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Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of mycobactericidal activity of chemical disinfectants and antiseptics used in the veterinary area — Test method and requirements (phase 2, step 1)

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National foreword

This British Standard is the UK implementation of EN 14204:2012. It supersedes BS EN 14204:2004 which is withdrawn.

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

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

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Foreword

This document (EN 14204:2012) has been prepared by Technical Committee CEN/TC 216 “Chemical disinfectants and antiseptics”, the secretariat of which is held by AFNOR.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by May 2013, and conflicting national standards shall be withdrawn at the latest by May 2013.

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

This document supersedes EN 14204:2004.

Data obtained using the former version of EN 14204 may still be used.

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

The following technical changes have been made: Membrane filtration (5.5.3) method and associated media and equipment have been added.

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

Introduction

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

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

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

1 Scope

This European Standard specifies a test method and the minimum requirements for mycobactericidal 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, 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 veterinary area – i.e. in the breeding, husbandry, production, transport and disposal of all animals except when in the food chain following death and entry to the processing industry.

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

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

NOTE 2 This method corresponds to a phase 2 step 1 test.

2 Normative references

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

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

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

3 Terms and definitions

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

4 Requirements

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

Table 1 - Obligatory and additional test conditions

Test conditions	Mycobactericidal activity
Test organism a) obligatory	*Mycobacterium avium*
b) additional	any relevant test organism
Test temperature a) obligatory	10°C ± 1°C
b) additional	4°C ± 1°C; 20°C ± 1°C; 40°C ± 1°C
Contact time a) obligatory	60 min ± 10 s[a]
b) additional	1 min ± 5 s; 5 min ± 10 s; 10 min ± 10 s, 15 min ± 10 s, 30 min ± 10 s, 120 min ± 10 s
Interfering substance a) obligatory low level soiling high level soiling	3,0 g/l bovine albumin 10 g/l yeast extract plus 10 g/l bovine albumin
b) additional	any relevant substance
NOTE For the additional conditions, the concentration defined as a result can be lower than the one obtained under the obligatory test conditions. [a] The obligatory contact time for disinfectants stated in Table 1 was chosen to enable comparison of standard conditions. The referenced test conditions are by no means intended as requirements for the use of a product. The recommended contact time for the use of the product is within the responsibility of the manufacturer.	

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

5 Test method

5.1 Principle

A sample of the product as delivered and/or diluted in hard water (or water for ready to use products) is added to a test suspension of mycobacteria in a solution of an interfering substance.

The mixture is maintained at (10 ± 1) °C for 60 min ± 10 s. At the end of this contact time, an aliquot is taken and the mycobactericidal action in this portion is immediately neutralized or suppressed by a validated method.

The number of surviving mycobacteria in each sample is determined and the reduction in viable counts calculated.

The test is performed using *Mycobacterium avium* as the test organism (Clause 4, Table 1).

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

5.2 Materials and reagents

5.2.1 Test organisms

5.2.1.1 The mycobactericidal activity shall be evaluated using the following strain :

Mycobacterium avium ATCC 15769[1)]

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

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

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

5.2.2 Culture media and reagents

5.2.2.1 General

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

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

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

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

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

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

5.2.2.2 Water

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

1) ATCC 15769 is the collection number of strain supplied by the American Type 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.

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 5.2.2.6 for the procedure to prepare hard water.

5.2.2.3 Middlebrook and Cohn 7H10 medium + 10 % oleic acid albumin dextrose complex (OADC) (reported as 7H10 in the text).

For performance of viable counts:

— Middlebrook 7H10 agar 19 g ;

— Glycerol 5 ml ;

— Water (see 5.2.2.2) to 895 ml.

Heat to boiling to dissolve completely. Sterilize for 10 min in the autoclave and cool to 50 °C to 55 °C. Add 100 ml Middlebrook OADC enrichment under aseptic conditions. Final pH = 6,6 ± 0,2 at 25 °C.

In case of encountering problems with neutralization (5.5.1.2), it may be necessary to add neutralizer to the 7H10. Annex B gives guidance on the neutralizers that may be used.

5.2.2.4 Diluent

Tryptone sodium chloride solution, consisting of:

— Tryptone, pancreatic digest of casein 1,0 g;

— Sodium Chloride (NaCl) 8,5 g;

— Water (see 5.2.2.2) to 1 000 ml.

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

5.2.2.5 Neutralizer

The neutralizer shall be validated for the product being tested in accordance with 5.5.1.2 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.

Sterilize in the autoclave.

5.2.2.6 Hard water for dilution of products

For the preparation of 1 000 ml of hard water, the procedure is as follows:

— prepare solution *A*: dissolve 19,84 g magnesium chloride ($MgCl_2$) and 46,24 g calcium chloride ($CaCl_2$) in water (5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration (5.3.2.7) or in the autoclave [5.3.2.1a)]. Store the solution at 2 °C to 8°C for no longer than one month.

NOTE 1 In the case of loss of volume during sterilization by autoclave, make up to 1 000 ml with water (5.2.2.2) under aseptic conditions before storage.

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

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

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

NOTE 2 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 is lower than 375 mg/l of calcium carbonate ($CaCO_3$) in the test tube.

5.2.2.7 Interfering substances

5.2.2.7.1 General

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

The interfering substance shall be sterile and prepared at 10 times its final concentration in the test.

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

NOTE The term “interfering substance” is used even if it contains more than one substance (5.2.2.8).

5.2.2.7.2 Low-level soiling (Bovine albumin solution)

— Dissolve 3 g of bovine albumin (Cohn fraction V for Dubos Medium) in 90 ml of water (5.2.2.2) in a 100 ml volumetric flask (5.3.2.12). Make up to the mark with water (5.2.2.2) ;

— sterilize by membrane filtration (5.3.2.7) keep in the refrigerator (5.3.2.8) and use within one month.

The final concentration of the bovine albumin in the test procedure (5.5.2) is 3 g/l.

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

a) dissolve 50 g yeast extract powder in 150 ml of water (5.2.2.2) in a 250 ml volumetric flask (5.3.2.12) and allow foam to collapse. Make up to the mark with water (5.2.2.2). Transfer to a clean dry bottle and sterilize in an autoclave [5.3.2.1a)]. Allow to cool to 20 °C ± 5 °C.

b) pipette 25 ml of this solution into a 50 ml volumetric flask and add 10 ml of water (5.2.2.2). Dissolve 5 g of bovine albumin fraction V (suitable for microbiological purposes) in the solution with shaking and allow foam to collapse. Make up to the mark with water (5.2.2.2) sterilize by membrane filtration and keep in a refrigerator (5.3.2.8) and use within one month.

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

5.2.2.8 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.

5.3 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 [5.3.2.1a)];

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

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 (4 ±1) °C, (10 ± 1) °C, (20 ± 1) °C, (40 ± 1) °C, (45 ± 1) °C and 50 °C to 55 °C.

5.3.2.3 CO_2 Incubator, capable of being controlled at either (36 ±1) °C or (37 ± 1) °C. An incubator at (36 ± 1) °C or (37 ± 1)°C may be used if a CO_2 incubator is not available.

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

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

5.3.2.5 Stopwatch

5.3.2.6 Shaker

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

b) Mechanical shaker

2) Disposable Sterile Equipment is an acceptable alternative to reusable glassware.

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

5.3.2.7 Membrane filtration apparatus, constructed of a material compatible with the substances to be filtered.

The apparatus shall have a filter holder of at least 50 ml volume. It shall be suitable for use with filters of diameter 47 mm to 50 mm and 0,45 µm pore size for filtration of hard water (5.2.2.6), bovine albumin (5.2.2.7.2 and 5.2.2.7.3) and if the membrane filtration method (5.5.3) is used.

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 °C to 8 °C.

5.3.2.9 Graduated pipettes, of nominal capacities 10 ml and 1 ml and 0,1 ml. Calibrated automatic pipettes may be used.

5.3.2.10 Petri dishes of size 90 mm to 100 mm.

5.3.2.11 Glass beads 3 mm to 4 mm in diameter.

5.3.2.12 Volumetric flasks.

5.3.2.13 High Speed homogenizer, e.g. Potter ® apparatus[4)]

5.3.2.14 Containers, test tubes, flasks or bottles of suitable capacity.

5.3.2.15 Coned bottom screw cap tube

5.4 Preparation of mycobacterial test suspension and product test solutions

5.4.1 Mycobacterial test suspensions (test and validation suspension)

5.4.1.1 General

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 of test organism

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

5.4.1.3 Working culture of test organism

In order to prepare a working culture of the test organism (5.2.1) subculture from the stock culture (5.4.1.2) by streaking on to at least two plates of 7H10 medium (5.2.2.3) and incubate (5.3.2.3). If a CO_2 incubator is not used, plates shall be placed into polyethylene bags or sealed with insulating tape to prevent drying of the medium.

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 the working culture(s). Never produce and use a third subculture.

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

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

5.4.1.4 Mycobacterial test suspension (N)

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

— either 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 (5.3.2.15) containing 6-7 g of dry glass beads (5.3.2.11). The test organisms together with 2 ml of water (5.2.2.2) 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 re-suspend them by mixing [5.3.2.6a)]. After 20 min sedimentation time the supernatant is transferred to a tube.

— or 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). Make three consecutive washings with water (5.2.2.2) (centrifuging each time at approximately 2 000 gN for 15 min). After each centrifuging, discard the supernatant, re-suspend 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 units per millilitre 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 3,0 x 10^8 cfu/ml[5)] to 8,0 x 10^8 cfu/ml using water (5.2.2.2). Maintain this test suspension in the water bath at (20 ± 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.

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.

NOTE A colorimeter is a suitable alternative.

c) For counting prepare 10^{-6} and 10^{-7} dilutions of the test suspension using diluent (5.2.2.4). 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 18 ml to 20 ml of 7H10 medium (5.2.2.3), i.e. four plates per duplicate 1,0 ml samples.

  For incubation and counting see 5.4.1.6.

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

5.4.1.5 Validation suspension (N_V)

a) To prepare the validation suspension dilute the mycobacterial test suspension (5.4.1.4) with the diluent (5.2.2.4) to obtain 3 x 10^2 cfu/ml to 1,6 x 10^3 cfu/ml.

b) For counting prepare a 10^{-1} dilution with diluent (5.2.2.4). 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 18 ml to 20 ml of 7H10 medium (5.2.2.3), i.e. four plates per duplicate 1,0 ml samples.

   For incubating and counting see 5.4.1.6.

5.4.1.6 Incubation and counting of the test and validation suspensions

a) Incubate (5.3.2.3) the plates for 21 days at (36 ±1) °C or (37 ± 1) °C. Discard any which are not countable. Count the plates and determine the number of colony forming units (cfu) for each plate.

b) Incubate the plates for a further 7 days. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.

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

c) Calculate the number of cfu/ml in the test suspension N and in the validation suspension N_V using the method given in 5.6.2.3 and 5.6.2.5. Verify according to 5.7.

5.4.2 Product test solutions

The concentration of a product test solution shall be 1,25 times the desired test concentration because it is diluted to 80 % during the test and the method validation (5.5.2).

Product test solutions shall be prepared in hard water (5.2.2.6) at a minimum three different concentrations to include one concentration in the active range and one concentration in the non-active range (5.8.2). The product as received may be used as one of the product test solutions, in this case the highest tested concentration is 80 %.

Dilutions of ready-to-use products, i.e. products that are not diluted when applied, shall be prepared in water (5.2.2.2).

For solid products, dissolve the product as received by weighing at least 1,0 g ± 10 mg of the product in a volumetric flask and filling up with hard water (5.2.2.6) or water (5.2.2.2) for ready to use products. Subsequent dilutions (lower concentrations) shall be prepared in volumetric flasks (5.3.2.12) on a volume/volume basis in hard water (5.2.2.6).

For liquid products, dilutions of the product shall be prepared with hard water (5.2.2.6) or water (5.2.2.2) for ready to use products 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 homogeneous preparation that is stable during the whole procedure. If during the procedure a visible inhomogeneity appears due to the formation of a precipitate or flocculent (for example, through the addition of the interfering substance), it shall be recorded in the test report.

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

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

5.5 Procedure for assessing the mycobactericidal activity of the product

5.5.1 General

5.5.1.1 Experimental conditions (obligatory and additional)

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

a) temperature: in θ °C;

— the obligatory and additional temperatures to be tested are specified in Clause 4, Table 1;

— the allowed deviation for each chosen temperature is ± 1 °C;

b) contact time: *t* in min;

— the obligatory and additional contact times to be tested are specified in Clause 4, Table 1;

— the allowed deviation for each chosen contact time is ± 10 s, (except for 1 min ± 5 s);

c) interfering substance;

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

d) test organism;

Mycobacterium avium (Clause 4, Table 1).

Additional test organisms may be tested.

5.5.1.2 Choice of test method (dilution-neutralization or membrane filtration)

The method of choice is the dilution-neutralization method (5.5.2). To determine a suitable neutralizer, carry out the validation of the dilution neutralization method (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.

In special circumstances it may be necessary to add neutralizer to 7H10 medium (5.2.2.3). If neutralizer is added to 7H10 medium, the same amount shall be added to 7H10 medium used in the test procedure.

5.5.1.3 General instructions for validation and control procedures

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 or filtration control and method validation) shall be performed at the same time with the test and with the same neutralizer – or rinsing liquid – used in the test.

If because of problems with neutralization, a neutralizer has been added to 7H10 medium (5.2.2.3) used for the validation and control procedures, the 7H10 medium 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), diluent (5.2.2.4), hard water (5.2.2.6) and interfering substance (5.2.2.7) to the test temperature [5.5.1.1a)] using the water bath (5.3.2.2) controlled at θ. Check that the temperature of the reagents is stabilized at θ.

The neutralizer (5.2.2.5) or the rinsing liquid (5.2.2.8) and water (5.2.2.2) shall be equilibrated at a temperature of (20 ± 1) °C.

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

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

5.5.2.1 General

The test and the control and validation procedures (5.5.2.3, 5.5.2.4, 5.5.2.5, 5.5.2.6) shall be carried out at the same time.

5.5.2.2 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 (5.4.1).

5.5.2.3 Dilution-neutralization method Test Na – Determination of mycobactericidal concentration

The procedure for determining mycobactericidal concentration is as follows:

a) Pipette 1,0 ml of the interfering substance (5.2.2.7) into a tube. Add 1,0 ml of the mycobacterial test suspension (5.4.1.4). Start the stopwatch (5.3.2.5) immediately, mix [5.3.2.6a)], and place the tube in a water bath controlled at the chosen test temperature θ [5.5.1.1a)] for 2 min ± 10 s.

   At the end of this time, add 8,0 ml of one of the product test solutions (5.4.2). Restart the stopwatch at the beginning of the addition. Mix [5.3.2.6a)] and place the tube in a water bath controlled at θ for the chosen contact time *t* [5.5.1.1b)]. Just before the end of *t*, mix [5.3.2.6a)] again.

b) At the end of *t*, take a 1,0 ml of the test mixture "Na" and transfer into a tube containing 8,0 ml neutralizer (5.2.2.5) and 1,0 ml water (5.2.2.2). Mix [5.3.2.6a)] and place in a water bath controlled at (20°C ± 1)°C. After a neutralization time of 5 min ± 10 s, immediately take a sample of 1,0 ml of the neutralized mixture "Na" (containing neutralizer, product test solution, interfering substance and test suspension) and transfer to a tube containing 9,0 ml diluent (5.2.2.4). Take a sample of 1,0 ml of the neutralized and ten-fold diluted test mixture "Na" in duplicate. Divide each sample in two portions of approximately equal size and inoculate 7H10 plates (5.2.2.3) using the spread plate technique.

   For incubation and counting see 5.5.2.7.

c) Perform the procedures a) and b) using the other product test solutions at the same time.

d) Perform the procedures a) to c) applying the other obligatory and – if appropriate – other additional experimental conditions (5.5.1.1).

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

5.5.2.4 Experimental conditions control "*A*" – Validation of the selected experimental conditions and/or verification of the absence of any lethal effect in the test conditions

To validate the selected experimental conditions and/or verify the absence of any lethal effect in the test conditions, the procedure is as follows.

a) Pipette 1,0 ml of the interfering substance used in the test 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 (5.2.2.6). 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 each sample in two portions of approximately equal size and inoculate 7H10 plates (5.2.2.3) using the spread plate technique.

For incubation and counting, see 5.5.2.7.

5.5.2.5 Neutralizer control "*B*" – Verification of the absence of toxicity of the neutralizer

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

a) Pipette 8,0 ml of the neutralizer – used in the test (5.5.2.1) — and 1,0 ml of water (5.2.2.2) into a tube. Add 1,0 ml of the validation suspension (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 ± 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 each sample in two portions of approximately equal size and inoculate 7H10 plates (5.2.2.3) using the spread plate technique.

For incubation and counting, see 5.5.2.7.

5.5.2.6 Method validation "*C*" – Dilution-neutralization validation

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

a) Pipette 1,0 ml of the interfering substance used in the test into a tube. Add 1,0 ml of the diluent (5.2.2.4) and then, starting a stopwatch, add 8,0 ml of the product test solution only of the highest concentration used in the test. 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* transfer 1,0 ml of the mixture into a tube containing 8,0 ml of neutralizer (5.2.2.5). Restart the stopwatch immediately at the beginning of the addition. Mix [5.3.2.6a)] and place the tube in a water bath controlled at (20 ± 1) C for 5 min ± 10 s. Add 1,0 ml of the validation suspension (5.4.1.5). Start a stopwatch (5.3.2.5) at the beginning of the addition and mix [5.3.2.6a)]. Place the tube in a water bath controlled at (20 ± 1) °C for the contact time *t*. 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 each sample in two portions of approximately equal size and inoculate 7H10 plates (5.2.2.3) using the spread plate technique.

For incubation and counting, see 5.5.2.7.

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

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

a) Incubate (5.3.2.3) the plates for 21 days at (36 ±1) °C or (37 ± 1) °C. Discard any plates which are not countable. Determine the number of colony forming units for each plate. Incubate the plates for a further 7 days. Do not recount plates which no longer show well-separated colonies. Recount the remaining plates. If the number of colony forming units has increased, use only the higher count for further evaluation.

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

c) Calculate the numbers of cfu/ml in the test mixture "Na" and the validation mixtures "*A*", "*B*" and "*C*" using the method given in 5.6.2.4 and 5.6.2.5. Verify according to 5.7.

5.5.3 Test procedure - Membrane filtration method

5.5.3.1 General

The test and the control and validation procedures (5.5.3.2 through 5.5.3.5) shall be carried out in parallel and separately for each experimental condition (5.5.1.1).

Each membrane filtration apparatus shall be equipped with a membrane of 0,45 µm pore size and 47 mm to 50 mm diameter (5.3.2.7) and filled with 50 ml of the rinsing liquid (5.2.2.8). The time required for filtering — if longer than one minute in exceptional cases — shall be recorded in the test report. When transferring the membranes to the surface of an agar plate, care should be taken to ensure that the test organisms are on the upper side of the membrane when placed on the plate, and to avoid trapping air between the membrane and agar surface.

5.5.3.2 Test "Na" – Determination of the mycobactericidal concentrations

The procedure for determining the bactericidal concentrations is as follows.

a) See 5.5.2.3a).

b) At the end of *t*, take a sample of 0,1 ml of the test mixture Na in duplicate and transfer each 0,1 ml sample into a separate membrane filtration apparatus (5.3.2.7). Filter immediately.

   Immediately take another sample of 1,0 ml of the test mixture Na and transfer the sample in a tube containing 9,0 ml diluent (5.2.2.4), mix, take a sample of 0,1 ml of the test mixture Na $^{-1}$ in duplicate and transfer each 0,1 ml sample into a separate membrane filtration apparatus (5.3.2.7).

   For all the filtrations, filter through at least 150 ml but no more than 500 ml of rinsing liquid (5.2.2.8). If the rinsing liquid is not water, complete the procedure by filtering 50 ml of water (5.2.2.2). Then transfer each of the membranes to the surface of separate 7H10 medium plates.

c) For incubation and counting, see 5.5.3.6.

d) See 5.5.2.3c).

e) See 5.5.2.3d).

5.5.3.3 Experimental conditions control "*A*" – Validation of the selected experimental conditions and/or verification of the absence of any lethal effect in the test conditions

To validate the selected experimental conditions and/or verify the absence of any lethal effect in the test conditions, the procedure is as follows.

a) See 5.5.2.3a).

b) At the end of *t*, take a sample of 1,0 ml of this mixture *A* in duplicate and transfer each 1,0 ml sample into a separate membrane filtration apparatus (5.3.2.7). Filter immediately and additionally with 50 ml of water (5.2.2.2). Then transfer each of the membranes to the surface of separate 7H10 medium plates (5.2.2.3).

For incubation and counting, see 5.5.3.6.

5.5.3.4 Filtration control "*B*" – validation of the filtration procedure

To validate the filtration procedure proceed as follows.

a) Take 0,1 ml of the validation suspension (5.4.1.5) in duplicate (suspension for control *B*) and transfer each 0,1 ml sample into a separate membrane filtration apparatus (5.3.2.7).

b) Filter immediately. Filter through the rinsing liquid (5.2.2.8) the same way as in the test [5.5.3.2b)]. If the rinsing liquid is not water, complete the procedure by filtering 50 ml of water (5.2.2.2). Then transfer each of the membranes to the surface of separate 7H10 medium plates (5.2.2.3).

For incubation and counting, see 5.5.3.6.

5.5.3.5 Method validation "*C*" – Validation of the membrane filtration method or counting of the bacteria on the membranes which have previously been in contact with the mixture of product and interfering substance

For validation of the membrane filtration method or counting of the bacteria on the membranes which have previously been in contact with the mixture of product and interfering substance, the procedure is as follows.

a) See 5.5.2.5a).

b) At the end of *t*, take 0,1 ml of the validation mixture *C* in duplicate and transfer each 0,1 ml sample into a separate membrane filtration apparatus (5.3.2.7). Filter immediately. Filter through the rinsing liquid (5.2.2.8) the same way as in the test [5.5.3.2b)], then cover the membranes with 50 ml of the rinsing liquid (5.2.2.8) and add 0,1 ml of the validation suspension (5.4.1.5). Filter immediately again and additionally with 50 ml of water (5.2.2.2), then transfer each of the membranes to the surface of separate 7H10 medium plates (5.2.2.3).

For incubation and counting, see 5.5.3.6.

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

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

a) Incubate (5.3.2.3) the plates for 21 days at (36 ± 1) °C or (37 ± 1) °C. Discard any plates which are not countable (for any reason). Count the colonies on the membranes.

Incubate the plates for a further 7 days. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. If the number has increased use only the higher number for further evaluation.

b) Note for each plate the exact number of colonies but record ">165" for any counts higher than 165 and determine the V_C values according to 5.6.2.2.

c) Calculate the numbers of colony forming unit per millilitre (cfu/ml) in the test mixture, Na, and in the validation mixtures *A*, *B* and *C* using the method given in 5.6.2.4 and 5.6.2.5. Verify according to 5.7.

5.6 Experimental data and calculation

5.6.1 Explanation of terms and abbreviations

5.6.1.1 Overview of the different suspensions and test mixtures

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

N, N_v, N_0, N_{V0}, N_a and *A*, *B* and *C* represent the number of cells counted per ml in the different test mixtures in accordance with Table 2.

Table 2 - 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 5 min (*B*) or 60 min (*C*)
Test	*N* Test suspension	N_0 (= *N*/10)	N_a (before neutralisation)
Controls	N_v Validation suspension	N_{V0} (= N_v/10)	*A, B, C*

5.6.1.2 V_C values

All experimental data are reported as V_c values:

— in the test and controls, a V_c value is the number of colony-forming units counted per 1,0 ml sample

5.6.2 Calculation

5.6.2.1 General

The first step in the calculation is the determination of the V_C values, the second the calculation of *N*, N_0, N_a, N_v, N_{V0}, *A*, *B* and *C*. The third step is the calculation of the reduction R (5.8.1).

5.6.2.2 Determination of V_C values

The V_c values are determined as follows.

a) The usual limits for counting mycobacteria on agar plates are between 15 and 300. In this standard a deviation of 10 % is accepted, so the limits are 14 and 330. On membranes the usual upper limit is 150, therefore, with the 10 % deviation, the limit is 165.

NOTE The lower limit (14) is based on the fact that the variability is increasing the smaller the number counted in the sample (1 ml or 0,1 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.

The upper limits (330, 165) reflect the imprecision of counting confluent colonies and growth inhibition due to nutriment depletion. They refer only to the counting on one plate and not necessarily to the sample.

b) For counting the test suspension N (5.4.1.4), the validation suspension N_v (5.4.1.5) and for all countings of the method (5.5.2), determine and record the V_c values according to the number of plates used per 1 ml sample (5.6.2.2).

If at least one of the duplicate plates shows a number higher than 330, report this V_c value as "> sum of the counts" (e.g. for ">330, 310, report ">640").

If a V_c value is lower than 14, report the number but substitute by "<14" for further calculation in the case of N_a (e.g. 5,8, report as <14).

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_0

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) are evaluated, calculate the number of cfu/ml as the weighted mean count using the following formula:

$$N = \frac{c}{(n_1 + 0{,}1 n_2) \times 10^{-6}} \quad (1)$$

where

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

n_1 is the number of V_c values (for 1 ml) taken into account in the lower dilution i.e. 10^{-6};

n_2 is the number of V_c values (for 1 ml) taken into account at the higher dilution i.e. 10^{-7};

10^{-6} is the dilution factor corresponding to the lower dilution.

Round off the results calculated to two significant figures. For this, if the last figure is below 5, the preceding figure is not modified; if the last figure is above 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

$$N = \frac{(430 + 405 + 37 + 25)}{(2 + 0{,}2 \times 1) \times 10^{-6}} = \frac{897}{2{,}2 \times 10^{-6}} = 4{,}0773 \times 10^{8} = 4{,}1 \times 10^{8} = \lg 8{,}6$$

N_0 is the number of cells per ml in the test mixture [5.5.2.3a)] at the beginning of the contact time (time "zero" = 0). It is one-tenth of the weighted mean of N due to the tenfold dilution by the addition of the product and interfering substance.

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

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

where

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

n is the 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 (4 plates of 0,5 ml) N_a^{-1}: 1,1; 8,8

$$N_a = \frac{(14+16)\times 10}{2} = <150\times 10^1 = <1500 = <1{,}5\times 10^3 = \lg(<1{,}5\times 10^3) = <3{,}18$$

duplicate V_C-values N_a^{-1} (4 plates of 0,5 ml): > 330,>330; > 330, >330

$$N_a = \frac{(660+>660)\times 10}{2} = >6600\times 10 = >66000 = >6{,}6\times 10^4 = \lg(>6{,}6\times 10^4) = >4{,}82$$

duplicate V_C-values N_a^0 (2 membranes) > 165,160

$$N_a = \frac{(165+160)\times 10}{2} = >1625\times 10^0 = >1625 = >1{,}6\times 10^3 = \lg(>1{,}6\times 10^3) = >3{,}21$$

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

EXAMPLE 2 If all subsequent dilutions of N_a show mean values of "more than”, take only the highest dilution (10^{-1}) as result for N_a.

	V_{C1}	V_{C2}		mean x 10	
N_a^0	> 660	> 660	=	> 6 600	
N_a^{-1}	> 660	> 660	=	> 6 600	N_a = > 6 600 x 10^1 = > 6,6 x 10^4

EXAMPLE 3 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.

	V_{C1}	V_{C2}		mean x 10	
N_a^0	< 14	18	=	< 160	N_a = < 160 x 10^0 = < 1,6 x 10^2
N_a^{-1}	< 14	< 14	=	< 140	

EXAMPLE 4 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.

	V_{C1}	V_{C2}		mean x 10	
N_a^0	> 660	> 660	=	> 6 600	
N_a^{-1}	96	107	=	1 015	N_a = 1 015 x 10^1 = 1,0 x 10^4

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

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 3:

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

where

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

z is the dilution factor corresponding to the lower dilution i.e. N_a^0 is the lower dilution in comparison with N_a^{-1}.

EXAMPLE 5 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 below.

	V_{C1}	V_{C2}	mean x 10
N_a^0	> 538	320	> 4 290
N_a^{-1}	46	57	515

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

$$> 4\,368{,}18 = > 4{,}4 \text{ x } 10^{3}$$

EXAMPLE 6 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.

	V_{C1}	V_{C2}		mean x 10
N_a^0	256	200	=	2 280
N_a^{-1}	< 14	26	=	< 200

$N_a = 2\,280 \text{ x } 10^0 = 2{,}3 \text{ x } 10^3$

5.6.2.4 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} (5.4.1.5). N_{V0} is the number of cells per ml in the mixtures *A*, *B* and *C* at the beginning of the contact time (time 0) (5.6.1.1). It is one-tenth of the V_C values of N_V (5.4.1.6) taken into account.

Calculate N_V and N_{V0} using the following formulae 4 and 5:

$N_V = 10c/n$

$N_{V0} = c/n$

where

c is the sum of V_C values taken into account (for 1 ml);

n is the number of V_C values taken into account.

5.6.2.5 Calculation of *A*, *B* and *C*

A, *B* and *C* are the numbers of survivors in the experimental conditions control *A* (5.5.2.4), neutralizer control *B* (5.5.2.5) and method validation *C* (5.5.2.6) at the end of the contact time *t* or the defined times 5 min (*B*) and 60 min (*C*). They correspond to the V_C values of the mixtures *A*, *B* and *C* taken into account.

Calculate *A*, *B* and *C* using the following formula 6:

A, *B*, *C* = *c*/*n*

where

c is the sum of V_C values taken into account (for 1ml);

n is the 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

— the requirements of 5.8.2 are fulfilled.

The test is also valid if none of the product dilutions achieve the required log reduction and all the results meet the criteria of 5.7.3. In that case the product does not pass the test at these concentrations.

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 mean of the two results shall be not higher than 15 and not lower than 5. Results below the lower limit are taken as the lower limit number (14). Results above the respective upper limit (330) are taken as the upper limit number.

EXAMPLE For N: 10^{-6} dilution: 84 (40 + 44) + 108 (58 + 50) cfu/1,0ml , 10^{-7} dilution: 20 (11 +9)+ < 14 (5 + 6) cfu/1,0ml; = 192/34 = 5,6 therefore between 5 and 15

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

5.7.3 Basic limits

Check that:

a) *N* is between 3,0 x 10^8 and 8,0 x 10^8 (8,48 ≤ lg *N* ≤ 8,90)

N_0 is between 3,0 x10^7 and 8,0 x 10^7 (7,48 ≤ lg N_0 ≤ 7,90)

b) N_{V0} is between 30 and 160 (3,0 x 10^1 and 1,6 x 10^2) (N_V is between 3,0 x10^2 and 1,6 x 10^3)

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

d) *B* is equal to or greater than 0,5 x N_{V0}

e) *C* is equal to or greater than 0,5 x N_{V0}

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

5.8 Expression of results

5.8.1 Reduction

The reduction ($R = N_0/N_a$) is expressed in logarithm.

Record the number of cfu/ml in the test suspension *N* (5.6.2.3) and the test N_a (5.6.2.4). Calculate N_0 (5.6.2.3).

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

$$\lg R = \lg N_0 - \lg N_a$$

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

At least one concentration per test (5.5.2) shall demonstrate a 4 lg or more reduction and at least one concentration shall demonstrate a lg reduction of less than 4.

5.8.3 Mycobactericidal concentration

Record the lowest concentration of the product which passes the test (lg $R \geq 4$).

5.9 Interpretation of results - conclusion

5.9.1 General

According to the chosen experimental conditions (obligatory or obligatory and additional) the mycobactericidal concentrations determined according to this standard may differ (Clause 4).

A product can only pass the test if the requirements of 5.8.2 are fulfilled.

5.9.2 Mycobactericidal activity for general purposes

The product shall be deemed to have passed the EN 14204 standard if it demonstrates in a valid test at least a 4 lg reduction within 60 min or less at 10 °C with the chosen interfering substance simulating low- or high-level soiling conditions defined by this standard when the test organism is *Mycobacterium avium*.

5.9.3 Qualification for certain fields of application

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

For more details see EN 14885.

5.10 Test report

The test report[7)] shall refer to this European Standard (EN 14204).

Any operation not included in the standard as well as any incidents which may have affected the results shall be reported.

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

a) identification of the laboratory;

b) identification of the client;

c) identification of the sample:

   1) name of the product;

   2) batch number;

   3) manufacturer;

   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) test method and its validation:

   Full details of the tests for validation of the neutralizer shall be given.

e) experimental conditions:

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

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

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

   4) appearance of product dilutions;

   5) contact time(s);

   6) test temperature(s);

   7) interfering substance;

   8) stability and appearance of the mixture during the procedure (note the formation of any precipitate or flocculant);

   9) temperature of incubation;

[7)] An example of a typical test report is given in Annex D.

10) neutralizer;

11) identification of the mycobacterial strain;

f) test results:

1) controls and validation;

2) evaluation of mycobactericidal 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 strain in national collections

Mycobacterium avium -	ATCC	15769
	CIP	105415

Abbreviations:

ATCC American Type Culture Collection

CIP Collection de l'Institut Pasteur

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 shall be validated according to the prescriptions of the standard.

Table B.1 - Neutralizers and rinsing liquids (*1 of 2*)

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[c)], Saponin, Polysorbate 80, Sodium dodecyl sulphate, Ethylene oxide condensate of fatty alcohol (non-ionic surfactants) [b)]	- 30 g/l Polysorbate 80 + 30 g/l saponin + 3g/l lecithin[c)] - 30 g/l Polysorbate 80 + 4 g/l sodium dodecyl sulphate + 3g/l.lecithin[c)] - 3 g/l Ethylene oxide condensate of fatty alcohol + 20g/l lecithin[c)] + 5g/l polysorbate 80 - *Rinsing liquid: 1g/l tryptone + 9 g/l NaCl + 5 g/l polysorbate 80.*
Biguanides and similar compounds	Lecithin[c)], Saponin, Polysorbate 80	- 30 g/l Polysorbate 80 + 30 g/l saponin + 3g/l lecithin[c)]. - *Rinsing liquid: 1g/l tryptone + 9 g/l NaCl + 5 g/l polysorbate 80.*
Oxidizing compounds (Chlorine, iodine, hydrogen peroxide, peracetic acid, hypochlorites, etc…)	Sodium thiosulphate [d)] Catalase [for hydrogen peroxide or products releasing hydrogen peroxide]	- 3 g/l to 20 g/l Sodium thiosulphae + 30 g/l polysorbate 80 + 3 g/l lecithin[c)]. - 50 g/l Polysorbate 80 + 0,25g/l catalase + 10 g/l lecithin[c)]. - *Rinsing liquid: 3 g/l sodium thiosulphate*
Aldehydes	L-histidine Glycine	- 30 g/l Polysorbate 80 + 3g/l lecithin[c)] + 1g/l L-histidine (or + 1 g/l glycine). - 30 g/l Polysorbate 80 + 30 g/l saponin + 1 g/l L-histidine (or + 1 g/l glycine). - *Rinsing liquid: 5 g/l polysorbate 80 + 0,5 g/l L-histidine (or + 1 g/l glycine).*

Table B.1 - Neutralizers and rinsing liquids *(2 of 2)*

Antimicrobial agent	**Chemical compounds able to neutralize residual antimicrobial activity**	**Examples of suitable neutralizers** [a]
Phenolic and related compounds: orthophenylphenol, phenoxyethanol, triclosan, phenylethanol, etc. Anilides	Lecithin[c] Polysorbate 80 Ethylene oxide condensate of fatty alcohol[b]	- 30 g/l Polysorbate 80 + 3g/l lecithin[c]. - 7 g/l Ethylene oxide condensate of fatty alcohol + 20 g/l lecithin[c] + 4 g/l polysorbate 80. - *Rinsing liquid: 1 g/l tryptone + 9 g/l NaCl + 5 g/l polysorbate 80.*
Alcohols	Lecithin[c], Saponin, Polysorbate 80 [e]	- 30 g/l Polysorbate 80 + 30 g/l saponin + 3 g/l lecithin[c]. - *Rinsing liquid: 1 g/l tryptone + 9 g/l NaCl + 5 g/l polysorbate 80.*

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 (KH2PO4) 34 g; distilled water (500 ml); adjusted to pH 7,2 ± 0,2 with sodium hydroxide (NaOH) 1 mol/l; distilled water up to 1000 ml].

b) The carbon chain-length varies from C12 to C18 carbon atoms.

c) Egg and soya; egg is preferable.

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

e) For the neutralization of short chain alcohols (less than C5), simple dilution may be appropriate. Care should be taken if the alcohol-based products contain additional antimicrobial agents.

Annex C
(informative)

Graphical representations of dilution-neutralization method

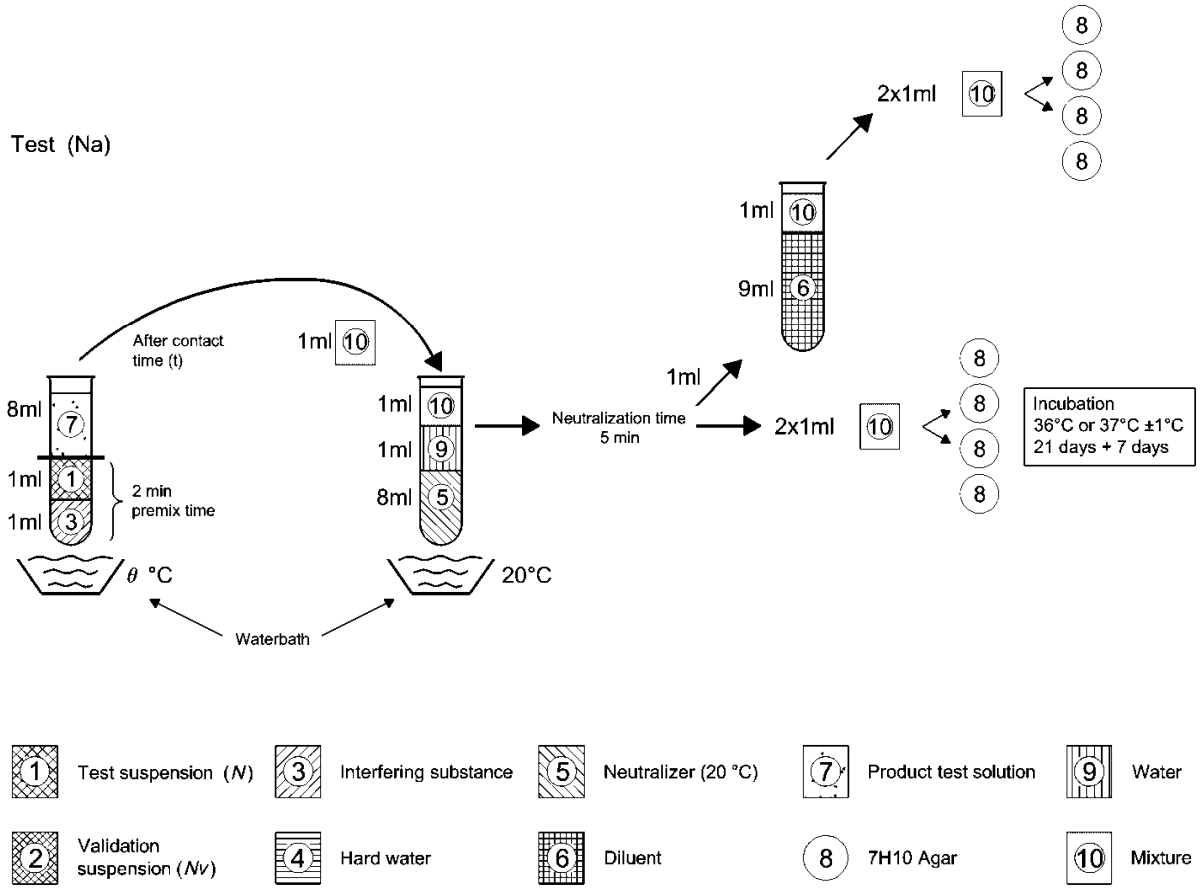

Figure C.1

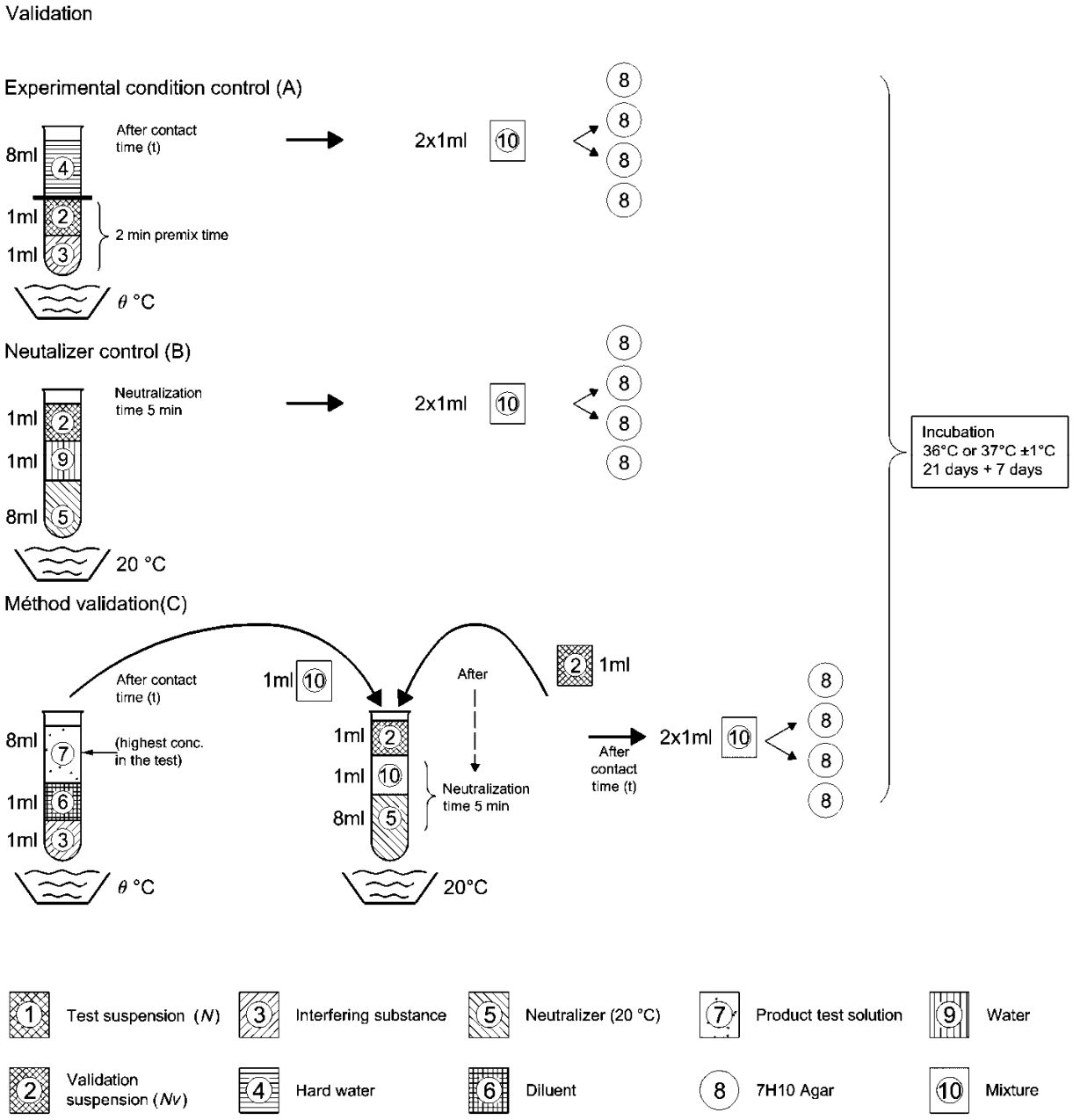
Validation
Experimental condition control (A)
8ml
1ml
1ml
After contact time (t)
2 min premix time
θ °C
2x1ml
Neutalizer control (B)
Neutralization time 5 min
20 °C
Incubation
36°C or 37°C ±1°C
21 days + 7 days
Méthod validation(C)
After contact time (t)
(highest conc. in the test)
1ml
1ml
1ml
θ °C
1ml
After
Neutralization time 5 min
8ml
20°C
After contact time (t)
2x1ml
Test suspension (N)
Validation suspension (Nv)
Interfering substance
Hard water
Neutralizer (20 °C)
Diluent
Product test solution
7H10 Agar
Water
Mixture

Figure C.2

Annex D
(informative)

Example of a typical test report

NOTE All names and examples in Annex D are fictitious apart from those used in the standard.

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TEST REPORT

EN 14204, MYCOBACTERICIDAL ACTIVITY

(obligatory and additional conditions)

Client: Centipede Formulations Inc., Mannheim / Euroland

Disinfectant sample

Name of the product: W Batch number: 11-47-013

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 its/their concentration(s): Not indicated

Product diluent recommended by the manufacturer for use: Potable water

Period of testing

Date of delivery of the product: 2010-10-06 **Dates of tests**: see "Test results" (attached)

Experimental conditions

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

Obligatory conditions:

Test organism: *Mycobacterioum avium* ATCC 15769

Test temperature: 10 °C Contact time: 60 min

Interfering substance: 3,0 g/l bovine albumin = low-level soiling;

Incubation temperature: 36 °C

Neutralizer: 30 g/l lecithin, sterilized in the autoclave

Additional conditions 1

Test temperature: 40 °C Contact time: 60 min

Interfering substance: 10,0 g/l bovine albumin + 10,0 g/l yeast extract = high level soiling

Temperature of incubation 36 °C

Bacterial strain used: *Mycobacterioum avium* ATCC 15769

Additional conditions 2

Test temperature: 4 °C Contact time: 60 min

Interfering substance: 10,0 g/l bovine albumin + 10,0 g/l yeast extract = high level soiling

Temperature of incubation: 36 °C

Bacterial strain used: *Mycobacterioum avium* ATCC 15769

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).

Conclusion:

According to EN 14204 (date of edition) the batch 11-47-013 of the product W possesses mycobactericidal activity for the reference strain of *Mycobacterium avium* at a concentration of 1,00 % (v/v) at 10 °C, with a 60 min contact time under high-level soiling conditions.

According to EN 14204 (date of edition) the batch 11-47-013 of the product W possesses mycobactericidal activity at a concentration of 0,25 % (v/v) at 40 °C, with a 60 min contact time under high-level soiling conditions.

According to EN 14204 (date of edition) the batch 11-47-013 of the product W possesses mycobactericidal activity at a concentration of 2,5 % (v/v) at 4 °C, with a 60 min contact time under high-level soiling conditions.

Antiseptville, 2010-11-07

Alexandra May, MD, PhD, Scientific Director

Test results (mycobactericidal quantitative suspension test)

EN 14204 (Phase 2. step.1) Product-name: .....W..... Batch No: 10-47-013

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

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

Dilution neutralization method

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

Pour plate Spread plate ✓ Number of plates: 2 / ml Neutralizer: 30 g/l lecithin.

Test temperature: 10 °C Interfering substances: 10,0 g/l bovine albumin + 10,0 g/l yeast extract

Test organism: *Mycobacterium avium* ATCC 15769 Incubation temp: 36 °C

Internal lab. no: Date of test: 2010-11-06 Responsible person: Fang Signature: Fang.

Validation and controls

Validation suspension (N_{V0})		Experimental Conditions control (*A*)		Neutralizer control (*B*)		Method validation (*C*) Product conc.: *10 ml/l*	
V_C	*86 (40 + 46),* 78 *(35 +43)* *= 82*	V_C	*79 (43 + 36),* 84 *(44 + 40)* *= 81,5*	V_{C1}	*86 (42 + 44),* *94* (48 + 46) = 90	V_{C1}	*75 (35 + 40),* 80 (35 + 45) = 77,5
14 ≤ V_C of N_{V0} ≤ 330 ? ☒ *yes* ☐ *no*		V_C of *A* is ≥ 0,5 x $\bar{x}$ of N_{V0}? ☒ *yes* ☐ *no*		V_C of *B* is ≥ 0,5 x $\bar{x}$ of N_{V0}*?* ☒ *yes* ☐ *no*		V_C of *C* is ≥ 0,5 x $\bar{x}$ of N_{V0}? ☒ *yes* ☐ *no*	

Test suspension and Test

Test-suspension		V_{C1}	V_{C2}	*N = 301,36* x 10^6 (lg *N* = 8,48)
(*N* and N_0):	10^{-6}	*(150 + 150) = 300*	*(150 + 148) =298*	N_0 = *N*/10 (lg N_0 = 7,48)
	10^{-7}	*(15, 15) =30*	*(20, 15) =35*	7,48 ≤ N_0 ≤ 7,90? ☒ *yes* ☐ *no*

Conc. of the product %	Dilution	V_{C1}	V_{C2}	N_a = $\bar{x}$ x10	lg N_a	lg*R* (N_0=7,48)	Contact time (min)
0,50	N_a^{-1} 10^{-1}	*(>330 +, >330) = >330*	*(>330 +, 330) = >330*	*>3300 x10*[1]	*>4,52*	*<2,96*	*60 min*
0,75	N_a^0 Neutralized mixture	*(>330 +, >330) = >330*	*(150 + 148) =298*	*>3140*	*>3,50*	*<3,98*	*60 min*
1,00	N_a^0 Neutralized mixture	*(125 + 115) =240*	*(110 + 130) =240*	*2400*	*3,38*	*4,10*	*60 min*

Remarks

Explanations:

V_C = count per 1,0 ml (two plates)

R = reduction (lg R = lg N_0 – lg N_a)

Bibliography

[1] European Pharmacopoeia (EP), Edition 1997 supplement 2000, Water for injections.

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