the water control N_W [5.6.2.3b)] and of the results of the test N_a (5.6.2.4).

For each product concentration and each experimental condition calculate and record the decimal log reduction separately using the formula (8):

$$R = \frac{N_W}{N_a} \quad or \quad \lg R = \lg N_W - \lg N_a \qquad (8)$$

For the controls and validation record N_{V0} (5.6.2.5), the results of *A*, *B* and *C* (5.6.2.6) and their comparison with N_{V0} [5.7.3 c)].

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

At least one concentration per test (5.5.2.2a) to d) 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 mycobactericidal / tuberculocidal concentration

5.8.3.1 Mycobactericidal concentration

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

The lowest concentration of the product active on the limiting test organism is the mycobactericidal concentration determined according to this European Standard.

5.8.3.2 Tuberculocidal concentration

Record the lowest concentration of the product which passes the test with *Mycobacterium terrae* (lg $R \geq 4$). The lowest concentration of the product active on *Mycobacterium terrae* is the tuberculocidal concentration determined according to this European Standard.

5.8.4 Precision, replicates

Taking into account the precision of the methodology determined by a statistical analysis based on data provided by a collaborative study for mycobacteria, replication of the test is recommended. Replication means the complete test procedure with separately prepared test – and validation suspensions. The replicate of the test may be restricted to the limiting test organism. The mean of the results of the replicates – not each single result – shall demonstrate at least a 4 lg reduction 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 mycobactericidal or tuberculocidal concentrations determined according to this European Standard may differ (see Clause 4).

5.9.2 Mycobactericidal activity

5.9.2.1 Mycobactericidal activity for general purposes

The product shall be deemed to have passed the EN 14563 standard if it demonstrates in a valid test at least a 4 lg reduction within 60 min or less at 20 °C with the chosen interfering substance (clean or dirty conditions) under the conditions defined by this European Standard when the test organisms are *Mycobacterium avium* and *Mycobacterium terrae*.

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

5.9.2.2 Mycobactericidal activity for specific purposes

The mycobactericidal 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 14563 standard (mycobactericidal activity) under the obligatory test conditions. The mycobactericidal concentration for specific purposes may be lower than the one determined for general purposes.

5.9.3 Tuberculocidal activity

5.9.3.1 Tuberculocidal activity for general purposes

The product shall be deemed to have passed the EN 14563 standard if it demonstrates in a valid test at least a 4 lg reduction within 60 min or less at 20 °C with the chosen interfering substance (clean and/or dirty conditions) under the conditions defined by this European Standard when the test organism is *Mycobacterium terrae.*

5.9.3.2 Tuberculocidal activity for specific purposes

The tuberculocidal concentration for a 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 14563 standard (tuberculocidal activity) under the obligatory test condition. The tuberculocidal concentration for specific purposes may be lower than the one determined for general purposes.

5.9.4 Claims

A product which passes the test is characterized as a chemical disinfectant for instruments possessing mycobactericidal or tuberculocidal activity under conditions representative of practical use.

5.10 Test report

The test report shall refer to this European Standard (EN 14563) mentioning if mycobactericidal activity or only tuberculocidal activity has been tested.

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 its/their concentration(s) (optional);
   8) appearance of the product.

d) selection of neutralizer:

full details of the test 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;
   4) appearance of the product dilutions;
   5) contact time(s);
   6) test temperature(s);
   7) interfering substance(s);
   8) stability and appearance of the mixtures during the procedure (note the formation of any precipitate or flocculant): interfering substance plus test suspension, neutralized test mixture N_a;
   9) temperature of incubation;
   10) neutralizer;
   11) identification of the test organisms used;
   12) drying time of the inoculated carriers;

f) test results:

   1) controls and validation;
   2) evaluation of mycobactericidal or tuberculocidal activity;
   3) number of replicates 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

—	*Mycobacterium avium*:	ATCC	15769
		DSM	44157
—	*Mycobacterium terrae*:	ATCC	15755
		DSM	43227

Annex B
(informative)

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

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

Antimicrobial agent	Chemical compounds able to neutralize residual antimicrobial activity	Examples of suitable neutralizers and of rinsing liquids (for membrane filtration methods)[a]
Quaternary ammonium compounds and fatty amines Amphoteric compounds	Lecithin, Saponin, Polysorbate 80, Sodium dodecyl sulphate, Ethylene oxide condensate of fatty alcohol (non-ionic surfactants)[b]	- 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. *Rinsing liquid : tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*
Biguanides and similar compounds	Lecithin[c], Saponin, Polysorbate 80	- Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. *Rinsing liquid : tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 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. *Rinsing liquid : sodium thiosulphate, 3 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). *Rinsing liquid : polysorbate 80, 5 g/l + L-histidine, 0,5 g/l (or + glycine, 1 g/l).*
Phenolic and related compounds: orthophenylphenol, phenoxyethanol, triclosan, phenylethanol, etc…. Anilides	Lecithin Polysorbate 80 Ethylene oxide condensate of fatty alcohol[b]	- Polysorbate 80, 30 g/l + lecithin, 3 g/l. - Ethylene oxide condensate of fatty alcohol, 7 g/l + lecithin, 20 g/l, + polysorbate 80, 4 g/l. *Rinsing liquid : tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*
Alcohols	Lecithin, Saponin, Polysorbate 80[e]	- Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. *Rinsing liquid : tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*

[a] According to the pH of the tested product, the pH of the neutralizer or the rinsing liquid 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 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.

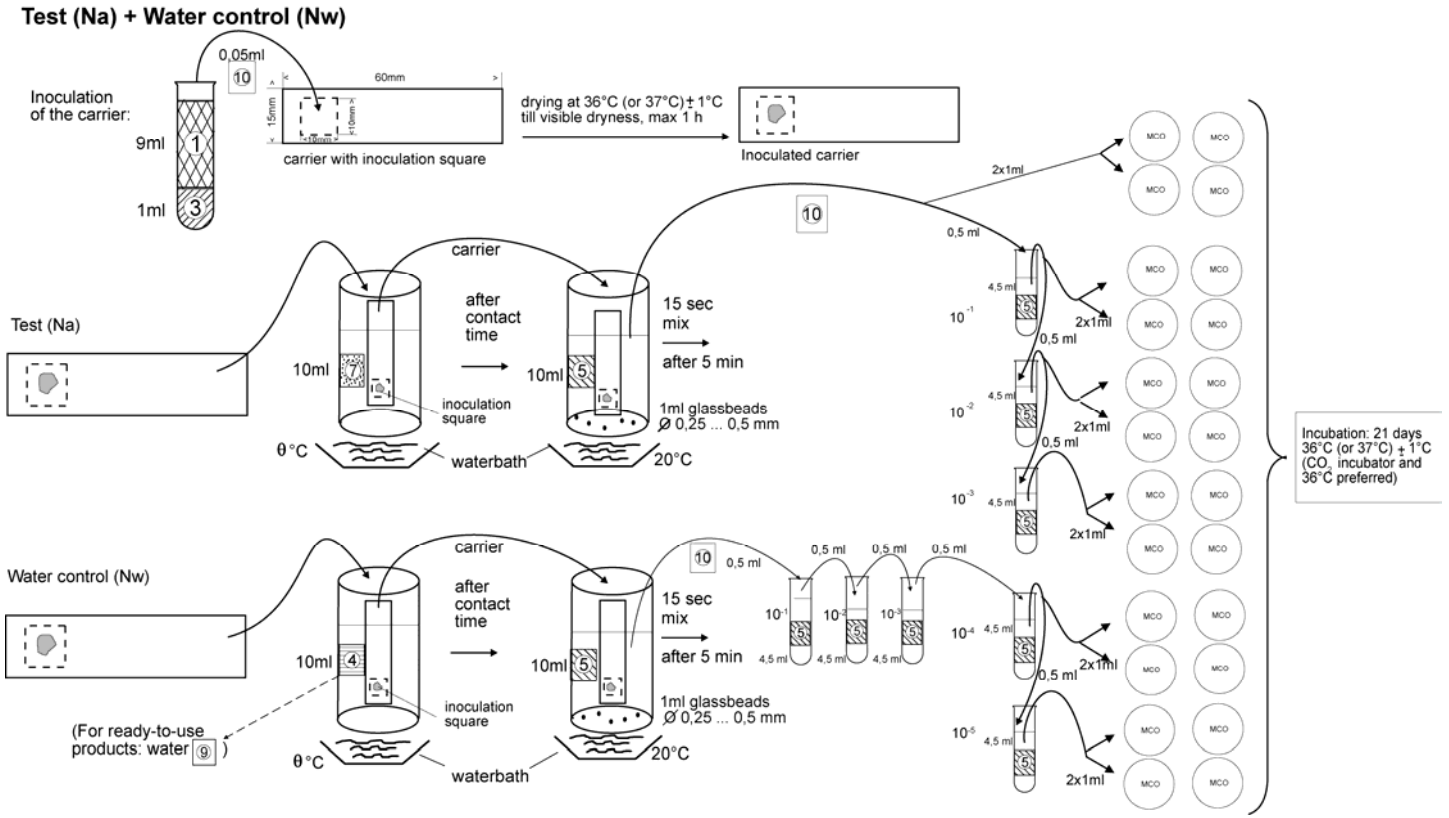
NOTE 1 Other neutralizer mixtures may be required for products containing more than one antimicrobial agent.

NOTE 2 The concentrations of the various neutralizing compounds or of the neutralizer as such may not be adequate to neutralize high concentrations of the products.

Annex C
(informative)

Graphical representations of the test method

For test (N_a) and water control (N_W) see Figure C.1.

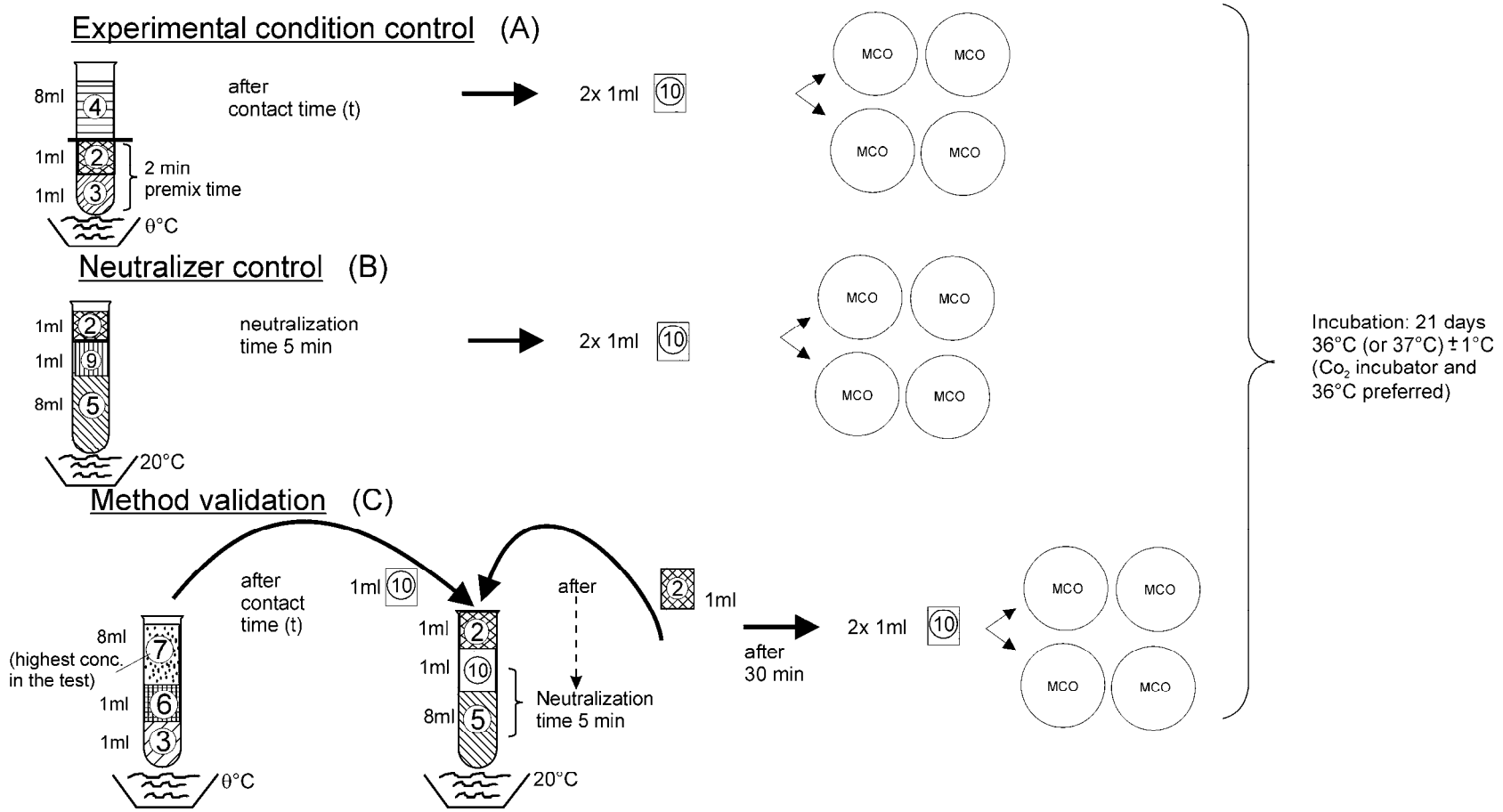


Key

1 Test suspension (*N*)
2 Validation suspension (N_V)
3 Interfering substance
4 Hard water
5 Neutralizer (20 °C)
6 Diluent
7 Product test solution
9 Water
10 Mixture

Figure C.1 — Test (N_a) and water control (N_W)

For validation see Figure C.2.



Key

1	Test suspension (N)	4	Hard water	7	Product test solution
2	Validation suspension (N_V)	5	Neutralizer (20 °C)	9	Water
3	Interfering substance	6	Diluent	10	Mixture

Figure C.2 — Validation

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 results of one replicate for *Mycobacterium avium* are given as an example.

NOTE 3 Test reports for tuberculocidal activity should be entitled “EN 14563, TUBERCULOCIDAL ACTIVITY” and be presented in the same format.

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TEST REPORT

EN 14563, MYCOBACTERICIDAL ACTIVITY
(obligatory and additional conditions)

1. Client : Centipede Formulations Inc., Mannheim / Euroland

2. Disinfectant-sample

Name of the product: Z **Batch number** : 91-71-51

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

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

Appearance of the product: Liquid, clear, yellowish

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

Product diluent recommended by the manufacturer for use: Potable water

3. Period of testing

Date of delivery of the product: 2005-01-26 **Dates of tests**: see “Test results” (attached)

4. Experimental conditions

Product diluent: hard water; concentrations of the product tested: see “Test results” (attached)

Obligatory conditions: test-organisms: *Mycobacterium avium ATCC 15769* and *Mycobacterium terrae ATCC 15755*; test temperature: 20 °C; contact time: 60 min; interfering substance: 0,3 g/l bovine albumin = clean conditions;

Incubation temperature: 36 °C

Additional conditions: test organism*: Mycobacterium avium ATCC 15769.*

Test temperature: 30 °C; contact time: 15 min; interfering substance: 3,0 g/l bovine albumin = dirty conditions;

Incubation temperature: 36 °C

Special remarks regarding the results:

All controls and validation were within the basic limits.

At least one concentration of the product demonstrated a lg reduction of less than 4 lg.

No precipitate during the test procedure (test mixtures were homogeneous).

5. Test results: see attached sheets

6. Conclusion:

For the product Z (batch 91-71-51), the mycobactericidal concentration for general purposes determined according to the EN 14563 standard (obligatory conditions) under clean conditions is:

0,75 % (v/v)

The mean reduction of six replicates with the limiting test organism *Mycobacterium avium* was $1,2 \times 10^4$. *Mycobacterium terrae* was tested 5 times and showed a mean lg reduction of 4,02 at a concentration of 0,5%.

The tuberculocidal concentration under clean conditions for general purposes is 0,5% (v/v).

For the product Z (batch 91-71-51), the concentration for specific purposes determined according to the EN 14563 standard at 30 °C, with 15 min contact time, under dirty conditions using *Mycobacterium avium ATCC 15769* as test organism is :

1,0% (v/v).

Antiseptville, 2006-05-01

Alexandra May, MD, PhD, Scientific Director

Test results (mycobactericidal quantitative carrier test)

EN 14563 (Phase 2. step 2) Product-name: .....*Z*..... Batch No: ..*91-71-51*..
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*....
Number of spread plates .*2.* / ml Neutralizer: .*Lecithin 3,0 g/l in diluent*……..
……..
Test temperature: *20°C* Interfering substances: .*bovine albumin 0,3 g/l*.....
Test organism: *M. avium ATCC 15769*. Drying time on carrier: *40* min (not > 60 min) Incubation temp.: *36°C*
Internal lab. no:...*58/00*.... Date of test:*2006-02-05.* Responsible person: *Fang*... Signature:.. *Fang*....

Validation and controls

Validation suspension (Nvo)				Experimental Conditions control (*A*)				Neutralizer 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	Counts per plate		Vc_1	Vc_2
19+19	*25+21*	*38*	*46*	*18+27*	*27+24*	*45*	*51*	*20+18*	*25+19*	*38*	*44*	*18+16*	*20+14*	*34*	*34*
$\overline{X}$ = *42*				$\overline{X}$ = *48*				$\overline{X}$ = *41*				$\overline{X}$ = *34*			
30 ≤ $\overline{X}$ of N_{vo} ≤ 160? ☒ yes ☐ no				$\overline{X}$ of *A* is ≥ 0,5x $\overline{X}$ of N_{vo}? ☒ yes ☐ no				$\overline{X}$ of *B* is ≥ 0,5x $\overline{X}$ of N_{vo}? ☒ yes ☐ no				$\overline{X}$ of *C* is ≥ 0,5x $\overline{X}$ of N_{vo}? ☒ yes ☐ no			

Test suspension

Water control

Test

Test suspension (*N*):	*N*	Counts per plate		Vc_1	Vc_2	$\overline{X}$ wm = *230,0* x 10^7 ; lg *N* = *9,36*
	10^{-7}	*92+108*	*118+131*	*200*	*249*	9,17 ≤ lg *N* ≤ 9,7 ? ☒ yes ☐ *no*
	10^{-8}	*14+11*	*14+18*	*25*	*32*	

Water control (N_W):	N_W	Counts per plate		Vc_1	Vc_2	$\overline{X}$ wm x 10 = *9750* x 10^4
	10^{-4}	*>330 + >330*	*>330 + >330*	*> 660*	*> 660*	6,15 ≤ lg N_w *=7,99* ≤ (lg *N* –1,3) ? ☒ yes ☐ *no*
	10^{-5}	*30 + 70*	*60 + 35*	*100*	*95*	

Conc. of the product %	Dilution step	Counts per plate		Vc_1	Vc_2	lg N_a = lg ($\overline{X}$ or $\overline{X}$ wm) + 1	lg *R* (lg *N*w = *7,99*)	Contact time (min)
0,5 %	10^0	*>330+>330*	*>330+>330*	*>660*	*>660*	*> 4,81* **used for calculation*	*< 3,18*	*60*
	10^{-1}	*>330+315*	*324+305*	*>645**	*629**			
	10^{-2}	*39+40*	*20+33*	*79**	*53**			
	10^{-3}	*3+5*	*2+4*	*< 14*	*< 14*			
0,75 %	10^0	*308+320*	*>330+>330*	*628**	*> 660**	*3,80*	*4,19*	*60*
	10^{-1}	*27+20*	*30+30*	*47**	*60**			
	10^{-2}	*1+0*	*3+1*	*< 14*	*< 14*			
	10^{-3}	*0+0*	*0+0*	*< 14*	*< 14*			
1,0 %	10^0	*70+66*	*59+55*	*136**	*114**	*3,10*	*4,89*	*60*
	10^{-1}	*4+3*	*7+13*	*< 14*	*20*			
	10^{-2}	*0+0*	*0+0*	*< 14*	*< 14*			
	10^{-3}	*0+0*	*0+0*	*< 14*	*< 14*			

Remarks:

Explanations:

V_C = count per ml (one plate or more)
$\overline{x}$ = average of Vc_1 and Vc_2
$\overline{x}$ wm = weighted mean of $\overline{x}$

R = reduction (lg *R* = lg N_W – lg N_a)
If N_a < 140 , lg *R* = > [lg N_W – 2,15]
See 5.6.2 for calculation rules!

Annex ZA
(informative)

Relationship between this European Standard and the Essential Requirements of EU Directive 93/42/EEC

This European Standard has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association to provide a means of conforming to Essential Requirements of the New Approach Medical Devices Directive 93/42/EEC.

Once this standard is cited in the Official Journal of the European Communities under that Directive and has been implemented as a national standard in at least one Member State, compliance with the clauses of this standard given in Table ZA confers, within the limits of the scope of this standard, a presumption of conformity with the corresponding Essential Requirements of that Directive and associated EFTA regulations.

Table ZA.1 — Correspondence between this European Standard and Medical Devices Directive 93/42/EEC

Clause(s)/sub-clause(s) of this EN	Essential Requirements (ERs) of Medical Devices Directive 93/42/EEC	Qualifying remarks/Notes
Scope	Article 1 and Annex IX, 4.3	
The whole standard	Annex 1, Clause 7 and 8	

WARNING — Other requirements and other EU Directives may be applicable to the product(s) falling within the scope of this standard.

Bibliography

[1] European Pharmacopeia (EP - edition 1997, suppl 2000): *Water for injections*.

[2] European Pharmacopeia (EP - edition 1997, suppl 2000): *Glycerol.*

[3] EN 14820, *Single-use containers for venous blood specimen collection.*

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