

PrEN12054

Chemical disinfectants and antiseptics

Products for hygienic and surgical hand rub and hand wash
bactericidal activity – Test method and requirements

DRAFT NATIONAL FOREWORD

This British Standard has been prepared by Technical Committee IH/57. It is the English language version of prEN 12054 ‘*Chemical disinfectants and antiseptics - Products for hygienic and surgical handrub and handwash - Bactericidal activity - Test method and requirements - (phase 2, step 1)*’, published by the European Committee for Standardization (CEN).

Cross-references

| International standard | Corresponding British Standard |
|------------------------|---|
| prEN 1040 | <i>Chemical disinfectants and antiseptics - Basic bactericidal activity - Test method and requirements (phase 1)</i> Draft for public comment was 93/502729 DC |
| prEN 1499 | <i>Chemical disinfectants and antiseptics - Hygienic handwash - Test method and requirements (phase 2, step 2)</i> Draft for public comment was 94/504445 DC |
| prEN 1500 | <i>Chemical disinfectants and antiseptics - Hygienic handrub - Test method and requirements (phase 2, step 2)</i> Draft for public comment was 94/504555 DC |

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EUROPEAN STANDARD

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Descriptors :

English version

Chemical disinfectants and antiseptics - Products
for hygienic and surgical handrub and handwash -
Bactericidal activity - Test method and
requirements - (phase 2, step 1)

Antiseptiques et désinfectants
chimiques - Produits pour le traitement
par frictions et le lavage hygiénique
et chirurgical des mains - Activité
bactéricide - Méthode d'essai et
prescriptions - (phase 2, étape 1)

Chemische Desinfektionsmittel und
Antiseptika - Produkte für die
hygienische und chirurgische
Händedesinfektion und Händewaschung -
Bakterizide Wirkung - Prüfverfahren
und Anforderungen - (Phase 2, Stufe 1)

This draft European Standard is submitted to the CEN members for CEN enquiry.
It has been drawn up by Technical Committee CEN/TC 216 .

If this draft becomes a European Standard, CEN members are bound to comply with
the CEN/CENELEC Internal Regulations which stipulate the conditions for giving
this European Standard the status of a national standard without any alteration.

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CEN

European Committee for Standardization
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Foreword

This Draft European Standard has been prepared by CEN Technical Committee 216 Chemical disinfectants and antiseptics for which the Secretariat is held by the Association Française de Normalisation (AFNOR).

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

This document is currently submitted to CEN enquiry ¹⁾. At the same time, a multicentre collaborative study of the method will be undertaken to establish the repeatability and reproducibility of the method. The findings of this study will be considered prior to preparation of the final draft.

If the final draft is accepted in accordance with the provisions of the common CEN/CENELEC rules, the following countries will be bound to implement the resulting European Standard (EN) : Austria, Belgium, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

¹⁾ This text is the agreed final technical document prepared by CEN/TC 216/WG 1 but may be subject to further modification to harmonise with the revised pr EN 1040 "Chemical disinfectants and antiseptics - Basic bactericidal activity - Test method and requirement".

0 Introduction

This European Standard describes a suspension test for establishing whether a handrub or handwash product used for postcontamination treatment of hands or for surgical hand disinfection has or does not have bactericidal activity under the laboratory conditions defined by this European Standard.

Any chemical disinfectant or antiseptic intended for use as a handrub or handwash should first meet the test requirements of pr EN 1040 (Chemical disinfectants and antiseptics - Basic bactericidal activity - Test method and requirements). If a handrub or handwash product then complies with the requirements of this test, it can be considered to possess bactericidal activity, but it should not necessarily be inferred that the product is acceptable for postcontamination treatment of hands or for surgical hand disinfection under practical conditions of use. Handrub and handwash products should be subjected to further tests (pr EN 1499, pr EN 1500, pr EN xxxx) which aim to simulate practical conditions of hand disinfection and thereby evaluate their bactericidal activity under conditions appropriate to their intended use ²⁾.

1 Scope

This European Standard specifies a test method and requirements for the minimum bactericidal activity of handwash and handrub products for postcontamination treatment of hands or for surgical hand disinfection that are intended to be used with water (handwash) or without water (handrub).

The field of application applies to areas and situations where disinfection is medically indicated. Such indications occur in patient care, for example :

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

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

NOTE 1 : Using this European Standard it is not possible to determine the bactericidal activity of the undiluted handrub product as some dilution is always produced by adding the inoculum.

NOTE 2 : Additional assessment of the performance of the product is necessary (see Introduction).

²⁾ CEN/TC 216 is preparing relevant tests.

2 Normative references

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this European Standard only when incorporated in it by amendment or revision. For undated references the latest editions of the publication referred to applies.

| | | |
|------------|------|---|
| pr EN 1040 | 1993 | Chemical disinfectants and antiseptics - Basic bactericidal activity - Test method and requirements (phase 1) |
| pr EN 1499 | 1994 | Chemical disinfectants and antiseptics - Hygienic handwash - Test method and requirements (phase 2/step 2) |
| pr EN 1500 | 1994 | Chemical disinfectants and antiseptics - hygienic handrub - Test method and requirements (phase 2/step 2) |
| pr EN xxxx | | Chemical disinfectants and antiseptics - Surgical hand disinfectants - Test method and requirements (phase 2 /step 2) ³⁾ |

3 Definitions

For the purposes of this standard the following definitions apply :

3.1 bactericide

A chemical agent or formulation which kills vegetative bacteria under defined conditions.

3.2 bactericidal activity

The capability of the product to reduce the number of viable bacterial cells of relevant organisms under the conditions defined by this European Standard.

3.3 hygienic handrub

Postcontamination treatment of hands using an appropriate microbicidal preparation directed against transient microorganisms to prevent their transmission regardless of the resident skin flora. This treatment involves rubbing a disinfectant solution into the hands without the addition of water before or during a procedure.

³⁾ CEN/TC 216 is preparing this test method.

3.4 hygienic handwash

Postcontamination treatment of hands using an appropriate microbicidal preparation directed against transient microorganisms to prevent their transmission regardless of the resident skin flora. This treatment involves washing the hands with a product for hygienic handwash.

3.5 surgical handrub

Preoperative treatment of hands using an appropriate microbicidal preparation directed against the microbial flora of hands to prevent the transmission of microorganisms into the surgical wound. This treatment involves was the hands with a product for surgical hand disinfection.

3.6 surgical handwash

Preoperative treatment of hands using an appropriate microbicidal preparation directed against the microbial flora of hands to prevent the transmission of microorganisms into the surgical wound. This treatment involves washing the hands with a product for surgical hand disinfection.

4 Requirements

The product, when tested in accordance with clause 5, shall demonstrate a reduction in viable counts as stated in clauses 4.1, 4.2, 4.3 or 4.4 for each of the following test organisms : *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus hirae* and *Staphylococcus aureus*.

The following specific requirements shall be met by the product according to its application.

4.1 Hygienic handrub : The product shall demonstrate a reduction in viable count from between 1×10^7 to 3×10^7 to no more than 3×10^2 colony forming units (cfu) per millilitre at 1 min. In addition, if the manufacturer recommends a shorter exposure time than 1 min, the product shall demonstrate a similar reduction in viable count at 30 s.

4.2 Hygienic handwash : The product shall demonstrate a reduction in viable count from between 1×10^7 to 3×10^7 to no more than 3×10^4 colony forming units (cfu) per millilitre at 1 min. In addition, if the manufacturer recommends a shorter exposure time than 1 min, the product shall demonstrate a similar reduction in viable count at 30 s.

4.3 Surgical handrub : The product shall demonstrate a reduction in viable count from between 1×10^7 to 3×10^7 to no more than 3×10^2 cfu/ml at 5 min. In addition, if the manufacturer recommends a shorter exposure time than 5 min, the product shall demonstrate a similar reduction in viable count at 3 min which is also the minimum exposure time to be recommended by the manufacturer.

4.4 Surgical handwash : The product shall demonstrate a reduction in viable count from between 1×10^7 to 3×10^7 to no more than 1×10^4 to 3×10^4 cfu/ml at 5 min. In addition, if the manufacturer recommends a shorter exposure time than 5 min, the product shall demonstrate a similar reduction in viable count at 3 min, which is also the minimum exposure time to be recommended by the manufacturer.

NOTE 1 : Products which have met the requirements for a hygienic handrub (4.1) or a hygienic handwash (4.2) and which are to be used additionally for surgical hand disinfection are not required to be tested further in phase 2/step 1.

NOTE 2 : The requirements of clauses 4.3 and 4.4 apply to products used solely for surgical hand disinfection and which have not previously been tested according to clauses 4.1 or 4.2.

5 Test method

5.1 Principle

A test suspension of bacteria is added to a prepared sample of the product under test. For handwash products only, the sample is prepared in hard water. The mixture is maintained at 20 °C. At specified times including any time stated in the manufacturer's instructions, but always at the times stated in table 1, an aliquot portion is taken; the bactericidal action of this portion is immediately neutralized or suppressed by a validated method. The method of choice is dilution-neutralization. However, if a suitable neutralization medium cannot be established, membrane filtration is used. The number of surviving bacteria is determined.

The number of bacteria in the test suspension is also determined and the reduction in viable count calculated.

Table 1 : Contact time for different categories of product

| | Manufacturer's recommended exposure time | Contact times "t" used in test |
|-------------------|--|--------------------------------|
| Hygienic handrub | 1 min < 1 min | 1 min 1 min and 30 s |
| Hygienic handwash | 1 min < 1 min | 1 min 1 min and 30 s |
| Surgical handrub | 5 min < 5 min | 5 min 5 min and 3 min |
| Surgical handwash | 5 min < 5 min | 5 min 5 min and 3 min |

The test is performed using *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus hirae*.

NOTE : *Pseudomonas aeruginosa* and *Staphylococcus aureus* may have previously been tested according to pr EN 1040 and the results obtained may be valid for use in this test.

5.2 Materials and reagents

5.2.1 Test organisms

The bactericidal activity shall be evaluated using four strains. The following strains shall be used :

| | |
|-------------------------------|------------|
| <i>Escherichia coli</i> (K12) | NCTC 10538 |
| <i>Pseudomonas aeruginosa</i> | ATCC 15442 |
| <i>Staphylococcus aureus</i> | ATCC 6538 |
| <i>Enterococcus hirae</i> | ATCC 10541 |

NOTE : See Annex E for corresponding strain number in other culture collections.

5.2.2 Culture media and reagents

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

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

5.2.2.1 Water

The water shall be free from substances that are toxic or inhibiting to the bacteria. It shall be freshly glass distilled and not demineralised water.

Sterilize at $121 \pm 3 \text{ } ^\circ\text{C}$ for 15 min.

NOTE 1 : If 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 injection EP (see annex F) can be used.

5.2.2.2 Tryptone Soya Agar (TSA)

For maintenance of bacterial strains and performance of viable counts.

| | |
|--|---------|
| Tryptone, pancreatic digest of casein, USP (see annex F) | 15 g |
| Soya peptone, papaic digest of soybean meal, USP | 5 g |
| NaCl, EP | 5 g |
| Water (5.2.2.1) | 1000 ml |

Sterilize at $121 \pm 3 \text{ } ^\circ\text{C}$ for 15 min. After sterilization the pH at 25 °C shall be $7,0 \pm 0,1$.

5.2.2.3 Diluent

| | |
|--|---------|
| Tryptone, pancreatic digest of casein, USP | 1 g |
| NaCl, EP | 8,5 g |
| Water (5.2.2.1) | 1000 ml |

5.2.2.4 Neutralization medium

The neutralization medium shall be validated for the product under test in accordance with Annex A. The neutralization medium shall be sterile.

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

5.2.2.5 Rinsing liquid (for membrane filtration)

The liquid shall be sterile, compatible with the filter membrane and capable of filtration through the filter membrane under the test conditions described in Annex A.

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

5.2.2.6 Hard water for dilution of the handwash product

Solution A : Dissolve 19,84 g anhydrous MgCl₂ and 46,24 g anhydrous CaCl₂ in purified water and dilute to 1 l. Sterilize at 121 °C for 20 min.

Solution B : Dissolve 35,02 g NaHCO₃ in distilled water and dilute to 1 litre. Sterilize by passing through a filter with maximum effective pore size of 0,45 µm.

Add at least 600 ml sterile purified water to 6 ml of Solution A in a sterile 1 litre flask, then add 8 ml Solution B and dilute to 1 litre with sterile purified water.

The final water hardness in the test shall be 300 ppm as CaCO₃. The pH of the hard water solution at 25 °C shall be 7,0 ± 0,2.

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) in the autoclave (5.3.2.1) by maintaining it at 121 +³₀ °C for 15 min ;

b) in the dry heat sterilizer (5.3.2.1) by maintaining it at 180 °C for at least 30 min, at 170 °C for at least 1 h or at 160 °C for at least 2 h.

5.3.2 Usual microbiological laboratory equipment ⁴⁾ and, in particular, the following.

- 5.3.2.1 Apparatus for moist heat sterilization (autoclave) or dry heat sterilization (oven).**
- 5.3.2.2 Water baths**, capable of being controlled at 20 °C ± 1 °C and 45 °C ± 1 °C.
- 5.3.2.3 Incubator**, capable of being controlled at 36 °C ± 1 °C. If an incubator at 36 °C ± 1 °C is not available, an incubator at 37 °C ± 1 °C may be used.
- 5.3.2.4 pH-meter**, having an accuracy of calibration of ± 0,1 pH units at 25 °C.
- 5.3.2.5 Spectrometer or nephelometer**, or any suitable means of estimating colony forming units in suspension in the range 1×10^8 to 3×10^8 cfu/ml.
- 5.3.2.6 Stopwatch.**
- 5.3.2.7 Vortex mixer (electromechanical agitator).**
- 5.3.2.8 Membrane filtration apparatus** (if this method is used for validation), constructed of a material compatible with the product under test, with a filter holder of usable volume 50 ml minimum, and suitable for use with filters of diameter 47 mm to 50 mm of 0,45 µm pore size.

The vacuum source used shall give an even filtration 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.
- 5.3.2.9 Test tubes**, or culture bottles or flasks of suitable capacity.
- 5.3.2.10 Graduated pipettes**, of nominal capacities 10 ml and 1 ml, graduated respectively in 0,5 ml and 0,1 ml divisions.
- 5.3.2.11 Petri dishes**, of small size (90 mm to 100 mm).

NOTE : For spread plate counting method, large size dishes (140 mm) may be used.

⁴⁾ Disposable equipment is an acceptable alternative to reusable glassware.

5.3.2.12 Glass beads (3 mm to 4 mm).

5.3.2.13 Volumetric flasks calibrated at 20 °C.

5.4 Preparation of test suspensions and test solutions

5.4.1 Bacterial suspension

5.4.1.1 Stock cultures of test organism

NOTE : Until an EN is available ⁵⁾, use the following method. (5.4.1.1 will be completed in a more advanced stage of the standardization work).

Maintain the stock cultures of the strains (5.2.1) on separate TSA slopes (5.2.2.2) in a refrigerator at 5 °C ± 1 °C. If prolonged storage is necessary, use lyophilized strains and store at - 18 °C or lower.

5.4.1.2 Working culture of the organisms

In order to prepare the working culture of the strains (5.2.1), subculture from the stock culture (5.4.1.1) by streaking onto TSA slopes (5.2.2.2) and incubate at 36 °C ± 1 °C [or 37 °C ± 1 °C (5.3.2.3)].

After 18 h to 24 h prepare a second subculture on a particular day, a 48 h subculture may be used for subsequent subculturing, providing the subculture has been kept in the incubator during the 48 h period. Under these circumstances prepare a further 24 h subculture before proceeding. Do not take a fourth subculture.

5.4.1.3 Bacterial test suspension

Take the working culture (5.4.1.2) and suspend the cells in the diluent (5.2.2.3) using glass beads. Remove the gross debris by sedimentation or by filtration through glass wool. Adjust the number of colony forming units in the final suspension to 1 x 10⁸ to 3 x 10⁸ cfu/ml using the diluent, estimating the number of units by any suitable means (5.3.2.5). Maintain this suspension at 20 °C ± 1 °C and use within 2 h.

Prepare four bacterial test suspensions, one using *Escherichia coli*, one using *Pseudomonas aeruginosa*, one using *Enterococcus hirae* and one using *Staphylococcus aureus*.

⁵⁾ CEN/TC 216 is preparing a European Standard on "Chemical disinfectants and antiseptics -Verification and preservation of stock cultures of test organisms".

5.4.1.4 Counting of bacterial test suspension

Dilute the adjusted suspensions (5.4.1.3) by 1×10^{-6} using diluent (5.2.2.3) so as to obtain suspensions of 1×10^2 cfu/ml to 3×10^2 cfu/ml. Homogenize the suspension using the vortex mixer. Take a sample of 1,0 ml of the 1×10^2 cfu/ml to 3×10^2 cfu/ml. Homogenize the suspension using the vortex mixer. Take a sample of 1,0 ml of the 1×10^2 cfu/ml to 3×10^2 cfu/ml suspension in duplicate and inoculate pour or spread plate.

When using :

- a) the pour plate technique, pipette each 1,0 ml sample into separate petri dishes and add 15 ml to 20 ml melted TSA (5.2.2.2), cooled to $45 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$;
- b) the spread plate technique, spread each 1,0 ml sample on an appropriate number of overdried plates containing TSA (5.2.2.2).

Incubate the plates at $36 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ (or at $37 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$) for 42 h to 48 h. Count each plate and record the number of colony forming units for each plate, and the average number of colony forming units (N) of the duplicates.

5.4.2 Product test solution

5.4.2.1 Handrub test solution

The handrub is used undiluted as the product test solution.

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

5.4.2.2 Handwash test solution

Using water of standard hardness (5.2.2.6) as the diluent, prepare a 55 % (V/V) dilution of the test handwash preparation. The product test solution shall be prepared freshly and used for not more than one working day.

5.5 Procedure

5.5.1 General

The inactivation of the bactericidal activity of the product shall be validated for each of the tested strains.

The dilution-neutralization method (5.5.2) is normally used, but if a suitable neutralization medium cannot be established, the membrane filtration method (5.5.3) is used in its place.

5.5.2 Dilution-neutralization method

5.5.2.1 General

Prior to testing, equilibrate all reagents (product test solution, bacterial test suspension, neutralization medium) to the test temperature of $20\text{ °C} \pm 1\text{ °C}$ using the water bath (5.3.2.2) controlled at $20\text{ °C} \pm 1\text{ °C}$. Check that the temperature of the reagents is stabilized at $20\text{ °C} \pm 1\text{ °C}$.

5.5.2.2 Test procedure for bactericidal activity of hygienic handrub or handwash products used for postcontamination treatment of hands

Pipette 9,0 ml of the hygienic handrub test solution (5.4.2.1) or handwash test solution (5.4.2.2) into a sterile container with a capacity of approximately 25 ml. Add 1,0 ml of one of the bacterial test suspensions (5.4.1.3). Immediately start the stopwatch, mix (5.3.2.7) and place the container in a water bath controlled at $20\text{ °C} \pm 1\text{ °C}$. The activity of the product shall be determined for a contact time of $1\text{ min} \pm 5\text{ s}$. If the manufacturer recommends a shorter exposure time than 1 min, the product shall also be tested at a contact time of $30\text{ s} \pm 5\text{ s}$.

Just before the end of the chosen contact time, mix (5.3.2.7). At the chosen contact time, pipette 1,0 ml of the test mixture into a tube containing 8,0 ml neutralization medium (5.2.2.4) and 1,0 ml water (5.2.2.1). Mix (5.3.2.7) and place in a water bath controlled at $20\text{ °C} \pm 1\text{ °C}$.

For the handrub test products : After the neutralization time of $1\text{ min} \pm 5\text{ s}$, prepare a series of ten-fold dilutions of the neutralized mixture in the diluent (5.2.2.3). Take a sample of 1,0 ml of the 10^{-2} and 10^{-3} dilutions of the neutralized mixture in duplicate and inoculate the pour or spread plates.

For the handwash test product : After the neutralization time of $1\text{ min} \pm 5\text{ s}$, prepare a series of ten-fold dilutions of the neutralized mixture in the diluent (5.2.2.3). Take a sample of 1,0 ml of the 10^{-2} and 10^{-3} dilutions of the neutralized mixture in duplicate and inoculate the pour or spread plates.

When using :

a) the pour plate technique, pipette each 1,0 ml sample of the mixture in separate petri dishes (5.3.2.11) and add 15 ml to 20 ml melted TSA (5.2.2.2) cooled to $45\text{ °C} \pm 1\text{ °C}$;

b) the spread plate technique, spread each 1,0 ml sample of the mixture on an appropriate number of overdried plates (5.3.2.11) containing TSA (5.2.2.2).

Incubate the plates at $36\text{ °C} \pm 1\text{ °C}$ (or $37\text{ °C} \pm 1\text{ °C}$) for 42 h to 48 h. Count each plate (or group of plates if several plates have been used for the spread plate method).

Record the number of colony forming units for each 1,0 ml sample of the neutralized test mixture (see Annex D table D.2).

Perform this procedure using the other bacterial test suspensions.

5.5.2.3 Test procedure for bactericidal activity of surgical handrub or surgical handwash products

Pipette 9,0 ml of the surgical handrub test solution (5.4.2.1) or surgical handwash test solution (5.4.2.2) into a sterile container with a capacity of approximately 25 ml. Add 1,0 ml of one of the bacterial test suspensions (5.4.1.3). Immediately start the stopwatch, mix (5.3.2.7) and place the container in a water bath controlled at $20\text{ °C} \pm 1\text{ °C}$. The activity of the product shall be determined for a contact time of $5\text{ min} \pm 10\text{ s}$. If the manufacturer recommends a shorter exposure time than 5 min, the product shall also be tested at a contact time of $3\text{ min} \pm 10\text{ s}$.

Just before the end of the chosen contact time, mix (5.3.2.7). At the chosen contact time, pipette 1,0 ml of the test mixture into a tube containing 8 ml neutralization medium (5.2.2.4) and 1,0 ml water (5.2.2.1). Mix (5.3.2.7) and place in a water bath controlled at $20\text{ °C} \pm 1\text{ °C}$.

For the handrub test products : After the neutralization time of $5\text{ min} \pm 10\text{ s}$, prepare a series of ten-fold dilutions of the neutralized mixture in the diluent (5.2.2.3). Take a sample of 1,0 ml of the mixture and its 10^{-1} dilution in duplicate and inoculate the pour or spread plates.

For the handwash test product : After the neutralization time of $5\text{ min} \pm 10\text{ s}$, prepare a series of ten-fold dilutions of the neutralized mixture in the diluent (5.2.2.3). Take a sample of 1,0 ml of the 10^{-2} and 10^{-3} dilutions of the neutralized mixture in duplicate and inoculate the pour or spread plates.

When using :

- a) the pour plate technique, pipette each 1,0 ml sample of the mixture in separate petri dishes (5.3.2.11) and add 15 ml to 20 ml melted TSA (5.2.2.2) cooled to $45\text{ °C} \pm 1\text{ °C}$;
- b) the spread plate technique, spread each 1,0 ml sample of the mixture on an appropriate number of overdried plates (5.3.2.11) containing TSA (5.2.2.2).

Incubate the plates at $36\text{ °C} \pm 1\text{ °C}$ (or $37\text{ °C} \pm 1\text{ °C}$) for 42 h to 48 h. Count each plate (or group of plates if several plates have been used for the spread plate method).

Record the number of colony forming units for each 1,0 ml sample of the neutralized test mixture (see Annex D).

5.5.3 Membrane filtration method

5.5.3.1 General

Prior to testing, equilibrate all reagents (product test solution, bacterial test suspension, neutralization medium) to the test temperature of $20\text{ °C} \pm 1\text{ °C}$ using the water bath (5.3.2.2) controlled at $20\text{ °C} \pm 1\text{ °C}$. Check that the temperature of the reagents is stabilized at $20\text{ °C} \pm 1\text{ °C}$.

5.5.3.2 Test procedure for bactericidal activity of hygienic handrub or hygienic handwash products

Pipette 9,0 ml of the hygienic handrub test solution (5.4.2.1) or handwash test solution (5.4.2.2) into a sterile container with a capacity of approximately 25 ml. Add 1,0 ml of one of the bacterial test suspensions (5.4.1.3). Immediately start the stopwatch, mix (5.3.2.7) and place the container in a water bath controlled at $20\text{ °C} \pm 1\text{ °C}$. The activity of the product shall be determined for a contact time of $1\text{ min} \pm 30\text{ s}$. If the manufacturer recommends a shorter exposure time than 1 min, the product shall also be tested at a contact time of $30\text{ s} \pm 5\text{ s}$.

Just before the end of the chosen contact time, mix (5.3.2.7). At the chosen contact time pipette two samples of 0,5 ml and transfer into two separate membrane filtration apparatus equipped with a membrane and containing 50 ml of the rinsing liquid (5.2.2.5). Filter immediately. The time required for transfer and filtration should not exceed 1 min. If greater than 1 min, this time shall be recorded in the test report (5.2.2.5). Then transfer the membranes to the surface of two separate TSA plates (5.3.2.11).

NOTE : When transferring membranes to TSA plates, ensure that the membrane is filtrate side uppermost and avoid trapping air between the membrane and agar surface.

Incubate the plates at $36\text{ °C} \pm 1\text{ °C}$ (or $37\text{ °C} \pm 1\text{ °C}$) for 42 h to 48 h. Count each plate and record the number of colony forming units for each 0,5 ml sample of the neutralised test mixture.

Perform this procedure using the other product test solutions and/or the other contact times and/or the other bacterial suspensions.

5.5.3.3 Test procedure for bactericidal activity of surgical handrub or surgical handwash products

Pipette 9,0 ml of the surgical handrub test solution (5.4.2.1) or surgical handwash test solution (5.4.2.2) into a sterile container with a capacity of approximately 25 ml. Add 1,0 ml of one of the bacterial test suspensions (5.4.1.3). Immediately start the stopwatch, mix (5.3.2.7) and place the container in a water bath controlled at $20\text{ °C} \pm 1\text{ °C}$. The activity of the product shall be determined for a contact time of $5\text{ min} \pm 10\text{ s}$. If the manufacturer recommends a shorter exposure time than 5 min, the product shall also be tested at a contact time of $3\text{ min} \pm 10\text{ s}$.

Just before the end of the chosen contact time, mix (5.3.2.7). At the chosen contact time, pipette two samples of 0,5 ml and transfer each sample onto two separate membrane filtration apparatus equipped with a membrane and containing 50 ml of the rinsing liquid (5.2.2.5). Filter immediately. The time required for transfer and filtration should not exceed one minute. If greater than one minute, this time shall be recorded in the test report. Rinse with at least 150 ml but no more than 500 ml of rinsing liquid (5.2.2.5). Then transfer the membranes to the surface of two separate TSA plates (5.3.2.11).

NOTE : When transferring membranes to TSA plates, ensure that the membrane is filtrate side uppermost and avoid trapping air between the membrane and agar surface.

Incubate the plates at $36\text{ °C} \pm 1\text{ °C}$ (or $37\text{ °C} \pm 1\text{ °C}$) for 42 h to 48 h. Count each plate and record the number of colony forming units for each 0,5 ml sample of the neutralized test mixture.

Perform this procedure using the other product test solutions and/or the other contact times and/or the other bacterial suspensions.

5.5.4 Validation of neutralization and membrane filtration procedures

The neutralization and membrane filtration procedures will have been previously validated for each of the four test organisms according to Annex A.

The validation test (Annex A) shall also be carried out at the same time as the procedure using the same conditions (bacterial test suspension, product test solution, hard water if used and neutralizing medium or rinsing liquid) used in the test (see 5.5.2.2 and 5.5.2.3).

5.6 Calculation and expression of results

5.6.1 Verification of methodology

5.6.1.1 Dilution-neutralization method

Check that :

- a) no individual plate count used for calculation of N, A and N' is greater than 300 cfu ;
- b) N, A and N' are between 100 and 300 cfu ;
- c) N' and A are equal to or greater than 0,5 times N ;
- d) n' is equal to or greater than 0,5 times N' ;

where :

- N is the average plate count of the test suspension control (in cfu) (5.4.1.4) ;
- A is the average plate count of the test suspension control for validation of the experimental (hard water) conditions (in cfu) (A.4.1.1) ;
- N' is the average plate count of the neutralization medium control (in cfu) (A.4.1.2) ;
- n' is the average plate count of the dilution neutralization test control (in cfu) (A.4.1.3).

5.6.1.2 Membrane filtration method

Check that :

- a) no individual plate count used for calculation of N and A is greater than 300 cfu and that N and A are between 100 and 300 cfu ;
- b) no individual plate count used for calculation of N' is greater than 150 cfu and that N' is between 50 and 150 cfu ;

c) n' is equal to or greater than 0,5 times N' ;

where :

N is the average plate count of the test suspension control (in cfu) (5.4.1.4).

A is the average plate count of the test suspension control for validation of the experimental (hard water) conditions (in cfu) (A.4.2.1) ;

N' is the average plate count of the filtration control (in cfu) (A.4.2.2) ;

n' is the average plate count of the filtration test control (in cfu) (A.4.2.3).

5.6.2 Expression of results

5.6.2.1 Dilution-neutralization method

Express the results from the test procedure (5.5.1) as the number of cfu per ml neutralized test mixture for each test organism at the test concentration and contact time.

Numbers (i.e. values) greater than 300 shall be reported as > 300 .

For handrub products, where all of the four values are 300 or less, use only the values of the undiluted sample to calculate the cfu/ml of the test mixture. Where only one of the values for the undiluted sample is 300 or less and both of the values for the 10^{-1} dilution are 300 or less, use only the values for the 10^{-1} dilution to calculate the cfu/ml of the test mixture.

For handwash products, calculate the average number of viable organisms in cfu/ml of the test mixture from the dilution(s) where the values of the duplicates are 300 or less.

Using the duplicate values chosen as stated above, calculate and record the average number of viable organisms in cfu/ml of the test mixture for each organism at the test concentration and contact times.

Where the calculated viable count is less than 3×10^2 , report as $< 3 \times 10^2$ cfu/ml (see Annex D).

5.6.2.2 Membrane filtration method

Express the results from the test procedure (5.5.1) as the number of cfu per 0,5 ml neutralized test mixture for each test organism at the test concentration and contact time.

Numbers (i.e. values) greater than 150 shall be reported as > 150 .

Where both values of the duplicates are 150 or less, calculate the average number of surviving test organisms in cfu/ml of test mixture for each organism at the test concentration and contact time.

Where the calculated viable count of the test mixture is less than $0,6 \times 10^2$, report as $< 0,6 \times 10^2$ cfu/ml.

Where any result above 150 is reported, express the viable count as $> 3 \times 10^2$ cfu/ml (see Annex D).

5.7 Conclusion

5.7.1 Hygienic handrub products which demonstrate a reduction in viable counts from 1×10^7 to 3×10^7 cfu/ml to no more than 3×10^2 cfu/ml within 1 min at 20 °C under the conditions defined by this test when the test organisms are *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus hirae* and *Staphylococcus aureus* are deemed to have passed the test. If the manufacturer recommends a shorter exposure time than 1 min, the product is deemed to pass the test if a similar reduction is also achieved at 30 s. Products which pass the test may be described as bactericidal preparation for hygienic hand disinfection and may proceed to further evaluation to qualify their efficacy under conditions simulating practical conditions on the hand (pr EN 1500).

5.7.2 Hygienic handwash products which demonstrate a reduction in viable counts from 1×10^7 to 3×10^7 cfu/ml to no more than 1×10^4 to 3×10^4 cfu/ml within 1 min at 20 °C under the conditions defined by this test when the test organisms are *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus hirae* and *Staphylococcus aureus* are deemed to have passed the test. If the manufacturer recommends a shorter exposure time than 1 min, the product is deemed to pass the test if a similar reduction is also achieved at 30 s. Products which pass the test may be described as bactericidal preparation for hygienic hand disinfection and may proceed to further evaluation to qualify their efficacy under conditions simulating practical conditions on the hand (pr EN 1499).

5.7.3 Surgical handrub products which demonstrate a reduction in viable counts from 1×10^7 to 3×10^7 cfu/ml to no more than 3×10^2 cfu/ml within 5 min at 20 °C under the conditions defined by this test when the test organisms are *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus hirae* and *Staphylococcus aureus* are deemed to have passed the test. If the manufacturer recommends a shorter exposure time than 5 min, the product is deemed to have passed the test if a similar reduction is also achieved at 3 min. Products which pass the test may be described as bactericidal preparations for surgical hand disinfection and may proceed to further evaluation to qualify their efficacy under conditions simulating practical conditions on the hand (pr EN xxxx).

5.7.4 Surgical handwash products which demonstrate a reduction in viable counts from 1×10^7 to 3×10^7 cfu/ml to 1×10^4 to 3×10^4 cfu/ml within 5 min at 20 °C under the conditions defined by this test when the test organisms are *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus hirae* and *Staphylococcus aureus* are deemed to have passed the test. If the manufacturer recommends a shorter exposure time than 5 min, the product is deemed to have passed the test if a similar reduction is also achieved at 3 min. Products which pass the test may be described as bactericidal preparations for surgical hand disinfection and may proceed to further evaluation to qualify their efficacy under conditions simulating practical conditions on the hand (pr EN xxxx).

5.7.5 Products which have met the requirements for a hygienic handrub (4.1) or a hygienic handwash (4.2) are deemed also to have met the requirements for surgical hand disinfection without the need for further testing.

5.8 Test report

The report shall refer to this European Standard.

The test report shall contain at least the following information :

a) laboratory ;

b) identification of the sample :

name of the product ;

batch number ;

manufacturer ;

date of delivery ;

recommended storage conditions ;

product diluent recommended by the manufacturer for use ;

c) experimental conditions :

period of analysis ;

test temperature ;

product test concentration ;

contact times ;

counting procedure ;

temperature of incubation ;

neutralization medium or rinsing liquid.

NOTE : If a spectrophotometer or nephelometer is used, details of the apparatus and the wavelength and optical path length at which the measurements are undertaken shall be included in the test report.

d) procedure for validation of test conditions :

If the dilution-neutralization method is used, full details of the tests for validation of the neutralization medium shall be given.

If the membrane filtration method is used, full details of the validation procedure together with the details of tests which were carried out to justify the use of the membrane filtration method shall be given.

e) test results :

See tables D.1 and D.2.

f) conclusion ;

g) date and signature.

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

Annex A (normative)

Test method for validation of dilution-neutralization and membrane filtration methods

A.1 Principle

A neutralization medium is chosen for each product in accordance with A.4.1 (see 5.5). If a suitable neutralization medium cannot be found, the membrane filtration method in accordance with A.4.2 is used.

A.2 Preparation of bacterial suspension

Dilute the bacterial test suspension (5.4.1.3) with the diluent (5.2.2.3) to obtain 1×10^2 to 2×10^2 cfu/ml. Determine the number of cfu for each plate and the average number of cfu (N), in accordance with 5.4.1.4.

A.3 Preparation of product test solution

See 5.4.2. For handrub products, the product is used undiluted as the product test solution. For handwash products, prepare a 55 % (V/V) dilution of the test handwash product in water of standard hardness (5.2.2.6) as the product test solution.

A.4 Test for validation

A.4.1 Dilution-neutralization method

Prior to testing, equilibrate all reagents (product test solution, water, bacterial test suspension, neutralization medium) to the test temperature of $20 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ using the water bath (5.3.2.2) controlled at $20 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$. Check that the temperature of the reagents is stabilized at $20 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$.

A.4.1.1 Validation of selected experimental (hard water) conditions

This validation test is performed for handwash products only, which are to be tested at a 55 % (V/V) dilution in water.

Place 9,0 ml of hard water (5.2.2.6) in a sterile test tube.

Add 1,0 ml of a diluted bacterial suspension containing 1×10^3 to 3×10^3 cfu/ml prepared in accordance with A.2. Immediately start the stopwatch and mix (5.3.2.7) for a few seconds.

Leave in the water bath at $20 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ for a contact time of 1 min (hygienic handwash) or 5 min (surgical handwash).

NOTE : Products intended for use both as a hygienic handwash or a surgical handwash need only be tested at the longer contact time of 5 min.

At the end of the contact time, mix (5.3.2.7) for a few seconds. Take two 1,0 ml samples of the mixture according to the chosen procedure (pour plate or spread plate method) and incubate 42 h to 48 h at $36\text{ °C} \pm 1\text{ °C}$ (or $37\text{ °C} \pm 1\text{ °C}$) (5.4.1).

Calculate A, the average number of cfu of the mixture.

Repeat the above procedure for each test organism.

A.4.1.2 Validation of non-toxicity of the neutralization medium

Place 9,0 ml of neutralization medium (5.2.2.4) in a sterile test tube.

Add 1,0 ml of a diluted bacterial suspension containing 1×10^3 to 3×10^3 cfu/ml prepared in accordance with clause A.2. Immediately start the stopwatch and mix (5.3.2.7) for a few seconds.

Leave in the water bath at $20\text{ °C} \pm 1\text{ °C}$ for a contact time of $1\text{ min} \pm 5\text{ s}$ (hygienic handwash or hygienic handrub) or $5\text{ min} \pm 10\text{ s}$ (surgical handwash or surgical handrub).

NOTE : Handrub and handwash products intended for use both for the postcontamination treatment of hands and for surgical hand disinfection need only be tested for the longer contact time of 5 min.

At the end of the contact time, mix (5.3.2.7) for a few seconds. Take two 1,0 ml samples of the mixture according to the chosen procedure (pour plate or spread plate method) and incubate for 42 h to 48 h at $36\text{ °C} \pm 1\text{ °C}$ (or $37\text{ °C} \pm 1\text{ °C}$) (5.4.1).

Calculate N', the average plate count of the neutralization medium control (in cfu).

Repeat the above procedure for each test organism.

A.4.1.3 Validation of the inactivation by the dilution neutralization method

Place 1,0 ml of the diluent for bacterial suspensions (5.2.2.3) in a sterile tube and then, starting a stopwatch, add 9,0 ml of the product test solution (5.4.2). Leave in the water bath at 20 °C for $5\text{ min} \pm 10\text{ s}$. Then, transfer 1,0 ml of the mixture into a test tube containing 8,0 ml of neutralization medium (5.2.2.4) previously kept in a water bath at $20\text{ °C} \pm 1\text{ °C}$. Leave in the water bath for $5\text{ min} \pm 10\text{ s}$.

Add 1,0 ml of a bacterial suspension containing 1×10^3 to 3×10^3 cfu/ml prepared in accordance with A.2. Immediately start the stopwatch and mix (5.3.2.7) for a few seconds.

Leave in the waterbath at $20\text{ °C} \pm 1\text{ °C}$ for a contact time of $1\text{ min} \pm 5\text{ s}$ (hygienic handwash or hygienic handrub) or $5\text{ min} \pm 10\text{ s}$ (surgical hand disinfection).

NOTE : Handrub and handwash products intended for use both for the postcontamination treatment of hands and for surgical hand disinfection need only be tested for the longer contact time of 5 min.

At the end of the contact time, mix (5.3.2.7) for a few seconds. Take two 1,0 ml samples of the mixture according to the chosen procedure (pour plate or spread plate method) and incubate 42 h to 48 h at $36\text{ °C} \pm 1\text{ °C}$ (or $37\text{ °C} \pm 1\text{ °C}$) (5.4.1).

Calculate n' , the average plate count of the dilution neutralization test control.

Repeat the above procedure for each test organism.

A.4.2 Membrane filtration method

Prior to testing, equilibrate all reagents (product test solution, water, bacterial test suspension, rinsing liquid) to the test temperature of $20\text{ °C} \pm 1\text{ °C}$ using the water bath (5.3.2.2) controlled at $20\text{ °C} \pm 1\text{ °C}$. Check that the temperature of the reagents is stabilized at $20\text{ °C} \pm 1\text{ °C}$.

A.4.2.1 Validation of selected experimental (hard water) conditions

This validation test is performed for handwash products only, which are to be tested at a 55 % (V/V) dilution in hard water.

Place 9,0 ml of hard water (5.2.2.6) in a sterile test tube.

Add 1,0 ml of a diluted bacterial suspension containing 1×10^2 to 3×10^2 cfu/ml prepared in accordance with A.2. Immediately start the stopwatch and mix (5.3.2.7) for a few seconds.

Leave in the water bath at $20\text{ °C} \pm 1\text{ °C}$ for a contact time of $1\text{ min} \pm 5\text{ s}$ (hygienic handwash) or $5\text{ min} \pm 10\text{ s}$ (surgical handwash).

NOTE : Products intended for use both as a hygienic handwash or a surgical handwash need only be tested at the longer contact time of 5 min.

At the end of the contact time, mix (5.3.2.7) for a few seconds. Take two 0,5 ml samples of the mixture and transfer into two separate membrane filtration apparatus equipped with a membrane and containing 50 ml of the rinsing liquid (5.2.2.5). Filter and rinse with 50 ml of the rinsing liquid (5.2.2.5) and then transfer the membranes to the surface of two separate TSA plates (5.2.2.2).

NOTE : When transferring membranes to TSA plates ensure that the membrane is filtrate side uppermost and avoid trapping air between the membrane and agar surface.

Incubate for 42 h to 48 h at 36 °C (or $37\text{ °C} \pm 1\text{ °C}$) (5.4.1).

Calculate A, the average number of cfu of the mixture.

Repeat the above procedure for each test organism.

A.4.2.2 Control of the filtration procedure

Take two 0,5 ml samples of a diluted bacterial suspension containing 1×10^2 to 3×10^2 cfu/ml prepared in accordance with A.2 and transfer into two separate membrane filtration apparatus equipped with a membrane and containing 50 ml of the rinsing liquid (5.2.2.5). Filter and rinse with 50 ml of the rinsing liquid (5.2.2.5) and then transfer the membranes to the surface of two separate TSA plates (5.2.2.2).

NOTE : When transferring membranes to TSA plates ensure that the membrane is filtrate side uppermost and avoid trapping air between the membrane and agar surface.

Incubate for 42 h to 48 h at $36 \text{ °C} \pm 1 \text{ °C}$ (or $37 \text{ °C} \pm 1 \text{ °C}$) (5.4.1).

Calculate N' , the average plate count of the filtration control (in cfu).

Repeat the above procedure for each test organism.

A.4.2.3 Validation of the inactivation by membrane filtration method

Place 1,0 ml of the diluent for bacterial suspensions (5.2.2.3) in a sterile tube and then, starting a stopwatch, add 9,0 ml of the product test solution (5.4.2). Mix (5.3.2.7) and leave in the water bath at 20 °C for the contact time of $5 \text{ min} \pm 10 \text{ s}$.

At the end of the contact time, mix (5.3.2.7) for a few seconds. Take two 0,5 ml samples of the mixture and transfer into two separate membrane filtration apparatus equipped with a membrane and containing 50 ml of the rinsing liquid (5.2.2.5).

Filter and rinse the membrane with at least 150 ml and not more than 500 ml of rinsing liquid (5.2.2.5). Then cover the membranes with 150 ml of rinsing liquid (5.2.2.5) and add 0,5 ml of the diluted bacterial suspension containing 0,5 ml of the diluted bacterial suspension containing 0,5 ml of a bacterial suspension containing 1×10^2 to 3×10^2 cfu/ml prepared in accordance with A.2.

Transfer to the surface of two separate TSA plates (5.2.2.2)..

NOTE : When transferring membranes to TSA plates ensure that the membrane is filtrate side uppermost and avoid trapping air between the membrane and agar surface.

Incubate for 42 h to 48 h at $36 \text{ °C} \pm 1 \text{ °C}$ (or $37 \text{ °C} \pm 1 \text{ °C}$) (5.4.1).

Calculate n' , the average plate count of the filtration test control (in cfu).

Repeat the above procedure for each test organism.

A.5 Validation

Check that the test results comply with the relevant requirements of 5.6.1.

Annex B (informative)

Neutralization media

Where a neutralization medium is specified, any of the following may be used.

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

| Disinfectant | Neutralizer (concentration per litre sampling fluid or diluent) |
|--|---|
| Chlorhexidine-gluconate | polysorbate (30 ml) + lecithin from eggs (3 g) + histidine (3 g) (higher concentrations, up to five times may be necessary for complete neutralization!) |
| Povidone iodine solution or liquid soap and chlorine compounds | polysorbate (30 ml) + lecithin from eggs (3 g) + histidine (1 g) + sodium thiosulphate (5 g) + bovine serum albumin, lyophilized (1 g) |
| Phenolic compounds | polysorbate (30 ml) + lecithin from eggs (3 g) + histidine (1 g) + sodium thiosulphate (5 g) |
| Quaternary ammonium | polysorbate (30 ml) + saponin (30 g) + histidine (1 g) + cystine (1 g) |

Annex C (informative)**Rinsing liquids**

Where a rinsing liquid is specified, any of the following may be used.

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

- Water (5.2.2.1) ;
- Diluent (5.2.2.3) ;
- Aqueous solution of 0,1 % (V/V) polysorbate 80 ⁶⁾ ;
- Aqueous solution of 0,5 % (V/V) polysorbate 80 ⁶⁾ ;
- Aqueous solution of 0,5 % (V/V) polysorbate 80 ⁶⁾ and 0,07 % lecithin ;
- Neutralization medium (5.2.2.4) ;
- Buffer solutions.

⁶⁾ Analytical quality, non-hydrolyzed (TWEEN 80) in accordance with EP volume 1.

Annex D (informative)

Example of a typical test report

Bactericidal activity of a hygienic handwash product intended for postcontamination treatment of hands

1. **Laboratory** Besson Test House
2. **Identification of the sample**

| | |
|---|----------------------------|
| Name of the product | Bacticen handwash |
| Batch number | 94-71-51 |
| Manufacturer | Centipede Formulations Inc |
| Date of delivery | 1994-05-11 |
| Storage conditions | those of the manufacturer |
| Product diluent recommended by the manufacturer for use | tap water |
| Contact time recommended by the manufacturer for use | 1 minute |
| Active substance(s) and its/their concentration(s) (optional) | not indicated |
3. **Experimental conditions**

| | |
|--|---|
| Period of analysis | 1994-02-20 to 1994-03-12 |
| Test temperature | 20 °C ± 1 °C |
| Product test concentration | product diluted to 55 % (V/V) in hard water |
| Contact time | 1 minute |
| Counting procedure | spread plate |
| Temperature of incubation | 36 °C ± 1 °C |
| Product diluent used during test | hard water |
4. **Procedure for validation of test conditions**

| | |
|-----------------------------|--|
| Neutralization medium | Polysorbate (30 ml/l) + lecithin (3 g/l) + histidine (1 g/l) + sodium thiosulphate (5 g/l) + bovine serum albumin, lyophilized (1 g/l) |
|-----------------------------|--|
5. **Test results**

See tables D.1 and D.2.

The product is bactericidal at 1 min contact time according to the requirements for a hygienic handwash product, for each organism tested.
6. **Conclusion**

The product possesses bactericidal activity under the conditions described in this European Standards.
7. **Date and signature**

Table D.1 : Verification of the methodology and validation of dilution-neutralization

A) Validation of dilution-neutralization method

| TEST ORGANISM | COLONY COUNTS PER PLATE (average number of cfu) | | |
|------------------------|--|------------------------------|------------------------|
| | Bacterial test suspension (5.4.1.4) | Validation of neutralization | |
| Staphylococcus aureus | | Control | Test |
| | | 149, 147 (N = 148) | 135, 143 (N' = 139) |
| Pseudomonas aeruginosa | 143, 149 (N = 146) | 134, 140 (N' = 137) | 128, 131 (n' = 130) |
| Escherichia coli | 142, 150 (N = 146) | 135, 139 (N' = 137) | 120, 124 (n' = 122) |
| Enterococcus hirae | 149, 141 (N = 144) | 133, 140 (N' = 137) | 143, 131 (n' = 137) |

For the strains tested :

N and N' are between 100 and 300 cfu ;

N' is greater than 0,5 times N ;

n' is greater than 0,5 times N'.

The neutralization is validated with the neutralization medium tested.

B) Validation of experimental (hard water) conditions

| TEST ORGANISM | COLONY COUNTS PER PLATE (average number of cfu) | |
|------------------------|--|-----------------------------------|
| | Bacterial test suspension (5.4.1.4) | Experimental test with hard water |
| Staphylococcus aureus | 149, 147 (N = 148) | 142, 148 (A = 145) |
| Pseudomonas aeruginosa | 143, 149 (N = 146) | 139, 147 (A = 143) |
| Escherichia coli | 142, 150 (N = 146) | 146, 142 (A = 144) |
| Enterococcus hirae | 149, 141 (N = 144) | 139, 145 (A = 142) |

For the four strains tested :

N and A are between 100 and 300 cfu ;

A is greater than 0,5 times N.

The method is validated for the experimental condition of product dilution 55 % (V/V) in tap water.

Table D.2 : Test results for handwash product diluted to 55 % (V/V) in hard water

| TEST ORGANISM | TEST SAMPLE | COLONY COUNT PER PLATE AFTER 1 MIN CONTACT | CFU/ML TEST MIXTURE | |
|------------------------|--|--|------------------------|---------------------------|
| | | | INITIAL COUNT (N) | AT 1 MIN CONTACT TIME (n) |
| Staphylococcus aureus | undiluted 10 ⁻¹ dilution | 110, 98 2, 0 | 1,48 x 10 ⁷ | 1,04 x 10 ³ |
| Pseudomonas aeruginosa | undiluted 10 ⁻¹ dilution | > 300, > 300 29, 29 | 1,46 x 10 ⁷ | 2,8 x 10 ⁴ |
| Escherichia coli | undiluted 10 ⁻¹ dilution | 114, 110 10, 9 | 1,46 x 10 ⁷ | 1,12 x 10 ³ |
| Enterococcus hirae | undiluted 10 ⁻¹ dilution | 143, 139 19, 11 | 1,44 x 10 ⁷ | 1,41 x 10 ³ |

Annex E (informative)**Corresponding reference strains**

| | | |
|---------------------------------|-------|--------|
| <i>Pseudomonas aeruginosa</i> : | ATCC | 15442 |
| | CIP | 103467 |
| | DSM | 939 |
| | NCIMB | 10421 |
| <i>Escherichia coli</i> : | CIP | 54.117 |
| | NCTC | 10538 |
| | NCIMB | 10083 |
| <i>Staphylococcus aureus</i> : | ATCC | 6538 |
| | CIP | 4.83 |
| | DSM | 10788 |
| | NCIMB | 9518 |
| <i>Enterococcus hirae</i> : | ATCC | 10541 |
| | CIP | 5855 |
| | DSM | 3320 |
| | NCIMB | 8192 |

Annex F (informative)

Bibliographical references

USP : United States Pharmacopoeia XXII

EP : European Pharmacopoeia (1993)