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Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of bactericidal activity against Legionella of chemical disinfectants for aqueous systems — Test method and requirements (phase 2, step 1)

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National foreword

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Chemische Desinfektionsmittel und Antiseptika - Quantitativer Suspensionsversuch zur Bestimmung der bakteriziden Wirkung gegen Legionella von chemischen Desinfektionsmitteln für wasserführende Systeme - Prüfverfahren und Anforderungen (Phase 2, Stufe 1)

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Foreword

This document (EN 13623:2010) 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of an identical text or by endorsement, at the latest by March 2011, and conflicting national standards shall be withdrawn at the latest by March 2011.

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Introduction

This European Standard specifies a suspension test for establishing whether a chemical disinfectant has a bactericidal activity against *Legionella pneumophila* in the fields described in the scope. This standard is specifically prepared for water treatment products, but it may also be possible to use it for other products.

Proliferation of *Legionella* only occurs in waters under certain conditions, and predominantly poses a risk when aerosolised. Many systems containing water do not require treatment. A decision to add chemical disinfectants to any water should be based on a risk assessment.

If the product complies with the requirements of this standard, it can be considered bactericidal against *Legionella pneumophila*, but it should not necessarily be inferred that the product is acceptable for a specific site of application without consideration of other relevant factors such as the pH, water, chemistry, temperature and degree of biological fouling at that site of application. It does not take into account the protective effect conveyed by biofilm on the organisms.

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

1 Scope

This European Standard specifies a test method and the minimum requirements for bactericidal activity of chemical disinfectant products intended to be used for treatment in aqueous systems against *Legionella pneumophila* that form a homogeneous, physically stable preparation when diluted with buffered ferrous hard water or hard water. Whenever *Legionella pneumophila* poses a risk to human health, this method is suitable for water used in cooling towers and water for general purposes, like spas, pools, showers and other uses. The method is not suitable for electro-chemical disinfection.

The European Standard applies to products used to treat water in order to kill *Legionella pneumophila.*

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

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

NOTE 3 This method does not take into account the fact that *Legionella pneumophila* is often found in cells of amoebae and/or biofilms and that thereby a product's activity against the bacteria may be reduced.

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

2 Normative references

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

EN 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 and the following apply.

3.1
cooling water
water used to remove heat from a process or environment

3.2
water for general purposes
water used in premises other than water used as cooling water

4 Requirements

The product shall demonstrate al least a four decimal log (lg) reduction, when diluted with buffered ferrous hard water (5.2.2.10) or hard water (5.2.2.7), and tested in accordance with Clause 5 under the obligatory test conditions (one selected test organism, at either 20 °C or 30 °C) within 60 min for rapid acting products or 15 h for slower acting products.

The bactericidal activity shall be evaluated using the following test organism: *Legionella pneumophila.*

Where indicated, additional specific bactericidal activity shall be determined applying other contact times and test organisms (in accordance with 5.2.1 and 5.5.1.1) in order to take into account intended specific use conditions.

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

5 Test methods

5.1 Principle

5.1.1 A sample of the product diluted with hard water (5.2.2.7 or 5.2.2.10) is added to a test suspension of bacteria in a solution of an interfering substance. The mixture is maintained at either (20 ± 1) °C or (30 ± 1) °C for 60 min ± 10 s or (15 ± 1) h (obligatory test conditions). At the end of the chosen contact time, an aliquot is taken, and the bactericidal and/or the bacteriostatic activity in this portion is immediately neutralized or suppressed by a validated method. The method of choice is dilution-neutralization. If a suitable neutralizer cannot be found membrane filtration is used. The numbers of surviving bacteria in each sample are determined and the reduction is calculated.

5.1.2 The test is performed using *Legionella pneumophila* as test organism (obligatory test conditions).

5.1.3 Additional and optional contact times are specified. Additional test organisms may be used.

5.2 Materials and reagents

5.2.1 Test organism

The bactericidal activity shall be evaluated using the following strain as test organism [1)]: *Legionella pneumophila*: serogroup 1, Philadelphia (NCTC 11192; ATCC 33152).

If required for specific applications, additional test organisms may be used, e.g. *Legionella pneumophila* serogroup 1 Benidorm (NCTC 12006, ATCC 43108).

The required incubation temperature for this test organism is (36 ± 1) °C or (37 ± 1) °C (5.3.2.3). The same temperature (either 36 °C or 37 °C) shall be used for all incubations performed during a test and its control and validation.

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

5.2.2 Culture media and reagents

5.2.2.1 General

Unless specifically stated, all weights of chemical substances given in this 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.

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

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

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

5.2.2.2 Water

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

Sterilize in the autoclave (5.3.2.1, a)). Sterilization is not necessary if the water is used e.g. for preparation of culture media and subsequently sterilized.

NOTE See 5.2.2.10 for the procedure to prepare buffered ferrous hard water.

5.2.2.3 Buffered Charcoal Yeast Extract (BCYE) Agar

BCYE agar, consisting of

—	yeast extract (bacteriological grade)	10,0 g;
—	agar	12,0 g;
—	activated charcoal	2,0 g;
—	alpha-ketoglutarate, monopotassium salt	1,0 g;
—	ACES buffer (N-2-acetamido-2-aminoethanesulfonic acid)	10,0 g;
—	potassium hydroxide (KOH) (pellets)	2,8 g;
—	L-cysteine hydrochloride monohydrate	0,4 g;
—	iron(III) pyrophosphate [Fe4(P207)3]	0,25 g;
—	distilled water	to 1 000,0 ml.

Preparation

a) Cysteine and iron solutions

Prepare fresh solutions of L-cysteine hydrochloride and iron(III) pyrophosphate by adding 0,4 g and 0,25 g respectively to 10-ml-volumes of water (5.2.2.2). Sterilize each solution by membrane filtration (5.3.2.7). Store in clean sterile containers at (20 ± 3) °C for not more than three months.

b) ACES buffer

Add the ACES granules to 500 ml of water (5.2.2.2) and dissolve by standing in a water bath at 45 °C to 50 °C. To a separate 480 ml of water (5.2.2.2), add all the potassium hydroxide pellets and dissolve with gentle shaking. To prepare the ACES buffer, mix the two solutions.

NOTE 1 ACES buffer can cause denaturation of the yeast extract if the following sequence is not followed.

c) Final medium

Add sequentially to the 980 ml of ACES buffer, the charcoal, yeast extract and α-ketoglutarate. Prepare a 0,1 mol/l solution of potassium hydroxide (KOH) by dissolving 5,6 g in 1 l of water (5.2.2.2). Prepare a 0,1 mol/l solution of sulphuric acid (H_2SO_4) by carefully adding 5,3 ml of H_2SO_4 to 1 l of water (5.2.2.2). Use the solutions of 0,1 mol/l potassium hydroxide or 0,1 mol/l sulphuric acid as appropriate to adjust the pH to 6,9 ± 0,2. Add the agar, mix and autoclave (5.3.2.1, a)). After autoclaving, allow to cool to (47 ± 2) °C in a water bath (5.3.2.2).

Add the L-cysteine and the iron(III) pyrophosphate solutions aseptically, mixing well between additions.

Dispense in 20 ml volumes into Petri dishes of 90 mm to 100 mm diameter. The pH of the final medium is 6,9 ± 0,2 at 25 °C. Allow excess moisture on the plates to dry and store at (4 ± 2) °C in airtight containers in the dark for up to four weeks.

Prolonged heating during sterilisation or heating at too high a temperature shall be avoided, as it can affect the nutritional qualities of BCYE medium. Batch-to-batch variation of the ingredients of the medium (particularly α-ketoglurarate) can also affect its performance. Therefore it is essential to check the quality of each newly prepared batch of media for its ability to support the growth of the test organism within three days of incubation using the validation suspension N_V (5.4.1.5).

NOTE 2 The ability of the media to support the growth of Legionella should be assessed quantitatively using either known quantities of the obligatory Legionella strain or by direct comparison to previous batches. Commercially supplied media may be used without testing if it has been performance tested in a laboratory accredited to EN ISO/IEC 17025:2005 for that purpose.

5.2.2.4 BCYE Broth

Prepared by the same method as BCYE agar (5.2.2.3), but omitting the addition of the agar.

5.2.2.5 Neutralizer

The neutralizer shall be validated for the product being tested in accordance with 5.5.1.2, 5.5.1.3 and 5.5.2. It shall be sterile.

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

5.2.2.6 Rinsing liquid (for membrane filtration)

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

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

5.2.2.7 Hard water for general purposes (HWGP)

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

a) 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 (5.3.2.1, a)). Autoclaving – if used – may cause a loss of liquid. In this case make up to 1 000 ml with water (5.2.2.2) under aseptic conditions. Store the solution in the refrigerator (5.3.2.8) for no longer than one month;

b) 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 the refrigerator (5.3.2.8) for no longer than one week;

c) place 600 ml to 700 ml of water (5.2.2.2) in a 1 000 ml volumetric flask (5.3.2.12) and add 6,0 ml (5.3.2.9) of solution A, then 8,0 ml of solution B. Mix and dilute to 1 000 ml with water (5.2.2.2). The pH of the hard water shall be 7,0 ± 0,2, when measured at (20 ± 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 1 When preparing the product test solutions (5.4.2), the addition of the product to the hard water produces a different final water hardness in each test tube. In any case the final hardness is lower than 300 mg/l of calcium carbonate ($CaCO_3$) in the test tube.

NOTE 2 This hard water represents typical conditions of non cooling water.

5.2.2.8 Interfering substance (yeast extract)

— yeast extract 0,5 g;

— water to 1 000,0 ml.

Sterilize in the autoclave (5.3.2.1, a)).

Final concentration of the yeast extract in the test is 0,000 5 %.

5.2.2.9 Page's Saline

Saline solution, consisting of:

— sodium chloride (NaCl) 0,120 g;

— magnesium sulphate ($MgSO_4 \times 7H_20$) 0,004 g;

— calcium chloride ($CaCl_2 \times 2H_20$) 0,004 g;

— disodium hydrogen phosphate (Na_2HPO_4) 0,142 g;

— potassium dihydrogenphosphate (KH_2PO_4) 0,136 g;

— water (5.2.2.2) to 1 000,0 ml.

Sterilize in the autoclave (5.3.2.1, a)).

NOTE To aid accurate preparation, it is recommended that a 10 l volume of Page's Saline is prepared and dispensed in smaller volumes as required for autoclaving. Alternatively the salt solutions may be made up individually in concentrated form for dilution when the product is required.

5.2.2.10 Buffered ferrous hard water for treatment of cooling water (BFHW)

For the preparation of 1 l of BFHW, the procedure is as follows:

a) 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 (5.3.2.1, a)). Autoclaving – if used – may cause a loss of liquid. In this case make up to 1 000 ml with

water (5.2.2.2) under aseptic conditions. Store the solution in the refrigerator (5.3.2.8) for no longer than one month;

b) 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 the refrigerator (5.3.2.8) for no longer than one week;

c) 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 70 ml of 0,2 M boric acid (13,6 g of Boric acid made up to 1 000 ml with distilled water), 30 ml of 0,05 M borax (19,07 g of Borax made up to 1 000 ml with distilled water) and 6,0 ml (5.3.2.9) of solution A, then 8,0 ml of solution B. Finally add 1,0 ml ferric sulphate solution ($3,0 \times 10^{-3}$ mol/l). Mix and dilute to 1 000 ml with water (5.2.2.2). The pH of the hard water shall be 8,0 ± 0,2, when measured at (20 ± 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). Sterilize by membrane filtration (5.3.2.7).

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

NOTE 1 When preparing the product test solutions (5.4.2), the addition of the product to the hard water produces a different final water hardness in each test tube. In any case the final hardness is lower than 300 mg/l of calcium carbonate ($CaCO_3$) in the test tube.

NOTE 2 This buffered ferrous hard water represents typical conditions of cooling water.

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 that are supplied sterile, by one of the following methods:

a) By moist heat, in the autoclave (5.3.2.1, a));

b) By dry heat, in the hot air oven (5.3.2.1, b)).

5.3.2 Usual microbiological laboratory equipment[2)]

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.

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

5.3.2.2 Water baths, capable of being controlled at (20 ± 1) °C, (30 ± 1) °C and at (47 ± 2) °C. An oven or incubator capable of being controlled at either of these temperatures may be the alternative.

5.3.2.3 Incubator, capable of being controlled at (36 ± 1) °C or (37 ± 1) °C. Plates will need to be incubated in sealed bags or containers to prevent drying out of the agar.

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

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

5.3.2.5 Stopwatch

5.3.2.6 Electromechanical Agitator (e.g. Vortex® mixer)[3)]

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 sterilization of solutions and suspensions and if the membrane filtration is used (5.5.3).

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

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

5.3.2.9 Graduated pipettes, of nominal capacities 10 ml, 1 ml and 0,1 ml, or calibrated automatic pipettes.

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

5.3.2.11 Centrifuge (2 000 g_N).

5.3.2.12 Volumetric flasks.

5.4 Preparation of test organism suspensions and test solutions

5.4.1 Test organism suspension (test and validation suspension)

5.4.1.1 General

Two different suspensions shall be prepared: the "test suspension" to perform the test and the "validation suspension" to perform the controls and method validation.

5.4.1.2 Preservation and stock cultures of test organism

The test organisms and their stock cultures shall be kept and reconstituted in accordance with the supplier's instructions.

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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.

Subculture onto BCYE agar (5.2.2.3). After incubation (5.3.2.3), make a suspension from the resulting growth just visible to the naked eye and dispense in Page's Saline (5.2.2.9) or water (5.2.2.2) for storage at a temperature equal or lower than − 70 °C. In case of no growth on BCYE agar a BCYE broth may be inoculated (5.2.2.4), and a subculture from this broth may be incubated (5.3.2.3) and prepared as described above.

NOTE Commercially available cryo-preservation systems may be used providing they produce satisfactory recovery of the organism.

5.4.1.3 Working cultures of test organism

In order to prepare the working culture of the test organism (5.2.1), prepare a subculture from the frozen suspension (5.4.1.2) by streaking onto BCYE plates (5.2.2.3) and incubate (5.3.2.3). This subculture is the working culture.

5.4.1.4 Test suspensions ("*N*")

a) Take the working culture (5.4.1.3) and remove growth from the plate by suspending with Page's saline (5.2.2.9). Aspirate the suspension and transfer into a tube. Mix (5.3.2.6) for 10 s, then centrifuge (5.3.2.11) for 15 min. Resuspend the pellet in Page's saline (5.2.2.9).

b) Adjust the number of cells in the suspension to 1,5 x 10^8 cfu/ml[4)] to 5 x 10^8 cfu/ml using Page's saline, (5.2.2.9), estimating the number of cfu by any suitable means. Maintain this test suspension in the water bath at either 20 °C for products intended for non cooling water applications or 30 °C for cooling water products (5.5.1.1, a)) and use within 2 h.

NOTE The use of spectrophotometer for adjusting the number of cells is highly recommended (about 620 nm wavelength - cuvette 10 mm path length). Each laboratory should therefore produce calibration data for each test organism knowing that suitable values of optical density are found between 0,150 and 0,400. A colorimeter is a suitable alternative.

c) For counting, prepare 10^{-6} and 10^{-7} dilutions of the test suspension using Page's saline (5.2.2.9). Mix (5.3.2.6). Take a sample of 1,0 ml of each dilution in duplicate and inoculate using the spread plate technique. Spread each 1,0 ml sample – divided into portions of approximately equal size (max. 0,2 ml) – on an appropriate number of plates containing BCYE (5.2.2.3).

For incubation and counting see 5.4.1.6.

5.4.1.5 Validation suspension ("N_V")

a) To prepare the validation suspension, dilute the test suspension (5.4.1.4) with Page's saline (5.2.2.9) to obtain 3,0 x 10^2 cfu/ml to 1,6 x 10^3 cfu/ml (about one fourth (1+3) of the 10^{-5} dilution).

b) For counting prepare a 10^{-1} dilution with Page's saline (5.2.2.9). Mix (5.3.2.6). Take a sample of 1,0 ml in duplicate and inoculate using the spread plate technique (5.4.1.4, c)).

For incubation 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 seven days. Discard any plates that are not countable for any reason. Count the plates and determine the number of cfu.

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

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

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

5.4.2 Product test solution

The concentration of a product test solution shall be ten times the desired test concentration because it is diluted to 10 % during the test and the method validation (5.5.2 or 5.5.3). Product test solutions shall be prepared in hard water at minimum three different concentrations to include one concentration in the active range and one concentration in the non-active range (5.8.2). The type of hard water is selected according to the intended use: BFHW (5.2.2.10) for products intended to be used for treatment of cooling water and HWGP (5.2.2.7) for products intended to be used for treatment of water for general purposes. The product as received may be used as one of the product test solutions, in this case the highest tested concentration is 10 %.

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 (5.2.2.7 or 5.2.2.10). Subsequent dilutions (lower concentrations) shall be prepared in volumetric flasks (5.3.2.12) on a volume/volume basis in hard water (5.2.2.7 or 5.2.2.10).

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

The product test solutions shall be prepared freshly and used in the test within 2 h. They shall give a physically 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 flocculant (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 flocculant 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 bactericidal activity of the product

5.5.1 General

5.5.1.1 Experimental conditions (obligatory and additional)

Besides the obligatory, contact time 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 θ (in degrees Celsius (°C)):

   1) the obligatory temperature to be tested for Cooling Water (3.1) is θ = 30 °C;

   2) the obligatory temperature to be tested for Water for General Purposes (3.2) is θ = 20 °C;

   3) the allowed deviation for the chosen temperature is ± 1 °C;

NOTE No additional temperatures are considered relevant to this test.

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

1) for fast acting products such as oxidising substances the obligatory contact time to be tested is t = 60 min;

2) for slow acting products the obligatory contact time is 15 h;

3) the additional contact times may be chosen from 2 h, 6 h, 15 h, 40 h, 48 h;

4) the allowed deviation for the chosen contact time of 60 min or less is ± 10 s, for contact times of 6 h or less it is ± 5 min and ± 1 h for all other contact times;

c) interfering substance: the obligatory interfering substance to be tested is 0,05 % yeast extract solution;

d) test organism:

1) the obligatory test organism is *Legionella pneumophila* (5.2.1);

2) additional test organisms (other strains of *Legionella pneumophila* or species of Legionella) may be used.

5.5.1.2 Choice of test method

The method of choice is the dilution-neutralization method. 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.

If both neutralizers are found to be invalid, the membrane filtration method (5.5.3) may be used.

5.5.1.3 General instructions for validation and control procedures

The neutralization and/or removal of the bactericidal or bacteriostatic 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 (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.

The same hard water (5.2.2.7 or 5.2.2.10) used in the test (5.5.2.2) shall be used in the validation and controls. The same agar (5.2.2.3) used in the test (5.5.2.2) shall be used in the validation and controls.

5.5.1.4 Equilibration of temperature

Prior to testing, equilibrate all reagents (product test solutions (5.4.2), test suspension (5.4.1.4), validation suspension (5.4.1.5) hard water (5.2.2.7 or 5.2.2.10) and interfering substance (5.2.2.8) to the test temperature of either 20 °C for products intended for non cooling water applications or 30 °C for cooling water products (5.5.1.1, a)) using the water bath (5.3.2.2) controlled at (20 ± 1) °C or (30 ± 1) °C.

Check that the temperature of the reagents is stabilized at the chosen temperature.

The neutralizer (5.2.2.5) or the rinsing liquid (5.2.2.6) and water (5.2.2.2) shall be equilibrated at a temperature of (20 ± 1) °C.

5.5.1.5 Precautions for manipulation of test organisms

Do not touch the upper part of the test tube sides when adding the test or the validation suspensions (5.4.1).

5.5.2 Dilution-neutralization method[5)]

5.5.2.1 General

The test and the control and validation procedures (5.5.2.2 to 5.5.2.5) shall be carried out at the same time.

5.5.2.2 Test "N_a" – determination of bactericidal concentrations

The procedure for determining bactericidal concentrations is as follows:

a) Pipette 0,1 ml of the interfering substance (5.2.2.8) and 7,9 ml BFHW (5.2.2.10) or HWGP (5.2.2.7) into a tube. Add 1,0 ml of the test suspension (5.4.1.4). Start the stopwatch (5.3.2.5) immediately, mix (5.3.2.6) and place the tube in a water bath controlled at either 20 °C for products intended for non cooling water applications or 30 °C for cooling water products (5.5.1.1, a)) for 2 min ± 10 s.

   At the end of this time, add 1,0 ml of one of the product test solutions (5.4.2). Restart the stopwatch at the beginning of the addition. Mix (5.3.2.6) and place the tube in a water bath controlled at either 20 °C for products intended for non cooling water applications or 30 °C for cooling water products for the chosen contact time *t* (5.5.1.1, b)). Just before the end of *t*, mix (5.3.2.6) again.

b) At the end of *t*, take a 1,0 ml sample of the test mixture N_a and transfer into a tube containing 8,0 ml neutralizer (5.2.2.5) and 1,0 ml water (5.2.2.2). Mix (5.3.2.6) and place in a water bath controlled at (20 ±1) °C. After a neutralization time of 5 min ± 10 s, immediately take four samples of 1,0 ml neutralized test mixture N_a (containing neutralizer, product test solution, interfering substance and test suspension). Inoculate two of the 1,0 ml samples using the spread plate technique divided into portions of approximately equal size (max. 0,2 ml) on an appropriate number of BCYE plates (5.2.2.3). Dilute the two remaining 1,0 ml samples with 9,0 ml of Page's saline (5.2.2.9) and inoculate 1,0 ml from each onto an appropriate number of BCYE plates (5.2.2.3).

   For incubation and counting, see 5.5.2.6.

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).

5.5.2.3 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) Pipette 0,1 ml of the interfering substance used in the test (5.5.2.2) into a tube. Add 1,0 ml of the validation suspension (5.4.1.5). Start the stopwatch immediately, mix (5.3.2.6) and place the tube in a water bath controlled at either 20 °C for products intended for non cooling water applications or 30 °C for cooling water products.for 2 min ± 10 s.

   At the end of this time, add 8,9 ml of hard water (5.2.2.10 or 5.2.2.7). Restart the stopwatch at the beginning of the addition. Mix (5.3.2.6) and place the tube in a water bath controlled at either 20 °C for products intended for non cooling water applications or 30 °C for cooling water products.for *t*. Just before the end of *t*, mix (5.3.2.6) again.

b) At the end of *t*, take a sample of 1,0 ml of this mixture *A* in duplicate and inoculate using the spread plate technique (5.5.2.2b)).

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5) For a graphical representation of this method, see C.1.

For incubation and counting, see 5.5.2.6.

5.5.2.4 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 (5.5.2.2) – 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 (5.3.2.6), and place the tube in a water controlled at (20 ± 1) °C for 5 min ± 10 s. Just before the end of this time, mix (5.3.2.6).

b) At the end of this time, take a sample of 1,0 ml of this mixture *B* in duplicate and inoculate using the spread plate technique (5.5.2.2, b)).

For incubation and counting, see 5.5.2.6.

5.5.2.5 Method validation *C* – dilution-neutralization validation

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

a) Pipette 0.1 ml of the interfering substance used in the test (5.5.2.2) into a tube. Add 7,9 ml of hard water (5.2.2.10 or 5.2.2.7) and then, starting a stopwatch, add 1,0 ml of the product test solution only of the highest concentration used in the test (5.5.2.2). Mix (5.3.2.6) and place the tube in a water bath controlled at either 20 °C for products intended for non cooling water applications or 30 °C for cooling water products for *t*. Just before the end of *t*, mix (5.3.2.6) again.

b) At the end of *t* transfer 1,0 ml of the mixture into a tube containing 8,0 ml of neutralizer (used in 5.5.2.2). Restart the stopwatch at the beginning of the addition. Mix (5.3.2.6) 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 at the beginning of the addition and mix (5.3.2.6). Place the tube in a water bath controlled at (20 ± 1) °C for (30 ± 1) min. Just before the end of this time, mix (5.3.2.6) again. At the end of this time, take a sample of 1,0 ml of the mixture *C* in duplicate and inoculate using the spread plate technique (5.5.2.2, b)).

For incubation and counting, see 5.5.2.6.

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

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 seven days. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units.

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 N_a and in the validation mixtures *A*, *B* and *C* using the method given in 5.6.2.4 and 5.6.2.6. Verify according to 5.7.

5.5.3 Membrane filtration method[6)]

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 (5.2.2.6). 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 "N_a" – determination of the bactericidal concentrations

The procedure for determining the bactericidal concentrations is as follows:

a) See 5.5.2.2, a).

b) At the end of *t*, take a sample of 0,1 ml of the test mixture "N_a" in duplicate and transfer each 0,1 ml sample into a separate membrane filtration apparatus (5.5.3.1). Filter immediately. Filter through at least 150 ml but no more than 500 ml of rinsing liquid (5.2.2.6). 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 BCYE plates (5.2.2.3).

For incubation and counting, see 5.5.3.6.

c) See 5.5.2.2, c).

d) See 5.5.2.2, d).

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 5.5.2.3, a).

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 (5.5.3.1). Filter immediately and additionally with 50 ml of water (5.2.2.2). Then transfer each of the membranes to the surface of separate BCYE 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 (5.5.3.1).

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6) For a graphical representation of this method, see C.2.

b) Filter immediately. Filter through the rinsing liquid (5.2.2.6) the same way as in the test (5.5.3.2, b)). 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 BCYE 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 5.5.2.5, a).

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 (5.5.3.1). Filter immediately. Filter through the rinsing liquid (5.2.2.6) the same way as in the test (5.5.3.2, b)), then cover the membranes with 50 ml of the rinsing liquid (5.2.2.6) 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 BCYE 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 seven days. Discard any plates that are not countable (for any reason). Count the colonies on the membranes.

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 cfu/ml in the test mixture "N_a" and in the validation mixtures "*A*", "*B*" and "*C*" using the method given in 5.6.2.4 and 5.6.2.6. Verify according to 5.7.

5.6 Experimental data and calculations

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 bacterial suspensions, N_a represents the bactericidal test mixture, *A* (experimental conditions control), *B* (neutralizer control), *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 millilitre in the different test mixtures in accordance with Table 1.

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

	Number of cells per millilitre in the bacterial suspensions	Number of cells per millilitre in the test mixtures at the beginning of the contact time (t = 0)	Number of survivors per millilitre in the test mixtures at the end of the contact time t or 5 min (B) or 30 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:

a) in the dilution-neutralization method (test and controls), a V_C value is the number of colony-forming units counted per 1,0 ml sample;

b) in the membrane filtration method, a V_C value is the number of colony-forming units counted per 0,1 ml sample of test mixture "N_a" and per 1,0 ml sample in the controls.

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).

5.6.2.2 Determination of V_C-values

The V_C-values are determined as follows:

a) The usual limits for counting bacteria 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 limits are different: 150, therefore with the 10 % deviation, the limit is 165.

NOTE 1 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), e.g. three plates per 1 ml sample with 3 cfu, 8 cfu and 5 cfu give a V_C-value of 16. The upper limits (330, 165) reflect the imprecision of counting confluent colonies and growth inhibition due to nutrient depletion. They refer only to the counting on one plate and not necessarily to the sample.

b) For counting the test suspension "N" (5.4.1.6*)*, the validation suspension "N_V" (5.4.1.6) and for all countings of the dilution-neutralization method (5.5.2.6), determine and record the V_C values according to the number of plates used per 1 ml sample (5.6.1.2).

NOTE 2 If more than one plate per 1 ml sample has been used to determine the V_C value, the countings per plate should be noted.

If the count on one plate is higher than 330, report the number as “> 330”. If more than one plate per 1 ml sample has been used and at least one of them shows a number higher than 330, report this V_C-value as "> sum of the counts" (e.g. for "> 330, 310, 302", report "> 942").

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).

For the membrane filtration method (5.5.3), the counts on the membranes are the V_C values (5.6.1.2).

Report the V_C values below the lower limit (14) or above the upper limit (165) as described above.

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 millilitre in the test suspension (5.4.1.4; 5.6.1.1).

Since the two dilutions of the test suspension (5.4.1.4 in connection with 5.4.1.6) are evaluated, calculate the number of cfu/ml as the weighted mean count using the following equation:

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

where:

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

n_1 is the number of V_C-values taken into account in the lower dilution, i.e. 10^{-6};

n_2 is the number of V_C-values taken into account in 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 more than 5, the preceding figure is increased by one unit; if the last figure is equal to 5, round off the preceding figure to the next nearest even figure. Proceed stepwise until two significant figures are obtained. As a result, the number of cfu/ml is expressed by a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

EXAMPLE

$$N = \frac{390 + 410 + 39 + 50}{(2 + 0{,}1 \times 2) \times 10^{-6}} = \frac{889}{2{,}2 \times 10^{-6}} = 4{,}040\,9 \times 10^{8} = 4{,}0 \times 10^{8}\ (in\ cfu/ml)$$

N_0 is the number of cells per millilitre in the test mixture (5.5.2.2, a)) at the beginning of the contact time (time "zero" $t = 0$). It is one-tenth of the weighted mean of *N* due to the tenfold dilution by the addition of the product and water.

5.6.2.4 Calculation of N_a

N_a is the number of survivors per millilitre in the test mixture (5.5.2.2, a)) at the end of the contact time and before neutralization or membrane filtration. It is tenfold higher than the V_C-values due to the addition of neutralizer and water (5.5.2.2, b)) or the sample volume of 0,1 ml (5.5.3.2, b)) in the membrane filtration method.

Calculate the mean for each dilution step $N_a^{\,0}$ and $N_a^{\,-1}$ using the following equations:

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

$N_a^{-1} = 100 \times c/n$ (3)

where:

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

n is the number of V_C-values taken into account.

When the values of N_a^{0} and N_a^{-1} lie between the upper and lower counting limits, the value of N_a is the mean of these two values.

a) If one or both of the duplicate V_C-values are either below the lower or above the upper limit, express the results as "less than" or "more than".

EXAMPLE 1

Duplicate V_C-values: 2, 16

$$N_a^{-1} = \frac{(<14+16)\times 10}{2} = <150\times 10^1 = <1\,500 = <1{,}5\times 10^3$$

EXAMPLE 2

Duplicate V_C-values N_a^{-2} (membrane filtration): > 165, > 165

$$N_a^{-2} = \frac{(>165+>165)\times 10}{2} = >1\,650\times 10^2 = >165\,000 = >1{,}6\times 10^5$$

EXAMPLE 3

Duplicate V_C-values (minimum of five spread plates per 1,0 ml sample): > 660, 600

$$N_a^{0} = \frac{(>660+600)\times 10}{2} = >6\,300 = >6{,}3\times 10^3$$

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

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

EXAMPLE 4

	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

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

EXAMPLE 5

	V_{C1}	V_{C2}		mean x 10	
N_a^{0}	< 14	< 14	=	< 140	
N_a^{-1}	< 14	< 14	=	< 140	N_a = < 140 x 10^0 = < 1,4 x 10^2

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

EXAMPLE 6

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

5.6.2.5 Calculation of N_V and N_{V0}

N_V is the number of cells per millilitre in the validation suspension (5.4.1.5, a)). 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, b)).

N_{V0} is the number of cells per millilitre 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 mean of the V_C-values of N_V (5.4.1.6, c)) taken into account.

Calculate N_V and N_{V0} using the following equations:

$$N_V = 10 \times c/n \quad (4)$$

$$N_{V0} = c/n \quad (5)$$

where:

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

n is the number of V_C-values taken into account.

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

A, *B* and *C* are the numbers of survivors in the experimental conditions control *A* (5.5.2.3 or 5.5.3.3), neutralizer control *B* (5.5.2.4) or filtration control (5.5.3.4) and method validation *C* (5.5.2.5 or 5.5.3.5) at the end of the contact time *t* (*A*) or the defined times 5 min (*B*) and 30 min (*C*). They correspond to the mean of the V_C-values of the mixtures *A*, *B* and *C* taken into account.

Calculate *A*, *B* and *C* using the following equation:

$$A, B, C = c/n \quad (6)$$

where

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

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:

a) all results meet the criteria of 5.7.3; and

b) the requirements of 5.8.2 are fulfilled.

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 (5.6.2.2, b)) are taken as the upper limit number.

EXAMPLE For *N*: 10^{-6} dilution: 168 + 215 cfu/ml; 10^{-7} dilution: 20 + < 14 cfu/ml; (168 + 215)/(20 + 14) = 383/34 = 11,26 = between 5 and 15.

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

5.7.3 Basic limits

For each test organism check that:

a) *N* is between $1,5 \times 10^8$ and $5,0 \times 10^8$ ($8,17 \leq lg\ N \leq 8,70$);

N_0 is between $1,5 \times 10^7$ and $5,0 \times 10^7$ ($7,17 \leq lg\ N_0 \leq 7,70$);

b) N_{V0} is between 30 and 160 ($3,0 \times 10^1$ and $1,6 \times 10^2$);

N_V is between $3,0 \times 10^2$ and $1,6 \times 10^3$);

c) *A*, *B*, *C* are equal to or greater than $0,5 \times N_{V0}$;

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

5.8 Expression of results and precision

5.8.1 Reduction

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

For each test organism record the number of cfu/ml in the test suspension *N* and N_0 (5.6.2.3) and of the results of the test N_a (5.6.2.4).

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

$$\lg R = \lg N_0 - \lg N_a \qquad (7)$$

For the controls and validation of the dilution-neutralization method or membrane filtration method, record N_{V0} (5.6.2.5), the results of *A*, *B* and *C* (5.6.2.6) and their comparison with N_{V0} (5.7.3, c)).

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

At least one concentration per test (5.5.2.2, a) to c)) 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 Bactericidal concentration

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

5.8.4 Precision, repetition

Taking into account the precision of other bactericidal phase 2 step 1 tests (e.g. EN 1276), repetition of the test is recommended. The number of repetitions shall be decided according to the required level of precision, taking into account the intended use of the test results.

Repetition means the complete test procedure with separately prepared test and validation suspensions. The mean of the results of the repetitions − not each single result − shall demonstrate at least a 4 lg reduction and shall also be calculated and recorded.

5.9 Interpretation of results – conclusion

5.9.1 General

According to the chosen experimental conditions (obligatory or obligatory and additional) the bactericidal concentrations determined according to this standard may differ (Clause 4). If the product is to be applied in cooling towers buffered ferrous hard water (BFHW) shall be used for diluting the product, for all other purposes hard water (HWGP) shall be used.

5.9.2 Bactericidal activity for general purposes

The product shall be deemed to have passed the EN 13623 standard if it demonstrates in a valid test at least a 4 lg reduction within 60 min or 15 h at 20 °C defined by this standard when the test organism is *Legionella pneumophila.* Depending on the water used for diluting the product (5.9.1) it is suitable for the treatment of cooling water or of water for general purposes.

5.9.3 Bactericidal activity for specific purposes

The bactericidal concentration for a specific purpose is the concentration of the tested product for which at least a 4 lg reduction is demonstrated in a valid test under the additional chosen test conditions. The product shall have passed the EN 13623 standard under the obligatory test conditions. The bactericidal concentration for specific purposes may be lower than the one determined for general purposes.

5.10 Test report

The test report shall refer to this European Standard (EN 13623). The test report shall state, at least, the following information:

a) identification of the testing laboratory;

b) identification of the client;

c) identification of the sample:

1) name of the product;

2) batch number and − if available − expiry date;

3) manufacturer – if not known: supplier;

4) date of delivery;

5) storage conditions;

6) product diluent recommended by the manufacturer for use;

7) active substance(s) and their concentration(s) (optional);

8) appearance of the product;

d) test method and its validation:

1) if the dilution-neutralization method is used, full details of the test for validation of the neutralizer shall be given;

2) 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;

e) experimental conditions:

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

2) diluent used for product test solution (BFHW or HWGP);

3) product test concentrations (i.e. desired test concentrations according to 5.4.2);

4) appearance of the product dilutions;

5) contact time(s);

6) test temperature;

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;

10) neutralizer or rinsing liquid;

11) identification of the bacterial strains used;

f) test results:

1) controls and validation;

2) evaluation of bactericidal activity;

3) number of repetitions;

g) special remarks;

h) conclusion;

i) locality, date and identified signature.

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

Annex A
(informative)

Determination of the bactericidal activity against *Legionella pneumophila*

a) Identification of the test laboratory .....BACS Laboratory

Identification of client…….A.N. Other Water Treatment Ltd

b) Identification of the sample

Name of the product .....Legionicide
Batch number 96-23-0250
Expiry Date…….2012-2-28
Manufacturer .....Centipide Formulations Inc.
Date of delivery 2010-02-03
Storage conditions .....Those of the manufacturer
Active substance(s) and its/their concentration(s) (optional) ....Not indicated

c) Test method and its validation

Method .....Dilution neutralization
Neutralizer .....Polysorbate 80 (30 ml/l), sodium thiosulphate (5 g/l), L-histidine (1 g/l) in 0,002 5 N phosphate buffer

d) Experimental conditions

Period of analysis .....2010-04-11 to 2010-05-28
Appearance of the products and its dilution .....Colourless clear liquid soluble in hard water
Product test concentrations .....0,1 g/l; 0,2 g/l; 0,3 g/l
Test temperature .....(30 ± 1) °C
Contact times .....60 min and 15 h
Interfering substances.....0,000 5 w/v % yeast extract;
Product diluent.....Hard water
Stability of the test mixture.....No precipitation of product throughout test
Temperature of incubation .....(36 ± 1) °C
Counting procedure.....Spread plate
Bacterial strain used.....*Legionella pneumophila* serogroup 1
.....NCTC 11192

e) Test results

See Tables A.1 and A.2.

f) Conclusion

According to EN 13623 (date of edition), the batch 96-23-0250 of the product "Legionicide", when diluted at 0,3 g/l in hard water, possesses bactericidal activity against the referenced strain of *Legionella pneumophila*, serogroup 1, NCTC 11192.

g) Locality, date and identified signature

Table A.1 — Verification of the methodology and validation of dilution neutralization for the test concentration of 0,3 g/l of the product received

Test organism	**Colony counts** (V_C-values per 1 ml sample)				
	Bacterial test suspension N (5.4.1.4)	Neutralization test suspension N_V (5.4.1.5)	Validation of neutralization and experimental conditions		
			Neutralizer toxicity control (B)	Dilution-neutralization control (C)	Experimental conditions control (A)
Legionella pneumophila	390, 410 at dilution of 10^{-6} 39, 50 at dilution of 10^{-6} ($N = 4{,}0 \times 10^8$) $N_0 = 4{,}0 \times 10^7$	50, 75 ($N_V = 625$) $N_{V0} = 62{,}5$	40, 44 ($B = 42$)	71, 59 ($C = 65$)	55, 60 ($A = 57{,}5$)
NOTE For the strain tested: — N is between $1{,}5 \times 10^8$ cfu/ml and 5×10^8 cfu/ml; — N_V is between $3{,}0 \times 10^2$ and $1{,}6 \times 10^3$ cfu/ml; — A, B, C are equal to or greater than $0{,}5 \times N_{V0}$.					

Table A.2 — Test results

Test organism	**Viable counts of test mixture at test concentration and contact time indicated** cfu/ml					
	0,1 g/l		0,2 g/l		0,3 g/l	
	60 min	15 h	60 min	15 h	60 min	15 h
V_C-values (N_a^0)	> 660, > 660	> 660, > 660	> 660, > 660	440, 408	17, 32	< 14, < 14, < 14
V_C-values (N_a^{-1})	> 660, > 660	408, 515	> 660, > 660	37, 59	< 14, < 14, < 14	< 14, < 14, < 14
Legionella pneumophila (N_a)	$> 6{,}6 \times 10^4$	$4{,}6 \times 10^4$	$> 6{,}6 \times 10^4$	$4{,}5 \times 10^3$	$2{,}5 \times 10^2$	$< 1{,}4 \times 10^2$
Calculation of lg reduction counts **lg R = lg N_0 – lg N_a** *lg N_0 = 7,32*	7,60 - 4,81 = 2,99	7,60 - 4,66 = 2,94	7,60 - 4,81 = 2,79	7,60 -3,65 = 3,95	7,60 - 2,40 = 5,2	7,60 - 2,15 = 5,45

Annex B
(informative)

Neutralizer

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

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

Table B.1

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[b], Saponin, Polysorbate 80, Sodium dodecyl sulphate, Ethylene oxide condensate of fatty alcohol (non-ionic surfactants)[c]	- 30 g/l Polysorbate 80 + 30 g/l saponin + 3 g/l lecithin. - 30 g/l Polysorbate 80 + 4 g/l sodium dodecyl sulphate + 3 g/l lecithin. - 3 g/l Ethylene oxide condensate of fatty alcohol + 20 g/l lecithin + 5 g/l polysorbate 80. *Rinsing liquid: 1 g/l tryptone + 9 g/l NaCl; 5 g/l polysorbate 80.*
Biguanides and similar compounds	Lecithin[b], Saponin, Polysorbate 80	- 30 g/l Polysorbate 80 + 30 g/l saponin + 3 g/l lecithin. *Rinsing liquid: 1 g/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 thiosulphate + 30 g/l polysorbate 80 + 3 g/l lecithin. - 50 g/l Polysorbate 80 + 0,25 g/l catalase + 10 g/l lecithin. *Rinsing liquid: 3 g/l sodium thiosulphate.*
Aldehydes	L-histidine Glycine	- 30 g/l Polysorbate 80 + 3 g/l lecithin + 1 g/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).*

Phenolic and related compounds: orthophenylphenol, phenoxyethanol, triclosan, phenylethanol, etc. Anilides	Lecithin [b] Polysorbate 80 Ethylene oxide condensate of fatty alcohol [c]	- 30 g/l Polysorbate 80 + 3 g/l lecithin. - 7 g/l Ethylene oxide condensate of fatty alcohol + 20 g/l lecithin + 4 g/l polysorbate 80. *Rinsing liquid: 1 g/l tryptone + 9 g/l NaCl; 5 g/l polysorbate 80.*
Alcohols [e]	Lecithin [b], Saponin, Polysorbate 80	30 g/l Polysorbate 80 + 30 g/l saponin + 3 g/l lecithin. *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 (e.g. phosphate buffer 0,25 mol/l: 34 g potassium dihydrogen phosphate (KH_2PO_4); 500 ml distilled water; adjusted to pH 7,2 ± 0,2 with sodium hydroxide (NaOH) 1 mol/l; distilled water up to 1 000 ml).

[b] Egg and soya; egg is preferable.

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

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

[e] For the neutralization of short chain alcohols (less than C_5), simple dilution may be appropriate. Care should be taken if the alcohol-based products contain additional antimicrobial agents.

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

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

Annex C
(informative)

Graphical representation of test procedures

C.1 Dilution-neutralization method

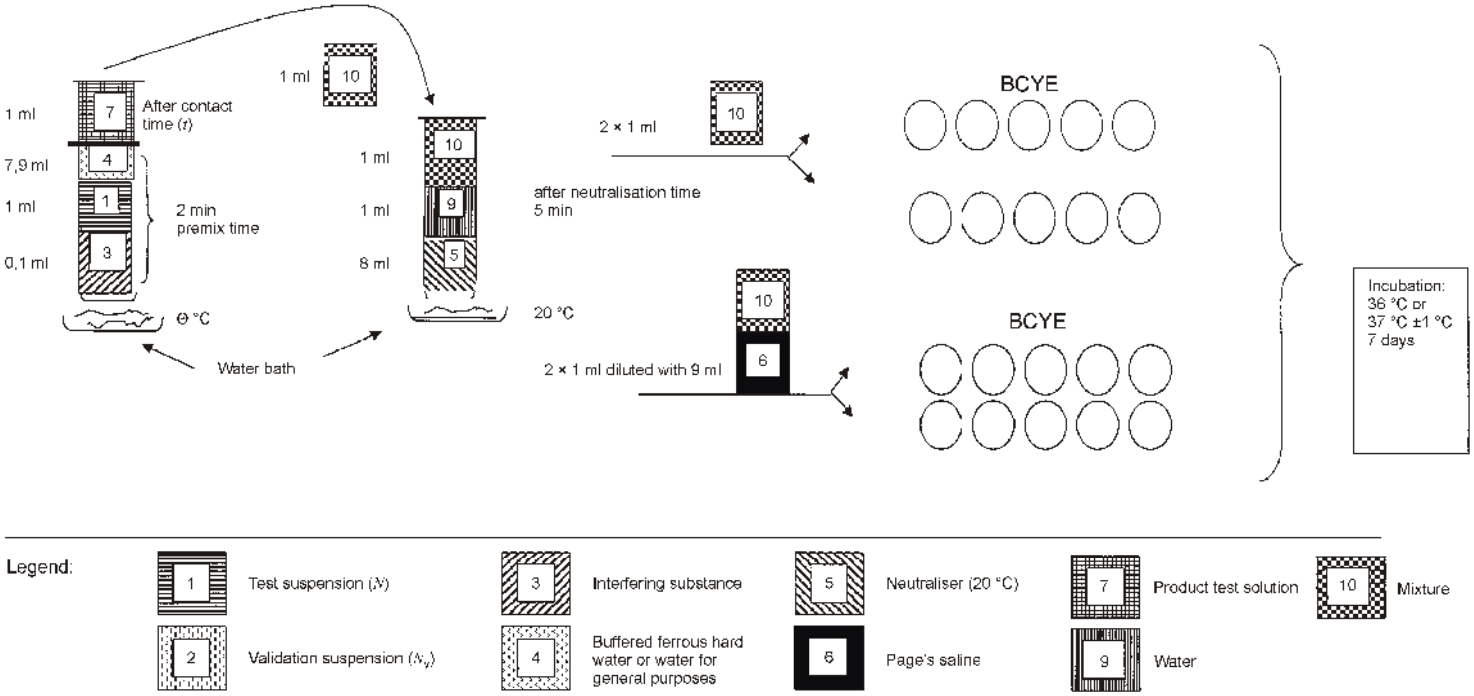

Figure C.1 — Test N_a

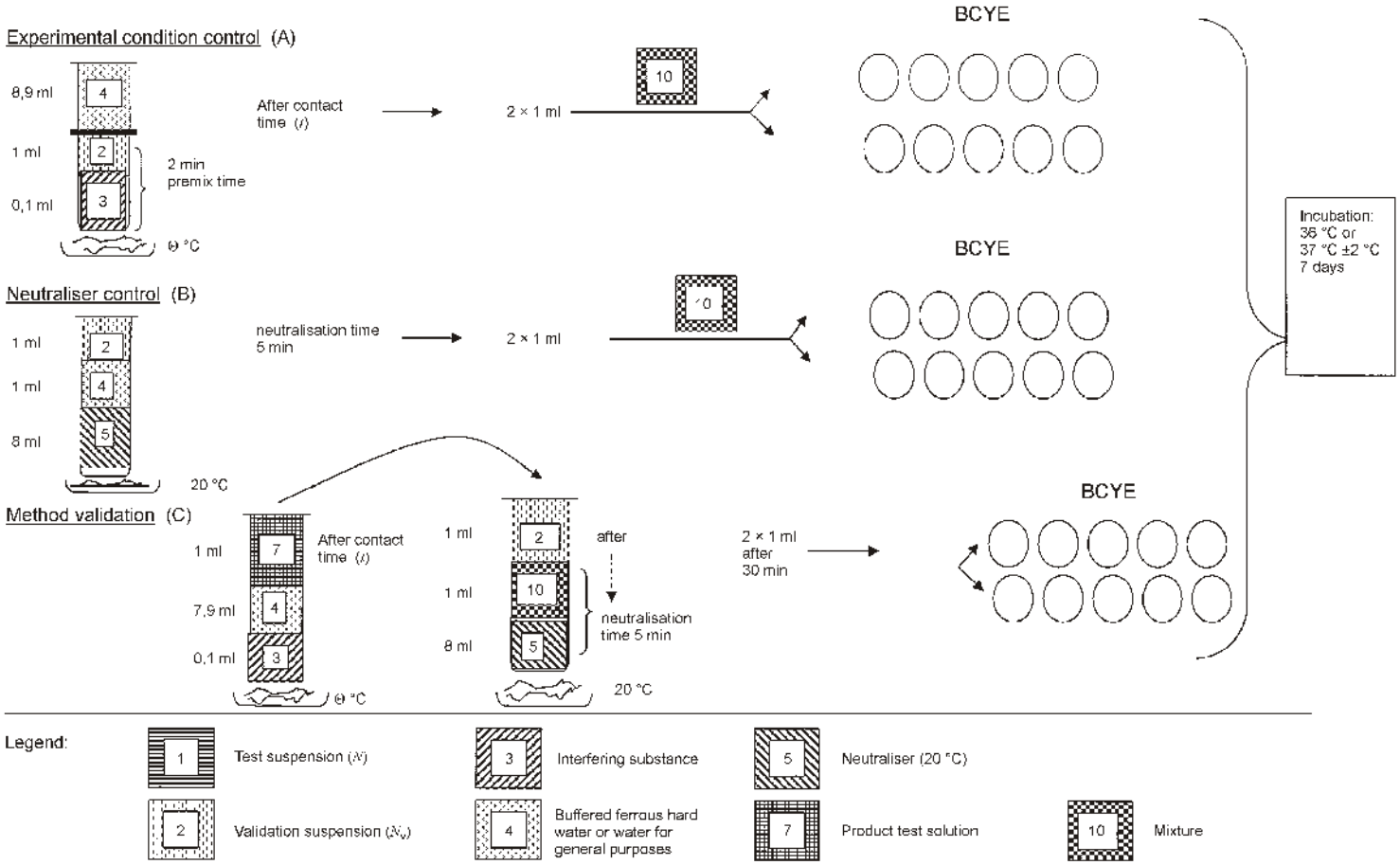

Figure C.2 — Validation

C.2 Membrane filtration method

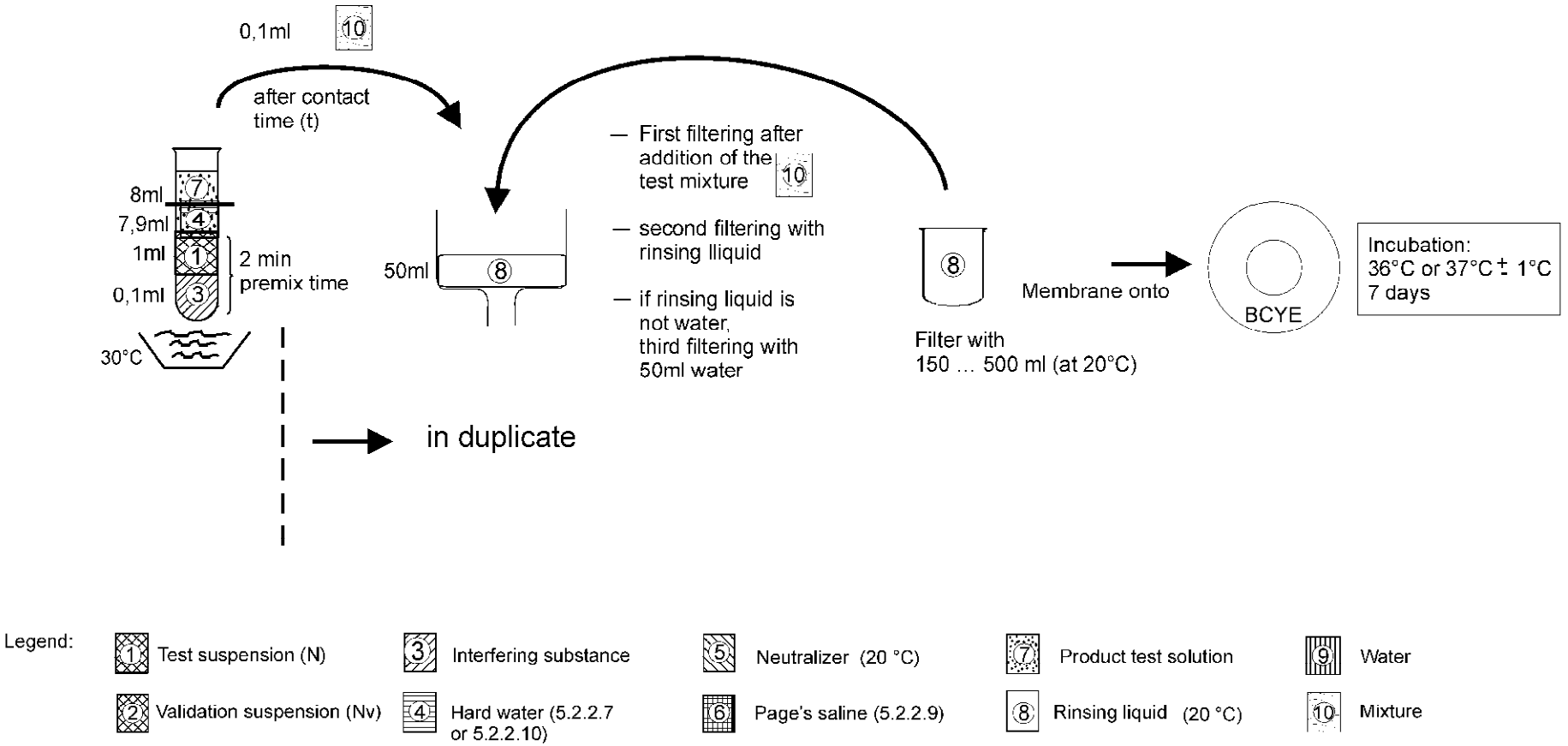

Figure C.3 — Test N_a

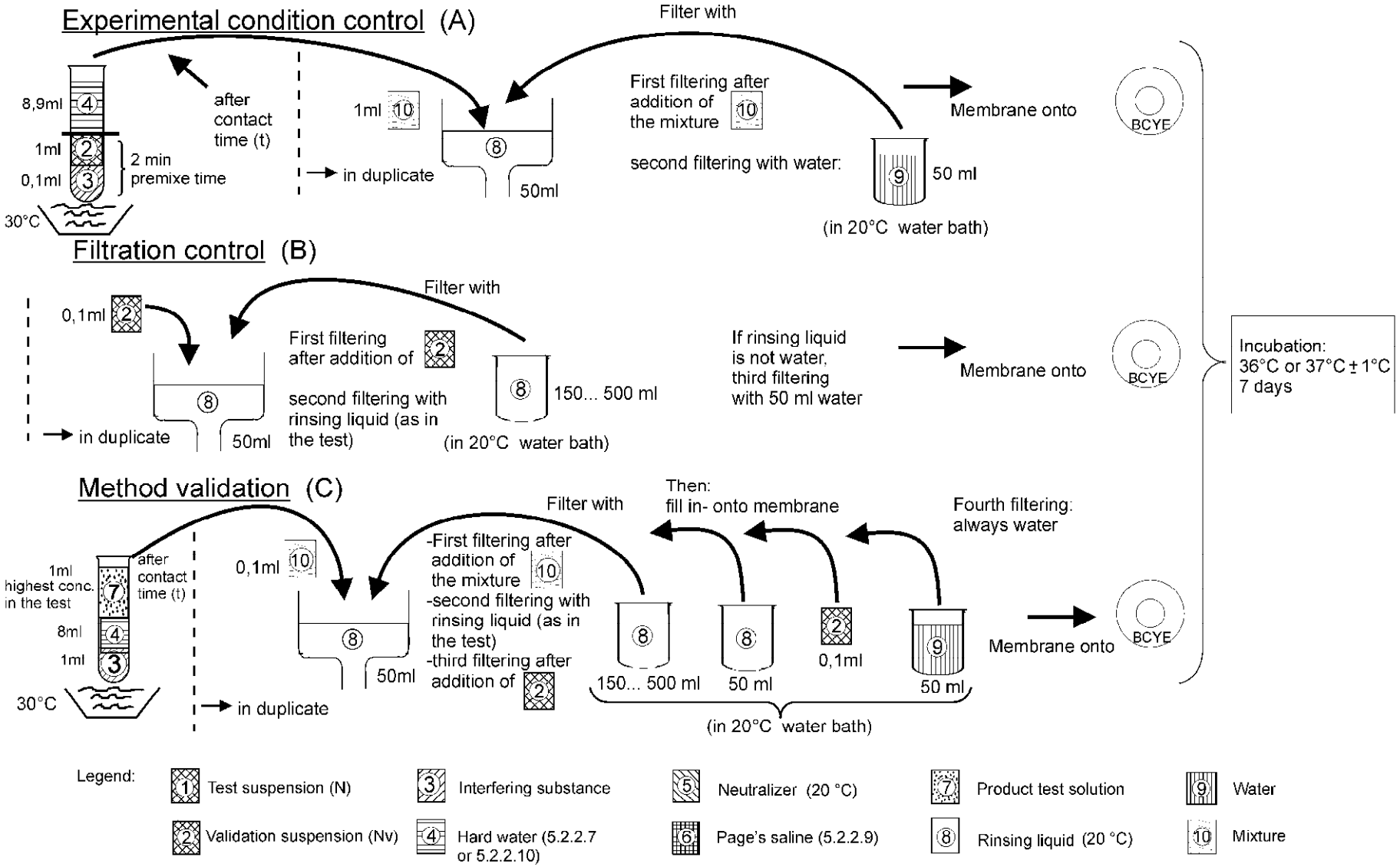

Figure C.4 — Validation

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[1] European Pharmacopeia (EP-Edition 2009): Water for injections.

[2] European Pharmacopeia (EP-Edition 2009): Glycerol.

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

[4] EN ISO/IEC 17025:2005, *General requirements for the competence of testing and calibration laboratories (ISO/IEC 17025:2005)*

[5] EN 1276, *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)*

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