Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas — Test method and requirements (Phase 2, step 1)
KS 929: 2015

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In order to keep abreast of progress in industry, Kenya standards shall be regularly reviewed. Suggestions for improvement to published standards, addressed to the Managing Director, Kenya Bureau of Standards, are welcome.
Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas — Test method and requirements (Phase 2, step 1)
Foreword

This Kenya Standard was developed by the Technical Committee on Disinfectants and Bleaches under the guidance of the Standards Project Committee, and it is in accordance with the procedures of the Kenya Bureau of Standards.

It describes a suspension test method for establishing whether a chemical disinfectant or antiseptic has or does not have a bactericidal activity in the fields described in clause 1. The laboratory test closely simulates practical conditions of application. Chosen conditions (contact time, temperature, organisms in suspension) reflect parameters which are found in practical situations including conditions which may influence the action of antiseptics or disinfectants. Each utilization concentration found from this test corresponds to defined experimental conditions. The conditions are intended to cover general purposes and to allow reference between laboratories and product types. However for some applications the recommendations of use of a product may differ and therefore additional test conditions need to be used.

These QAC based aromatic disinfectants are used in households and domestic service disinfecting.

During the preparation of this standard, reference was made to the following documents:


BS 6471: 1984   Method for determination of the antimicrobial value of QAC disinfectant formulation.


BS EN 1276:1997 Chemical disinfectants and antiseptics. Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas Test method and requirements (phase 1, step 1).

Acknowledgement is hereby made for the assistance received from these sources.
Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas — Test method and requirements (phase 2, step 1)

1 Scope

This Kenya Standard specifies a test method (phase 2/step 1) and the minimum requirements for bactericidal activity of chemical disinfectant and antiseptic products that form a homogeneous, physically stable preparation in hard water and that are used in food, industrial, domestic and institutional areas, excluding areas and situations where disinfection is medically indicated and excluding products used on living tissues except those for hand hygiene in the above considered areas.

This Standard applies at least to the following:

a) processing, distribution and retailing of:

1) food of animal origin:
   — milk and milk products;
   — meat and meat products;
   — fish, seafood, and related products;
   — eggs and egg products;
   — animal feeds;

2) food of vegetable origin:
   — beverages;
   — fruits, vegetables and derivatives (including sugar, distillery;
   — flour, milling and baking;
   — animal feeds;

b) institutional and domestic areas:
   — catering establishments;
   — public areas;
   — public transport;
   — schools;
   — nurseries;
   — shops;
   — sports rooms;
   — waste containers (bins);
   — hotels;
   — dwellings;
   — clinically non sensitive areas of hospitals;
   — offices;

c) other industrial areas:
KS 929: 2015

— packaging material;
— biotechnology (yeast, proteins, enzymes);
— pharmaceutical;
— cosmetics and toiletries;
— textiles;
— space industry, computer industry;

By using this Kenya Standard, it is not possible to determine the bactericidal activity of undiluted product as some dilution is always produced by adding the inoculum and interfering substance. Products can only be tested at a concentration of 80 % or less.

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

2 Normative references

The following referenced documents are indispensable for the application of this publicly available standard. 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 microbial strains used for the determination of bactericidal and fungicidal activity

EN 1040, Chemical disinfectants and antiseptics - Basic bactericidal activity - Test method and requirement

3 Definitions

For the purpose of this specification, the following definitions shall apply:

3.1 product (for chemical disinfection and/or antisepsis)
chemical agent or formulation used as a chemical disinfectant or antiseptic

3.2 bactericide
product which kills vegetative bacteria under defined conditions

NOTE The adjective derived from ‘bactericide’ is ‘bactericidal’.

3.3 bactericidal activity
capability of the product to produce at least a $10^5$ reduction in the number of viable bacterial cells belonging to reference strains of Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus and Enterococcus hirae under conditions defined by this standard

3.4 clean conditions
conditions representative of surfaces which have received a satisfactory cleaning programme and/or are known to contain minimal levels of organic and/or inorganic materials

3.5 dirty conditions
conditions representative of surfaces which are known to or may contain, organic and/or inorganic materials
4.0 Requirements

The product, when diluted in hard water and tested in accordance with clause 5 under simulated clean conditions (0.3 g/L bovine albumin see 3.4) or dirty conditions (3 g/L bovine albumin see 3.5) according to its practical applications and under the required test conditions (20 ºC, 5 min, 4 selected reference strains), shall demonstrate at least a $10^5$ log reduction in viable counts.

The bacterial activity shall be evaluated using the following four test organisms: *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus hirae*.

The determined bactericidal concentration of the test product is suggested as being suitable for practical situations of use.

Where appropriate, additional specific bactericidal activity shall be determined under other conditions of time, temperature, additional strains and interfering substances (see 5.2.1 and 5.5.1) in accordance with 5.5.1 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 initial test conditions of 20 ºC, 5 min, 4 selected reference strains.

4.1 Test method

Principle

4.1.1 A test suspension of bacteria in a solution of interfering substances is added to a prepared sample of the product under test diluted in hard water. The mixture is maintained at 20 ºC ± 1 ºC for 5 min ± 10 s (required test conditions).

At this contact time, an aliquot is taken; the bactericidal and/or the bacteriostatic action in this portion is immediately neutralized or suppressed by a validated method. The method of choice is dilution-neutralization. If a suitable neutralizer cannot be found, membrane filtration is used. The number of surviving bacteria in each sample are determined and the reduction in viable counts is calculated.

4.1.2 The test is performed using *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus hirae*. Additional and optional exposure times, temperatures, strains, and interfering substances are specified.

4.2 Materials and reagents

4.2.1 Test organisms

The bactericidal activity shall be evaluated using the following four strains:

- *Pseudomonas aeruginosa* ATCC 15442;
- *Escherichia coli* ATCC 10536;
- *Staphylococcus aureus* ATCC 6538;
- *Enterococcus hirae* ATCC 10541.

If required, for specific applications, additional strains may be chosen for example from;

- *Salmonella typhimurium* ATCC 13311;
- *Lactobacillus brevis* DSM 6235;
- *Enterobacter cloacae* DSM 6234

The determined bactericidal concentration of the test product is suggested as being suitable for practical situations of use. Where appropriate, additional specific bactericidal activity shall be determined under other conditions of time, temperature, additional strains and interfering substances (see 4.2.1 and 4.5.1) in accordance with 4.5.1 in order to take into account intended specific use conditions.

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NOTE For these additional conditions, the concentration defined as a result can be lower than the one obtained under the initial test conditions of 20 °C, 5 min, four selected reference strains.

NOTE See annex E for corresponding strain numbers in some other culture collections.

If additional strains are used, they shall be incubated under optimum growth conditions (temperature, time, atmosphere) and noted in the test report. If the additional strains selected do not correspond to the specified strains, their suitability for supplying inocula of sufficient concentration shall be verified. If the additional strains tested 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 under a reference for 5 years.

4.2.2 Culture media and reagents

4.2.2.1 General

The reagents shall be of analytical grade and/or appropriate for microbiological purposes.

NOTE 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.

4.2.2.2 Water

The water shall be free from substances that are toxic or inhibiting to the bacteria. It shall be freshly glass distilled water and not demineralized water. Sterilize in the autoclave (see 4.3.1 a).

NOTE 1 If the water is sterilized during the sterilization of the reagents, this is not necessary.

NOTE 2 If distilled water of adequate quality is not available, water for injections can be used.

4.2.2.3 Tryptone Soya Agar (TSA)

Tryptone soya agar, consisting of:

- Tryptone, pancreatic digest if casein 15.0 g
- Soya peptone, papaic digest of soybean meal 5.0 g
- Sodium chloride (NaCl) 5.0 g
- Agar 15.0 g
- Water (4.2.2.2) 1000 ml

Sterilize in the autoclave (4.3.2.1 a). After sterilization the pH of the medium shall be equivalent to 7.2 ± 0.2 when measured at 20 ± 1 °C.

NOTE: In case of encountering problems with neutralization (4.5.1.2 and 4.5.1.3), it may be necessary to add neutralizer to the TSA. Annex B give guidance on the neutralizers that may be used.

5.2.2.4 Diluent

Tryptone sodium chloride solution, consisting of:

- Tryptone, pancreatic digest if casein 1.0 g
- Sodium chloride (NaCl) 8.5 g
- Water (4.2.2.2) to 1000 ml

Sterilize in the autoclave (4.3.2.1 a). After sterilization the pH of the medium shall be equivalent to 7.2 ± 0.2 when measured at 20 ± 1 °C.

4.2.2.5 Neutralizer

The neutralizer shall be validated for the product being tested in accordance with 4.5.1.3, 4.5.1.3 and 5.2.2. It shall be sterile.

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

4.2.2.6 Rinsing liquid (for membrane filtration)

The rinsing liquid shall be validated for the product being tested in accordance with 4.5.1.2, 4.5.1.3 and 4.5.3.
It shall be sterile, compatible with the filter membrane and capable of filtration through the filter membrane under the test conditions described in 4.5.3.

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

4.2.2.7 Hard water for dilution of products

For the preparation of 1000 ml of hard water, the procedure is as follows:

— prepare solution A: Dissolve 19.84 g anhydrous MgCl₂ and 46.24 g anhydrous (CaCl₂) in water (see 4.2.2.2) and dilute to 1000 ml; Sterilize by membrane filtration (4.3.2.7) or in the autoclave (4.5.3.2.1 a). Autoclaving, if used, may cause a loss of liquid. In this case, make up to 1000 ml with water (4.2.2.2) under aseptic conditions. Store the solution in the refrigerator (4.3.2.8) for no longer than one month.

— prepare solution B: Dissolve 35.02 g sodium bicarbonate (NaHCO₃) in water (see 4.2.2.2) and dilute to 1000 ml. Sterilize by membrane filtration (4.3.2.7). Store the solution in the refrigerator (4.3.2.8) for no longer than a week.

— place 600 ml to 700 ml of water (see 4.2.2.2) in a 1000 ml volumetric flask (4.3.2.12) and add 6.0 ml (4.3.2.9) of solution A, then add 8.0 ml solution B. Mix and dilute to 1000 ml with water (see 4.2.2.2). The pH of the hard water shall be 7.2 ± 0.2 when measured at 20 ± 1 °C (4.3.2.4). If necessary, adjust the pH by using a solution of approximately 40 g/l (about 1mol/l) of sodium hydroxide (NaOH) or approximately 36.5 (about 1mol/l) of hydrochloric acid.

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

NOTE When preparing the product test solutions (see 4.4.2) the addition of the product in this hard water solution produces a different final water hardness in each test tube. In any case, the final hardness is lower than 300 mg/kg of CaCO₃ in the test tube.

4.2.2.8 Interfering substances

4.2.2.8.1 General

The interfering substances 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 (pH, calcium and/or magnesium hardness) and chemical composition (mineral substances, protein, glucides, lipids, and detergents) shall be fully defined.

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

4.2.2.8.2 Clean solutions (Bovine albumin solution – low concentration)

Bovine albumin solutions for the test conditions shall be prepared as follows:

dissolve 0.30 g of bovine albumin V (suitable for microbiological purposes) in 100 ml of water (see 4.2.2.2); sterilize by membrane filtration (4.3.2.7), keep in the refrigerator (4.3.2.8) and use within one month;

The final concentration of the bovine albumin in the test procedure (see 4.5) is 0.3 g/l;

4.2.2.8.3 Dirty conditions (bovine albumin – high concentration)

Dissolve 3.0 g of bovine albumin fraction V (suitable for microbiological purposes) in 100 ml of water (see 4.2.2.2).
Sterilize by membrane filtration (4.3.2.7), keep in the refrigerator (4.3.2.8) and use within one month.

The final concentration of bovine albumin in the test procedure (see 4.5.2) is 3 g/l.

4.2.2.8.4 Milk (dairies)

Skimmed milk, guaranteed free of antibiotics and additives and reconstituted at a rate of 100 g powder per litre of water (see 4.2.2.2), shall be prepared as follows:
prepare a solution of 100 g milk-powder in 1000 ml water (see 4.2.2.2). Heat for 30 min at (105 ± 3)ºC (or 5 min at (121 ± 3)ºC)

The final concentration of reconstituted milk in the test procedure (see 4.5.) is 10.0 g/l of reconstituted milk.

4.2.2.5 Yeast extract (breweries)

Dehydrated yeast extract for bacteriology, shall be prepared as follows:

prepare a 100 g/l solution in water (see 4.2.2.2), adjust to pH 7.0 ± 0.2 with sodium hydroxide;
sterilize in the autoclave (see 4.3.2.1 a).

The final concentration of yeast extract in the test procedure (see 4.5) is 10.0 g/l.

4.2.2.6 Sucrose (beverage, soft drink industries)

Prepare a 100 g/l solution of sucrose in water (see 4.2.2.2), sterilize by membrane filtration (15 ± 3ºC).

The final concentration of sucrose in the test procedure (see 4.5.2) is 10.0 g/l.

4.2.2.7 pH 5.0 and pH 9.0 buffer solutions (cleaning in place)

The buffer solution used shall be described in the test report and pH values shall be recorded. The final pH in test tubes (together with test organisms and product) shall be controlled and found equal to 5.0 ± 0.2 or 9.0 ± 0.2.

4.2.2.8 Sodium lauryl sulfate (cosmetic area, e.t.c)

Prepare a 50 g/l solution of sodium lauryl sulfate in water (see 4.2.2.2). Sterilize in the autoclave (see 4.3.1).

The final concentration of sodium lauryl sulfate in the test procedure (see 4.5.2) is 5.0 g/l.

4.3 Apparatus and glassware

4.3.1 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 (see 4.3.2.1 a)
b) by dry heat, (see 4.3.2.1 b)

4.3.2 Usual microbiological laboratory equipment and in particular, the following:

4.3.2.1 Apparatus for sterilization

a) for moist heat sterilization, an autoclave capable of being maintained at (121 ± 3)ºC for a minimum holding time of 15 min;
b) for dry heat sterilization, a hot air oven capable of being maintained at (160 ± 5)ºC for a minimum holding time of 30 min, at (170 ± 6)ºC for a minimum holding time of 1 h or at (180 ± 6)ºC for a minimum holding time of 2 h.

4.3.2.2 Water baths, capable of being controlled at 20 ºC ± 1 ºC, at 45 ºC ± 1 ºC and at additional test temperatures ± 1 ºC (see 4.5.1).

4.3.2.3 Incubator, capable of being controlled at either 36 ºC ± 1 ºC or 37 ºC ± 1 ºC. An incubator at 37 ºC ± 1 ºC may be used if an incubator at 36 ºC ± 1 ºC is not available.

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4.3.2.4 pH-meter, having an accuracy of calibration of ± 0.1 pH units at 25 °C.

NOTE: A puncture electrode or a flat membrane electrode should be used for measuring the pH of the agar media (4.2.2.3).

4.3.2.5 Stopwatch.

4.3.2.6 Shaker
   a) Electromechanical agitator e.g. Vortex® mixer
   b) Mechanical shaker

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

The apparatus shall have a filter holder which shall have a usable volume 50 ml minimum, and suitable for use with filters of diameter 47 mm to 50 mm, of 0.45 µm pore size for sterilization of hard water (4.2.2.7), bovine albumin (4.2.2.8.2) and (4.2.2.8.3), and if the membrane filtration method is used (4.5.3).

The vacuum source used shall give an even filtration flow rate. In order to obtain a uniform distribution of the microorganisms over the membrane and in order 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.

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

4.3.2.9 Graduated pipettes, of nominal capacities 10 ml and 1 ml and 0.1 ml. Calibrated automatic pipettes may be used.

4.3.2.10 Petri dishes, of size 90 mm to 100 mm.

4.3.2.11 Glass beads, (diameter: 3 mm to 4 mm).

4.3.2.12 Volumetric flasks

4.4 Preparation of test organism suspensions and product test solutions

4.4.1 Bacterial suspensions

4.4.1.1 Stock cultures of test organisms

Stock cultures shall be kept in accordance with the requirements of EN 12353.

4.4.1.2 Working culture of test organisms

In order to prepare a working culture of strains (see 4.2.1), subculture from the stock culture (see 4.4.1.1) by streaking onto TSA slopes and incubate (see 4.3.2.3). After 18 h to 24 h prepare a second subculture from the first subculture in the same way and incubate for 18 h to 24 h. From this second subculture, a third subculture may be produced in the same way. The second and (if produced) third subcultures are the working cultures.

If it is not possible to prepare the second subculture on a particular day, a 48 h subculture may be used for subsequent subculturing, provided that the subculture has been kept in the incubator during the 48 h period.

In these circumstances, prepare a further 24 h subculture after proceeding. Do not take a fourth subculture.

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

4.4.1.3 Bacterial test suspensions

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1) Vortex in 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 KEBS of this product.

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a) Take 10 ml of diluent (see 4.2.2.4) and place in a 100 ml flask with 5 g of glass beads (see 4.3.2.11).

Take the working cultures (see 4.4.1.2) and transfer loopfuls of the cells into the diluent. The cells should be suspended in the diluent by immersing the loop in the diluent and rubbing it against the side of the flask to dislodge the cells. Shake the flask for 3 min using a mechanical shaker (see 4.3.2.13).

Aspirate the suspension from the glass beads and transfer to another tube.

Adjust the number of cells in the suspension to \((1.5 \times 10^6)\) cfu/ml to \(5 \times 10^8\) cfu/ml using the diluent (4.2.2.4), estimating the number of cfu by any suitable means. Maintain this suspension in the water bath at 20 ºC ± 1 ºC and use within 2 h.

b) For counting of the bacterial test suspension, prepare \(10^{-6}\) and \(10^{-7}\) dilutions of the test suspension using diluent (see 4.2.2.4). Mix (see 4.3.2.6 a). Take a sample of 1.0 ml of each dilution in duplicate and inoculate using the pour plate or the spread plate technique.

1) when using the pour plate technique, spread 1.0 ml sample into separate Petri dishes and add 15 ml to 20 ml melted TSA cooled to 45 ºC ± 1 ºC.
2) when using the spread plate technique, spread each 1.0 ml sample – divided into portions of approximately equal size – on an appropriate number (at least two) of surface dried plates containing TSA.

4.4.1.4 Validation suspension (“\(N_v\)”)  

a) To prepare the validation suspension, dilute the test suspension with the diluent (4.2.2.4) to obtain the bacterial count of \((3.0 \times 10^2)\) cfu/ml to \((1.6 \times 10^3)\) cfu/ml \{about one fourth \((1 + 3)\) of the \(10^{-5}\) dilution\}.

b) For counting, prepare a \(10^{-1}\) dilution with the diluent (4.2.2.4). Mix (4.3.2.6 a). Take a sample of 1.0 ml in duplicate and inoculate using the pour plate or spread plate technique.

4.4.1.5 Incubation and counting of bacterial test suspension  

For incubation and counting of the test and validation suspension, the procedure is as follows:

a) Incubate the plates at 36 ºC ± 1 ºC (or 37 ºC ± 1 ºC) (see 4.3.2.3) for 24 h. Discard any plates that are not countable (for any reason). Count the cfu on the plates to determine the number of colony forming units. Incubate the plates for a further 24 h. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.

b) Note for each plate the exact number of colonies but record “>330” for any counts higher than 330 and determine \(V_c\) values according to 4.6.2.2.

c) Calculate the number of cfu/ml in the test suspension (“\(N\)”) using the method given in 4.6.1.1.

4.4.2 Product test solution

Details of samples of the product as received shall be recorded.

Product test solutions shall be prepared in hard water (see 4.2.2.7) at three different concentrations to include one concentration in the active range and one concentration in the non active range.

The concentration of the product test solution shall be 1.25 times the required test concentration. 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 (see 4.2.2.7). Subsequent dilutions shall be prepared in volumetric flasks (see 4.3.2.12) on a volume/volume basis in hard water (see 4.2.2.7). For liquid products, dilutions of the product shall be prepared in hard water (see 4.2.2.7) on a volume/volume basis using volumetric flasks (4.3.2.12). For products supplied in a ready to use state, water (see 4.2.2.2) shall be used to prepare dilutions.

2) cfu/ml: Colony Forming Unit per ml.
When the product is diluted in hard water it shall give a physically homogeneous stable preparation. The product test solutions and dilutions of it shall be prepared freshly and used within 60 min.

NOTE If the product is of low stability this period should be shortened.

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

4.5 Procedure

4.5.1 Choice of experimental conditions

The selection of contact temperature, contact time and interfering substances shall be carried out according to the practical use considered for the product (see clause 4) as follows:

a) temperature: $\theta$ (in °C):
   - the temperature to be tested is $\theta = 20 \text{ °C} \pm 1 \text{ °C}$;
   - the additional temperatures may be chosen from $4 \text{ °C} \pm 1 \text{ °C}$, $10 \text{ °C} \pm 1 \text{ °C}$, $30 \text{ °C} \pm 1 \text{ °C}$ or $40 \text{ °C} \pm 1 \text{ °C}$;

b) contact time: $t$ (in min):
   - the contact time to be tested is $t = 5 \text{ min} \pm 10 \text{ s}$ or;
   - the additional contact times may be chosen from $1 \text{ min} \pm 10 \text{ s}$, $15 \text{ min} \pm 10 \text{ s}$, $30 \text{ min} \pm 10 \text{ s}$ or $60 \text{ min} \pm 10 \text{ s}$;

c) strains:
   - strains shall be as stated in 4.2.1;

d) interfering substances:
   - the interfering substance to be tested is the bovine albumin solution (see 4.2.2.8.2) under clean conditions (0.3 g/l bovine albumin) or for dirty (3 g/l bovine albumin) conditions according to practical applications (see clause 4);
   - in the case of additional requirements, interfering substances as given in 4.2.2.8 may be tested. Those interfering substances shall be chosen according to the application field specified for the product. The product shall not cause the formation of any precipitate in the experimental conditions used. Each selected experimental condition ($u$, $t$, strains and interfering substance) shall be validated in accordance with Annex A.

4.5.2 Test procedure for assessing the bactericidal effect of the product

4.5.2.1 General

The method of choice is the dilution-neutralization method. To determine a suitable neutralizer, the following procedure shall be adopted. Carry out the validation of the dilution neutralization method (see A.4.1) using a suitable neutralizer chosen according to laboratory experience and published data. If this neutralizer is not valid, repeat the validation test using an alternative neutralizer containing a combination of polysorbate 80 30 g/l, saponin 30 g/l, L-histidine 1 g/l, lecithin 3 g/l, sodium thiosulfate 5 g/l in either diluent (4.2.2.4) or in phosphate buffer 0.0025 mol/l. If both neutralizers are found to be invalid, the membrane filtration may be used in place of the dilution-neutralization method. The inactivation of the bactericidal and/or bacteriostatic activity of the product shall be validated for each of the tested strains and for each of the chosen experimental conditions (see 5.5.1).

4.5.2.2 Dilution-neutralization method

4.5.2.2.1 General

Prior to testing, equilibrate all reagents (product test solution, bacterial test suspension, interfering substances) to the test temperature of $u \text{ °C} \pm 1 \text{ °C}$ using the water bath (see 4.3.2.2) controlled at $u \text{ °C} \pm 1 \text{ °C}$.
temperature of the reagents is stabilized at $u \pm 1 ^\circ C$. The neutralizer and water (see 4.2.2.2) shall be equilibrated at a temperature of $20 ^\circ C \pm 1 ^\circ C$.

4.5.2.2.2 Test procedure for bactericidal activity of products

Pipette 1.0 ml of interfering substance (see 4.2.2.8) into a test tube. Add 1.0 ml of one of the bacterial test suspensions containing $(1.5 \times 10^8)$ cfu/ml to $5 \times 10^8$ cfu/ml. Start the stopwatch immediately, mix (see 4.3.2.6) and place the test tube in the water bath at $\Theta ^\circ C \pm 1 ^\circ C$ for 2 min $\pm 10$ s. At the end of this time, add 8.0 ml of one of the product test solutions. Restart the stopwatch, mix (see 5.3.2.6) and place the test tube in a water bath controlled at $u ^\circ C \pm 1 ^\circ C$ for the appropriate contact time $t \pm 10$ s.

NOTE When adding bacterial suspensions, care should be taken to avoid touching the upper part of the test tube sides.

Just before the end of the contact time, mix (see 4.3.2.6). At the end of the contact time pipette 1.0 ml of the test mixture into a tube containing 8.0 ml neutralizer (see 4.2.2.5) and 1.0 ml water (see 4.2.2.2). Mix (see 4.3.2.6) and place in a water bath controlled at $20 ^\circ C \pm 1 ^\circ C$. After a neutralization time of 5 min $\pm 10$ s, immediately take a sample of 1.0 ml of neutralized mixture (neutralizer, product test solution, interfering substance, bacterial test suspension) in duplicate and transfer each 1.0 ml sample into separate petri dishes (see 4.3.2.10) and quickly add 2 ml to 15 ml melted TSA (see 4.2.2.3), cooled to $45 ^\circ C \pm 1 ^\circ C$. In exceptional cases neutralizer can be added to TSA (see Annex C). Perform this procedure using the other product test solutions and the other bacterial test suspensions.

5.5.2.2.3 Counting of the test mixture

Incubate the plates at $36 ^\circ C \pm 1 ^\circ C$ (or $37 ^\circ C \pm 1 ^\circ C$) (see 4.3.2.3) for 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units for each plate. Incubate the plates for a further 24 h. Do not recount plates which no longer show well-separated colonies. Recount the remaining plates. Determine the higher number of colonies $V_c$ for each plate. Calculate the number of cfu/ml in the test mixture ($N_a$) using the method given in 4.6.1.2. For calculating the viable count of the text mixture, the dilution factor is $10^{21}$.

4.5.2.3 Membrane filtration method

4.5.2.3.1 General

Prior to testing, equilibrate all reagents (product test solutions, bacterial test suspension, interfering substances) to the test temperature of $\Theta ^\circ C \pm 1 ^\circ C$ using the water bath (see 4.3.2.2) controlled at $\Theta ^\circ C \pm 1 ^\circ C$. Check that the temperature of the reagents is stabilized at $\Theta ^\circ C \pm 1 ^\circ C$. The rinsing liquid shall be equilibrated at $20 ^\circ C \pm 1 ^\circ C$.

4.5.2.3.2 Test procedure for bactericidal activity of products

Pipette 1.0 ml of interfering substance (see 4.2.2.8) into a test tube. Add 1.0 ml of one of the bacterial test suspension (see 4.4.1.3).

Start the stopwatch immediately, mix (see 4.3.2.6) and place the test tube in the water bath at $\Theta ^\circ C \pm 1 ^\circ C$ for 2 min $\pm 10$ s. At the end of the contact time, add 8.0 ml of one of the product test solutions. Restart the stopwatch, mix (see 4.3.2.6) and place the test tube in a water bath controlled at $\Theta ^\circ C \pm 1 ^\circ C$ for the appropriate contact time $t \pm 10$ s.

Just before the end of the chosen contact time, mix (see 4.3.2.6). At the chosen contact time pipette two samples of 0.1 ml of the test mixture and transfer each sample into a separate membrane filtration apparatus equipped with a membrane containing 50 ml of the rinsing liquid (see 4.2.2.6). Filter immediately. The time required for transfer and filtration should not exceed 1 min. If greater than 1 min, this time shall be

The validation test (see Annex A) shall also be carried out at the same time as the test procedure (see 4.5.2) using only the highest product concentration and the same conditions (bacterial test suspension, product test solution, neutralizer or rinsing liquid, interfering substances, hard water) as used in the test (see 4.5.2.2 or 4.5.2.3).

4.5.2.3.3 Counting of test mixture

Incubate the plates at $36 ^\circ C \pm 1 ^\circ C$ (or $37 ^\circ C \pm 1 ^\circ C$) (see 4.3.2.3) for 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units for each plate. Incubate the plates for a further 24 h. Do not recount plates which no longer show well-separated colonies. Recount the remaining
plates. Determine the higher number of colonies \( V \) for each plate. Calculate the number of cfu/ml in the test mixture \((N_c)\) using the method given in 4.6.1.2.

4.5.3 Validation of dilution-neutralization method and membrane filtration method

\[
c = \text{the sum of the colonies counted on all the plates taken into account; } n_1 \text{ is the number of plates taken into account at the first dilution; } n_2 \text{ is the number of plates taken into account at the second dilution; } \\
d = \text{the dilution factor corresponding to the first dilution taken into account.}
\]

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.

**b)** 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:** The dilution-neutralization and membrane filtration methods shall be validated for each of the test organisms and experimental conditions (see 4.5.1)

\[
N = \frac{100 - \text{recorded mean count}}{1} - 1.56 \times 10^6 - 1.0 \times 10^8 \text{ (in cfu/ml) according to Annex A.}
\]

4.6 Calculation and expression of results

4.6.1 Calculation of viable counts (cfu/ml)

**4.6.1.1 Bacterial test suspension**

Viable counts of the bacterial test suspension (see 4.4.1.4) should be calculated according to the principles described in ISO 7218:1985 (see NOTE) as follows:

a) only colony counts which are less than 300 cfu/plate should be used for calculation of viable counts. For a result to be valid at least one plate has to contain 15 or more colonies; viable counts should be calculated using at least one pair of plates, where one or both plates contain more than 15 colonies and both plates contain less than 300 colonies. If plates from two dilutions fall within this range calculate the number of cfu/ml as the weighted mean count. If plates from only one dilution fall within this range calculate the arithmetic mean.

**NOTE** ISO 7218: 1985 Microbiology Ð General guidance for microbiological examinations.

For calculation of the weighted mean count in cfu/ml use the following formula (1):

\[
\text{recorded in the test report. Rinse with at least 150 ml but not more than 500 ml of rinsing liquid (see 4.2.2.6). If the rinsing liquid is not water, complete the procedure by filtering 50 ml of water (n_1 + 0.1 n_2) / d}
\]

where:

(1) (see 4.2.2.2). Then transfer the membranes to the surface of two separate TSA plates. In exceptional cases neutralizer can be added to TSA (see Annex C).

**NOTE** When transferring, care should be taken to ensure that the bacteria are on the upper side of the membrane when placed on the TSA and to avoid trapping air between the membrane and agar surface.

Perform this procedure using the other product test solutions and the other bacterial test suspensions.

**4.6.1.2 Test procedure and test for validation**
For the test procedure (see 4.5.2.2.2 and 4.5.2.3.2) and test for validation (see A.2, A.4.1 and A.4.2) the viable count should be calculated using the following method. Only colony counts which are less than 300 cfu per plate should be used for calculation of viable counts. Viable counts should be calculated using colony counts from both plates.

When at least one plate contains 15 or more colonies, use the following formula (2) for calculation of the viable counts in cfu/ml:

\[ c \times d \times V \]

where:
- \( c \) is the sum of the colonies counted on both plates;
- \( n \) is the number of plates taken into account;
- \( d \) is the dilution factor corresponding to the dilution taken into account. For the dilution neutralization test procedure (see 5.5.2.2) and the bacterial suspension (see A.2) the dilution factor is 10^2;
- \( V \) is the sample volume. For the dilution neutralization and validation procedure (see 4.5.2.2.3 and A.4.1) and the bacterial suspension (see A.2) the sample volume is C is the number of cfu/ml of the dilution-neutralization validation (see A.4.1c) or of the filtration test control (see A.4.2c);
- \( A \) is the number of cfu/ml of the experimental conditions validation (see A.4.1a or A.4.2a).

### 4.6.3 Expression of results

For each test organism record the number of cfu/ml in the bacterial test suspension (N) (see 4.4.1.4) and after the test procedure for bactericidal activity of the product (N_a) (see 4.5.2.2.3 or 4.5.2.3.3).

For the validation of neutralization (see Annex A) record the number of cfu/ml (N_v) in the bacterial suspension (see A.2).

For validation of the dilution neutralization method (see A.4.1) record the number of cfu/ml in the neutralizer toxicity control (B) and the dilution neutralization control (C).

For validation of the membrane filtration method (see A.4.2), record the number of cfu/ml in the filtration control (B) and the filtration test control (C). For each test organism and product test concentration calculate and record the reduction in viability as follows:

\[ R = \frac{N}{3 \times 10^2} \times 10^{21} \]

1.0 ml. For the membrane filtration test and validation procedure (see 4.5.2.3.3 and A.4.2) the sample volume is 0.1 ml.

\[ R \] = reduction in viability

### 4.7 Conclusion

For the test procedure (see 4.5) where the number of cfu on all plates counted is lower than 15, record the viable count of the test mixture as lower than 1.5 x 10^8 cfu/ml (< 1.5 x 10^8 cfu/ml). Where the number of cfu on all plates counted is higher than 300 record the viable count of the test mixture as higher than 3 x 10^3 cfu/ml (> 3 x 10^3 cfu/ml).

### 4.6.2 Verification of methodology

For each test organism check that:

a) \( N \) is between 1.5 x 10^8 cfu/ml and 5 x 10^8 cfu/ml;

b) \( N_v \) is between 6 x 10^2 and 3 x 10^3 cfu/ml;
c) $B$ is equal to or greater than 0.05 times $N_v$;

d) $C$ is equal to or greater than 0.5 times $B$;

e) $A$ is equal to or greater than 0.05 times $N_v$.

where:

$N$ is the number of cfu/ml of the bacterial test suspension (see 4.4.1.4);

$N_v$ is the number of cfu/ml of the bacterial suspension (see A.2);

$B$ is the number of cfu/ml of the neutralizer toxicity validation (see A.4.1b) or of the filtration control (see A.4.2b);

Bactericidal activity for general purposes is characterized by the concentration of the tested product for which criteria 4.6.1 and 4.6.2 are met and for which a $10^5$ or more reduction in viability is demonstrated under the required test conditions: 5 min $\pm$ 10 s, 20 $^\circ$C $\pm$ 1 $^\circ$C and clean or dirty conditions (see clause 4), and when the test organisms are Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus and Enterococcus hirae.

Bactericidal activity for specific purpose is characterized by the concentration of the tested product for which criteria 4.6.1 and 4.6.2 are met and for which a $10^5$ or more reduction in viability is demonstrated, when the test organisms are Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus and Enterococcus hirae and if required (see clause 4) additional test organisms under additional conditions: $t$ in min, $\theta$ in $^\circ$C and interfering substances. It is accepted that for certain applications, this suspension test may provide sufficient information for the particular application and that additional surface test may not be relevant (see annex G). For applications where this suspension test without surface test is used to support use recommendations, the justification for omitting surface test should be given.

4.8 Test report

Refer to Annex A
A.1  General

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

a) identification of the laboratory;

b) 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

c) test method and its validation:

If the dilution-neutralization method is used full details of the test for validation of the neutralizer shall be given; If the membrane filtration method is used, full details of the procedure which was carried out in order to justify the use of the membrane filtration method shall be given;

d) experimental conditions:
   1) period of analysis (dates of test);
   2) product diluent used during the test;
   3) product test concentrations;
   4) appearance product dilutions;
   5) contact time(s);
   6) test temperature(s);
   7) interfering substance;
   8) stability of the mixture (interfering substance and product diluted in hard water);
   9) temperature of incubation;
   10) neutralizer or rinsing liquid;
   11) identification of the bacterial strains used;

e) test results:
   1) validation tests;
   2) evaluation of bactericidal activity (see table D.1);
   f) conclusion;
   g) locality, date and identified signature.

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

A.2  Validation of dilution-neutralization and membrane filtration methods

A.2.1  Principle

A neutralizer is chosen for each product in accordance with A.4.1 (see 4.5). If a suitable neutralizer cannot be found the membrane filtration method in accordance with A.4.2 is used.
**A.2.2 Preparation of bacterial suspension**

To prepare the bacterial suspension, dilute the bacterial test suspension (see 4.4.1.3) with the diluents (see 4.2.2.4) to obtain the bacterial count of $6 \times 10^2 \text{ cfu/ml}$ to $3 \times 10^3 \text{ cfu/ml}$.

For counting of the suspension prepare a $10^2$ dilution with the diluent (see 4.2.2.4). Mix (see 4.3.2.6). Take a sample of 1.0 ml of the $10^2$ dilution in duplicate and transfer each 1.0 ml sample into separate petri dishes (see 4.3.2.10) and add 12 ml to 15 ml melted TSA (see 4.2.2.3), cooled to 45 $^\circ\text{C} \pm 1 ^\circ\text{C}$. Incubate the plates at 36 $^\circ\text{C} \pm 1 ^\circ\text{C}$ (or 37 $^\circ\text{C} \pm 1 ^\circ\text{C}$) (see 4.3.2.3) for 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units. Incubate the plates for a further 24 h. Do not recount plates which no longer show well separated colonies. Recount the remaining plates.

Determine the higher number of colonies for each plate. Calculate the number of cfu/ml in the test suspension ($N_v$) using the method given in 4.6.1.2 (dilution factor $10^2$ and volume 1 ml).

**A.3 Preparation of product test solution**

Prepare (see 4.4.2) a product test solution of the highest strength used in the test (1.25 C).

**A.4 Test for validation**

**A.4.1 Dilution-neutralization method**

Perform the following procedure for each experimental conditions (strains, interfering substance, temperature, contact time).

Prior to testing, equilibrate all reagents (product test solutions, diluent, bacterial suspension, interfering substances, hard water (see 4.2.2.7)) to the test temperature of $\theta ^\circ\text{C} \pm 1 ^\circ\text{C}$ using the water bath (see 4.3.2.2) controlled at $\theta ^\circ\text{C} \pm 1 ^\circ\text{C}$. Check that the temperature of the reagents is stabilized at $\theta ^\circ\text{C} \pm 1 ^\circ\text{C}$. The neutralizer and the water (see 4.2.2.2) shall be equilibrated at 20 $^\circ\text{C} \pm 1 ^\circ\text{C}$.

a) Validation of selected experimental conditions (or verification of the absence of any lethal effect in the test conditions).

Place in a sterile test tube 1.0 ml of the selected interfering substance (see 4.2.2.8) and 1.0 ml of the bacterial suspension containing $6 \times 10^2$ to $3 \times 10^3 \text{ cfu/ml}$ prepared in accordance with A.2. Mix (see 4.3.2.6) for a few seconds and leave in the water bath at $\theta ^\circ\text{C} \pm 1 ^\circ\text{C}$ for 2 min $\pm 10$ s. At the end of this time, add 8.0 ml of hard water (see 4.2.2.7). Start the stopwatch at the beginning of the addition and mix (see 4.3.2.6) for a few seconds.

Leave in the water bath at $\theta ^\circ\text{C} \pm 1 ^\circ\text{C}$ for the time $t \pm 10$ s. Immediately before the end of the contact time, mix (see 4.3.2.6). At the end of the contact time, take a sample of 1.0 ml of the mixture in duplicate and transfer into separate Petri dishes (see 4.3.2.10). Add 12 ml to 15 ml melted TSA (see 4.2.2.3) cooled to 45 $^\circ\text{C} \pm 1 ^\circ\text{C}$. Incubate the plates at 36 $^\circ\text{C} \pm 1 ^\circ\text{C}$ (or 37 $^\circ\text{C} \pm 1 ^\circ\text{C}$) (see 4.3.2.3) for 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units. Incubate the plates for a further 24 h. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. Determine the higher number of colonies for each plate. Calculate the number of cfu/ml in the experimental conditions validation (A) using the method given in 4.6.1.2.

b) Neutralizer toxicity validation

Place in a sterile test tube 8.0 ml of neutralizer (see 4.2.2.5) and 1.0 ml of water (see 4.2.2.2).

Introduce 1.0 ml of the bacterial suspension containing $6 \times 10^2$ to $3 \times 10^3 \text{ cfu/ml}$ prepared in A.2.

Start a stopwatch at the beginning of the addition and mix (see 4.3.2.6), leave in contact in the water bath at 20 $^\circ\text{C} \pm 1 ^\circ\text{C}$ for 5 min $\pm 10$ s. Immediately before the end of the contact time, mix (see 4.3.2.6). At the end of the contact time take a sample of 1.0 ml of the mixture in duplicate and transfer into separate Petri dishes (see 5.3.2.10).

Add 12 ml to 15 ml melted TSA (see 4.2.2.3) cooled to 45 $^\circ\text{C} \pm 1 ^\circ\text{C}$.
Incubate the plates at 36 °C ± 1 °C (or 37 °C ± 1 °C) (see 4.3.2.3) for 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units. Incubate the plates for a further 24 h. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. Determine the higher number of colonies for each plate.

Calculate the number of cfu/ml in the neutralizer toxicity conditions validation (B) using the method given in 4.6.1.2.c) Dilution-neutralization validation.

Place 1.0 ml of interfering substance (see 4.2.2.8) in a sterile tube, add 1.0 ml of the diluent (see 4.2.2.4) and then, starting a stopwatch, 8.0 ml of the product dilution prepared in A.3. Leave in contact in the water bath for the time t ± 10 s at a temperature °C ± 1 °C. Then, transfer 1.0 ml of the mixture into a test tube containing 8.0 ml of neutralizer (see 4.2.2.5) previously kept in the water bath at 20 °C ± 1 °C. Leave in contact in this water bath for 5 min ± 10 s. Add 1.0 ml of the bacterial suspension containing 6 x 10^2 cfu/ml to 3 x 10^3 cfu/ml prepared in accordance with A.2.

Start a stopwatch at the beginning of the addition and mix (see 4.3.2.6) for a few seconds. Leave in contact in the water bath at 20 °C ± 1 °C for 30 min ± 1 min.

Immediately before the end of the contact time, mix (see 4.3.2.6). At the end of the contact time, take a sample of 1.0 ml of the mixture in duplicate and transfer into separate Petri dishes (see 4.3.2.10). Add 12 ml to 15 ml melted TSA (see 4.2.2.3) cooled to 45 °C ± 1 °C.

Incubate the plates at 36 °C ± 1 °C (or 37 °C ± 1 °C) (see 4.3.2.3) for 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units. Incubate the plates for a further 24 h. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. Determine the higher number of colonies for each plate.

Calculate the number of cfu/ml in the dilution-neutralization validation (C) using the method given in 4.6.1.2.

A.4.2 Membrane filtration method

Perform the following procedure for each experimental conditions (strains, interfering substance, diluent, contact time, temperature). Prior to testing, equilibrate all reagents (product test solutions, diluent, bacterial suspension, interfering substances, hard water (see 4.2.2.7)) to the test temperature of °C ± 1 °C using the water bath (see 4.3.2.2) controlled at °C ± 1 °C. The rinsing liquid shall be equilibrated at 20 °C ± 1 °C.

a) Validation of selected experimental conditions (or verification of the absence of any lethal effect in the test conditions). Place in a sterile tube 1.0 ml of the selected interfering substance (see 4.2.2.8) and 1.0 ml of the bacterial suspension containing 6 x 10^2 to 3 x 10^3 cfu/ml prepared in accordance with A.2. Mix (see 4.3.2.6) for a few seconds and leave in the water bath at °C for 2 min ± 10 s. At the end of this time, add 8.0 ml of hard water (see 4.2.2.7). Start a stopwatch at the beginning of the addition and mix (see 4.3.2.6) for a few seconds. Leave in contact in the water bath at °C for the time t ± 10 s. Immediately before the end of the contact time, mix (see 4.3.2.6). At the end of the contact time, transfer in duplicate 1.0 ml of the mixture and transfer into two separate membrane filtration apparatus equipped with a membrane and containing 50 ml of the rinsing liquid (see 4.2.2.6). Filter and rinse with 50 ml of water (see 4.2.2.2) and then transfer the membranes to the surface of two separate TSA plates (see 4.2.2.3). In exceptional cases a neutralizer can be added to TSA (see Annex C).

NOTE 1. When transferring membrane to TSA plates, care should be taken to ensure that the membrane is filtrate side uppermost and to avoid trapping air between the membrane and agar surface.

Incubate the plates at 36 °C ± 1 °C (or 37 °C ± 1 °C) (see 4.3.2.3) for 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units. Incubate the plates for a further 24 h. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. Determine the higher number of colonies for each plate.

Calculate the number of cfu/ml in the experimental conditions control (A) using the method given in 4.6.1.2.

b) Validation of the filtration procedure. Sample in duplicate 0.1 ml of the bacterial suspension containing 6 x 10^2 to 3 x 10^3 cfu/ml prepared in accordance with A.2. Transfer into two separate membrane filtration apparatus equipped with a membrane and containing 50 ml of the rinsing liquid (see 4.2.2.6).
Filter and rinse with 50 ml of water (see 4.2.2.2) and then transfer the membranes to the surface of two separate TSA plates (see 5.2.2.3).

NOTE 2  When transferring membrane to TSA plates, care should be taken to ensure that the membrane is filtrate side uppermost and to avoid trapping air between the membrane and agar surface.

Incubate the plates at 36 °C ± 1 °C (or 37 °C ± 1 °C) (see 4.3.2.3) for 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units. Incubate the plates for a further 24 h. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. Determine the higher number of colonies for each plate.

Calculate the number of cfu/ml in the filtration control (B) using the method given in 4.6.1.2.

c) Validation of the filtration method (or counting of the bacteria on the membranes which have previously been in contact with the mixture of product and interfering substance).

Place 1.0 ml of interfering substance (see 4.2.2.8) in a sterile tube. Add 1.0 ml of the diluent (see 4.2.2.4) and then, starting a stopwatch, 8.0 ml of the product dilution prepared in A.3. Mix (see 4.2.3.6).

Leave in contact in the water bath for the time t ± 10 s at the temperature u ± 1 °C.

Immediately before the end of the contact time, mix (see 4.3.2.6). At the end of the contact time sample in duplicate 0.1 ml of the mixture and transfer into two separate membrane filtration apparatus equipped with a membrane and containing 50 ml of the rinsing liquid (see 4.2.2.6). Filter and rinse the membranes with at least 150 ml and not more than 500 ml per 50 ml or 100 ml measure of rinsing liquid (see 4.2.2.6).

Then cover the membranes with 50 ml of rinsing liquid (see 4.2.2.6) and add 0.1 ml of the bacterial suspension containing $6 \times 10^{2}$ to $3 \times 10^{3}$ cfu/ml prepared in A.2.

Filter and rinse with 50 ml of water (see 4.2.2.6) and then transfer the membranes to the surface of two separate TSA plates (see 4.2.2.3).

NOTE 3  When transferring membrane to TSA plates, care should be taken to ensure that the membrane is filtrate side uppermost and to avoid trapping air between the membrane and agar surface.

Incubate the plates at 36 °C ± 1 °C (or 37 °C ± 1 °C) (see 4.3.2.3) for 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units. Incubate the plates for a further 24 h. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. Determine the higher number of colonies for each plate.

Calculate the number of cfu/ml in the membrane filtration test validation (C) using the method given in 4.6.1.2.

A.5 Validation

Check that the test results comply with the relevant requirements of 4.6.1.2 or 4.6.2.
Any of the following neutralizers may be used: ± lecithin 3 g/l; polysorbate 80, 30 g/l (v/v); sodium thiosulfate 5 g/l; L-histidine 1 g/l; saponin 30 g/l indiluent (4.2.2.4) or in phosphate buffer 0.25 mol/l at 1 % (v/v); — phosphate buffer 0.25 mol/l: K$_2$HPO$_4$ 34 g water (see 5.2.2.2) 500 ml adjusted to pH 7.2 ± 0.2 with NaOH 1 mol/l water (see 4.2.2.2) up to 1000 ml sterilized in an autoclave (see 4.3.1); — fresh egg yolk diluted to 5 % (v/v) or 0.5 % (v/v); — 30 g/l polysorbate 80$^3$; 4 g/l sodium lauryl sulfate; lecithin 3 g/l; — 5 % (v/v) fresh egg yolk; 40 g/l polysorbate 80$^3$; — 7 % (v/v) ethylene oxide condensate of fatty alcohol; 20 g/l lecithin; 4 % (v/v) polysorbate 80$^3$; — 4 % (v/v) ethylene oxide condensate of fatty alcohol; 4 g/l lecithin; — 30 g/l polysorbate 80; lecithin 3 g/l; L-histidine 1 g/l; — glycine as a function of concentration of product; — 30 g/l polysorbate 80$^3$; lecithin 3 g/l; — phospholipid emulsion (commercial) at 50 mg/ml (dilute 1 to 10); — sodium thioglycollate at 0.5 g/l or 5 g/l; — L-cysteine at 0.8 g/l or 1.5 g/l; — thiomalic acid at 0.075 % (v/v) (adjusted to pH 7 with NaOH); — sodium thiosulfate at 5 g/l; — catalase or peroxidase: one unit of these enzymes catalyzes the decomposition of 1 mol of hydrogen peroxide per minute at 25 °C and at pH 7; — polysorbate 80$^3$, 30 g/l; saponin 30 g/l; L-histidine 1 g/l; L-cysteine 1 g/l.

NOTE The following list is not exhaustive and other media may be used.

$^3$) Analytical quality, non-hydrolyzed. TWEEN 80 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 KEBS of this product.
Annex C
(informative)
Rinsing liquids

Any of the following rinsing liquids may be used:

— water (see 4.2.2.2);
— diluent (see 4.2.2.4);
— aqueous solution of 0.1 % (v/v) polysorbate 80;
— aqueous solution of 0.5 % (v/v) polysorbate 80;
— aqueous solution of 0.5 % (v/v) polysorbate 80 and 0.7 g/l lecithin;
— neutralizer (see 4.2.2.5);
— buffer solutions.

NOTE The following list is not exhaustive and other liquids can be used.

Neutralizer added to the agar for counting:

— 10 % (v/v) of a solution containing 0.7 g/l lecithin and 5 % (v/v) polysorbate 80;
— 10 % (v/v) of a solution containing 10 g/l lecithin and 5 % (v/v) polysorbate 80;
— 10 % (v/v) of a solution containing fresh egg yolk 1.5 % (v/v), and 5 % (v/v) polysorbate 80.
Annex D
(informative)
Example of a typical test report

Bactericidal activity in general use conditions (for clean condition)
a) identification of the test laboratory

b) identification of the sample

Name of the product .............................................. Z
Batch number .......................................................... 91-71-51
Manufacturer ..................................................... Centipede Formulations Inc
Date of delivery ...................................................... 1991-02-11
Storage conditions ........................................... room temperature and darkness
Product diluent recommended by the manufacturer for use ........................................ Potable water
Active substance(s) and its (their) concentration(s)
(optional) .......................................................... Not Indicated

d) test method and its validation

Method .......................................................... Dilution-neutralization
Neutralizer ......................................................... 30 g/l, lecithin, sterilized in the autoclave

e) test results (see table D.1)

f) conclusion

According to EN 1276 (date of edition), the batch 91.71.51 of product Z, when diluted at 1% (V/V), in hard water, possesses bactericidal activity in five minutes at 20°C under clean condition (0.3 g/l bovine albumin) for referenced strains Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Enterococcus hirae.

g) locality, date and identified signature.
<table>
<thead>
<tr>
<th>Test organisms</th>
<th>Validation test:</th>
<th>Bacterial test suspension (5.4.1.4)</th>
<th>Test procedure at concentration % (w/v) (see 5.6.2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria suspension (see A.2)</td>
<td>Experimental conditions (see A.4.1.b and A.4.2.a)</td>
<td>Neutrophil Toxicity control (see A.4.1.b) or filtration control (see A.4.2.b)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa ATCC 15442</td>
<td>Vc: 190 x 10^5</td>
<td>A: 2 x 10^6</td>
<td>B: 1,5 x 10^8</td>
</tr>
<tr>
<td>Escherichia coli ATCC 15597</td>
<td>Vc: 200 x 10^5</td>
<td>Vc: 190 x 10^5</td>
<td>Vc: 180 x 10^6</td>
</tr>
<tr>
<td>Staphylococcus aureus ATCC 9005</td>
<td>Vc: 215 x 10^6</td>
<td>Vc: 190 x 10^6</td>
<td>Vc: 180 x 10^6</td>
</tr>
<tr>
<td>Enterococcus hirae ATCC 15541</td>
<td>Vc: 250 x 10^6</td>
<td>Vc: 200 x 10^6</td>
<td>Vc: 180 x 10^6</td>
</tr>
</tbody>
</table>

Vc = viable count
Nc = number of cfu/ml of the bacterial test suspension (5.4.1.4)
Na = number of cfu/ml of the bacterial test suspension (A.2)
R = reduction in viability
Na = number of cfu/ml in the test mixture (see 5.5.2.2.3 or 5.5.2.3.3)
A = number of cfu/ml of the experimental conditions validation (A.4.1.a or A.4.2.a)
B = number of cfu/ml of the neutralizer toxicity validation (A.4.1.b) or of the filtration validation (A.4.2.b)
C = the number of cfu/ml of the dilution-neutralization validation (A.4.1.c) or of the membrane filtration test validation (A.4.2.c)
Annex E
(ininformative)

Information on the application and interpretation of standards on chemical disinfectants and antiseptics

E.1 General guidelines for the application and interpretation of test methods in accordance with European Standards for chemical disinfectants and antiseptics

a) All ‘use recommendations’ for chemical disinfectant and antiseptic products should be supported by results of bactericidal, fungicidal, sporicidal and virucidal KS tests which are appropriate to the intended field and method of application.

b) To achieve this, chemical disinfectant and antiseptic products should be subjected to a specified programme of testing which will include phase 1, phase 2 step 1 and phase 2 step 2 tests, except for situations as given in points e), f) and g).

c) ‘Use recommendations’ may be supported by results of phase 3 tests which are appropriate to the intended field and method of application.

d) The various steps and phases are defined as follows:

Phase 1 suspension tests for the basic activity of the product;

Phase 2 step 1 suspension tests under conditions representative of practical use;

Phase 2 step 2 other laboratory tests e.g. handwash, handrub and surface tests simulating practical conditions.

Phase 3 field test under practical conditions.

e) It is accepted that for certain applications, the phase 2 step 1 and phase 2 step 2 tests may provide sufficient information for the particular application and that additional phase 1 tests may not be relevant.

For applications where phase 2 step 1 and phase 2 step 2 tests without phase 1 tests are used to support use recommendations, the justification for omitting phase 1 tests should be given. Such applications will be indicated either in the standard itself or in the additional standard which specifies guidelines for the application and interpretation of the tests.

f) It is accepted that for certain applications, the phase 2 step 1 suspension tests may provide sufficient information for the particular application and that additional phase 2 step 2 tests may not be relevant. For applications where phase 2 step 1 tests without phase 2 step 2 tests are used to support use recommendations the justification for omitting phase 2 step 1 tests should be given. Such applications will be indicated either in the standard itself or in the additional standard which specifies guidelines for the application and interpretation of the tests.

g) It is accepted that for certain applications the phase 2 step 2 together with phase 1 tests may provide sufficient information for the particular application and that additional phase 2 step 1 tests may not be relevant. For applications where phase 2 step 2 tests without phase 2 step 1 tests are used to support product claims, the justification for omitting phase 2 step 2 tests should be given. Such applications will be indicated either in the standard itself or in the additional standard which specifies guidelines for the application and interpretation of the tests.

h) All bactericidal, fungicidal and sporicidal claims for ‘bioactive substances’ should be supported by appropriate phase 1 tests.