RECKITT BENCKISER
PHARMACEUTICALS INC.

6753 '00 SEP 13 P2:06

September 1, 2000

Dockets Management Branch (HFA-305)
Food & Drug Administration
5600 Fishers Lane
Rockville, MD 20857

Dear Sir or Madam:

The attached report is submitted to Docket No. 75N-183F for Topical Antimicrobial Drug Products for Over-the Counter Human Use.

This study complies with the European Standard EN1040 for chemical disinfectants and antiseptics, a copy of which is appended to the report. Although FR 56, No. 140, pg 33658 states "While the data are considered sufficient to support in vitro and in vivo effectiveness for the finished product (references cited all refer to Dettol) the available data are inadequate to show the contribution of the chloroxylenol." This is difficult to accomplish because PCMX is a dry powder that must be formulated in a vehicle. Secondly, it is sparingly soluble in water; hence the formulation of Dettol also contains water, castor oil soap, alcohol, and pine oil. Justifiably, there is concern that these components of the vehicle may contribute to the effectiveness of the product. The enclosed test report shows quite clearly, however, that PCMX, and only PCMX, in the Dettol formulation has any antimicrobial effect. Thus, this study demonstrates the effectiveness of PCMX and should permit the elevation of PCMX to Category I (monograph.)

We request a reopening of the administrative record, if appropriate, in order that this report may be entered into Docket No. 75N-183F.

Sincerely,

Alan N. Young

Alan N. Young
Director, Regulatory Affairs

75N-183F

RPT 1

1909 HUGUENOT ROAD RICHMOND,VIRGINIA 23235 TELEPHONE (804) 379-1090 FAX (804) 379-1215

RECKITT & COLMAN

MEMORANDUM

TO: D. Payne

CC: K.Kilvington, W.Peers,

SUBJECT: Antimicrobial Efficacy of Dettol liquid formulations without PCMX and Pine Oil

FROM: B.Timmins

DATE: 15 October 1999

Background

Pine Oil, a constituent of Dettol Liquid in some markets is listed as an active ingredient. It is desirable to remove this active agent listing.
To demonstrate PCMX is the active agent, and that pine oil plays little part in the antimicrobial properties of the product, Dettol Liquid has been formulated without i) PCMX and ii) PCMX and pine oil. It was not possible to test a Dettol liquid formulation omitting only pine oil. The antimicrobial properties of these samples were compared to the complete Dettol liquid formulation in the EN 1040 test. The EN 1040 test establishes whether a product has any bactericidal activity.

Protocol

Samples of Dettol Liquid were assessed using EN 1040 for developmental samples protocol (21287v.3) at a final concentration of 2.5% (v/v) with a contact time of 5 minutes.

Sample	Batch / Lot Number	Description
1	03041/029	minus PCMX
2	03041/028	minus PCMX and pine oil
3	BN 9195002B Exp 1 Jul 2002	complete formulation

see Appendix 1 for details

Aliquots of test product and organism were mixed, after 5 minutes an aliquot was removed, activity arrested by the addition of a neutraliser, and the surviving bacteria enumerated.

The Microbicidal Effect (ME) was calculated:

$$ME = Log_{10} \text{ Control Count} - Log_{10} \text{ Survivor Count}$$

Results / Discussion

Sample	Description	In-test Conc. % (v/v)	ME Value					
			Pseudomonas aeruginosa (NCIMB 10421)			*Staphylococcus aureus* (NCTC 10788)		
1	-PCMX	2.5	<2.63	<2.63	<2.63	<2.94	<2.94	<2.94
2	-PCMX -Pine Oil	2.5	<2.63	<2.63	<2.63	<2.94	<2.94	<2.94
3	Complete	2.5	>6.46	>6.46	>6.46	>6.77	>6.77	>6.77

The EN 1040 test establishes whether a product displays basic bactericidal activity, and requires a reduction in bacterial recovery of $\log_{10}$ 5 cycles.
Dettol formulations lacking PCMX and both PCMX and pine oil displayed no detectable antimicrobial activity in this test. In comparison, the complete Dettol formulation displayed high efficacy.

This demonstrates that pine oil does not significantly contribute to the antimicrobial properties of Dettol liquid. The complete Dettol liquid formulation displays excellent antimicrobial activity.

Summary

Dettol liquid formulations omitting i) PCMX and ii) PCMX and pine oil failed to fulfil the requirements of the EN 1040 test and therefore are considered not to display basic bactericidal activity. No difference in the negligible efficacy of these formulations was discernible.

Appendix 1 Formulation Details

Ingredient	03041/028	03041/029
PCMX	0	0
Castor Oil Soap	144.2	144.2
Isopropyl Alcohol	94.3	94.3
Pine Oil	0	16.68
Caramel	0.3	0.3
Softened water	to 1000ml	to 1000ml

BRITISH STANDARD

BS EN
1040 : 1997

Chemical disinfectants and antiseptics — Basic bactericidal activity — Test method and requirements (phase 1)

The European Standard EN 1040 : 1997 has the status of a British Standard

ICS 11.080; 67.020

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Committees responsible for this British Standard

The preparation of this British Standard was entrusted to Technical Committee IH/57, Chemical disinfectants and antiseptics, upon which the following bodies were represented:

Association of British Health-care Industries
Association of Public Analysts
British Association for Chemical Specialities
Campden and Chorleywood Food Research Association
Consumer Policy Committee of BSI
Department of Health
Institute of Trading Standards Administration
Ministry of Agriculture, Fisheries and Food
Ministry of Defence
Public Health Laboratory Service
Society for Applied Bacteriology
Society of Chemical Industry

This British Standard, having been prepared under the direction of the Health and Environment Sector Board, was published under the authority of the Standards Board and comes into effect on
15 September 1997

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Amendments issued since publication

Amd. No.	Date	Text affected

The following BSI references relate to the work on this standard:
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National foreword

This British Standard has been prepared by Technical Committee IH/57 and is the English language version of EN 1040 : 1997 *Chemical disinfectants and antiseptics — Basic bactericidal activity — Test method and requirements (phase 1)*, published by the European Committee for Standardization (CEN).

Compliance with a British Standard does not of itself confer immunity from legal obligations.

Summary of pages

This document comprises a front cover, an inside front cover, pages i and ii, the EN title page, pages 2 to 18, an inside back cover and a back cover.

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EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 1040

February 1997

ICS 11.080; 67.020

Descriptors: Disinfectants, chemical compounds, tests, determination, antibacterial activity, preparation, filtration analysis, neutralization method, dilution, culture media

English version

Chemical disinfectants and antiseptics — Basic bactericidal activity — Test method and requirements (phase 1)

Antiseptiques et désinfectants chimiques — Activité bactéricide de base — Méthode d'essai et prescriptions (phase 1)

Chemische Desinfektionsmittel und Antiseptika — Bakterizide Wirkung (Basistest) — Prüfverfahren und Anforderungen (Phase 1)

This European Standard was approved by CEN on 1997-01-05. 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.

Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the Central Secretariat or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the Central Secretariat has the same status as the official versions.

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CEN

European Committee for Standardization
Comité Européen de Normalisation
Europäisches Komitee für Normung

Central Secretariat: rue de Stassart 36, B-1050 Brussels

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Foreword

This European Standard has been prepared by Technical Committee CEN/TC 216, Chemical disinfectants and antiseptics, the secretariat of which is held by AFNOR.

Other methods to evaluate the efficacy of chemical disinfectants and antiseptics for different fields of application are in preparation.

Annex A is normative.

Annexes B, C, D, E, F and G are informative.

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 August 1997, and conflicting national standards shall be withdrawn at the latest by August 1997.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

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Introduction

This European Standard describes a suspension test method for establishing whether a chemical disinfectant or antiseptic has or does not have a bactericidal activity under the laboratory conditions defined by this European Standard. If a product complies with the test requirements, it can be considered as possessing a bactericidal activity. The acceptibility of a product as a chemical disinfectant or antiseptic for a defined purpose cannot be determined from this test method. Chemical disinfectants and antiseptics are subjected to further testing by relevant tests according to European Standards to evaluate their activity under conditions appropriate to their intended use[1].

There is no evidence that the strains used in this standard are virulent.

1 Scope

This European Standard specifies a test method (phase 1) and the minimum requirements for bactericidal activity of chemical disinfectant and antiseptic products that form a homogeneous physically stable preparation in water. This European Standard is applicable to products for use in agricultural (but not crop protection), domestic service, food hygiene and other industrial fields, institutional, medical and veterinary applications.

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

NOTE 2. This European Standard does not evaluate the activity of a product for an intended use. More specific test methods described in European Standards (see introduction) are used for further assessment of the efficacy of chemical disinfectants and antiseptics for a defined purpose.

NOTE 3. This method corresponds to a phase 1 test (see annex G).

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 edition of the publication referred to applies.

prEN 12353 *Chemical disinfectants and antiseptics — Preservation of microbial strains used for the determination of bactericidal and fungicidal activity*

3 Definitions

For the purposes of this European Standard the following definitions apply.

3.1 product (for chemical disinfection and/or antisepsis)

Chemical agent or formulation used as a chemical disinfectant or antiseptic.

3.2 bactericide

Product which kills vegetative bacteria under defined conditions.

NOTE. The adjective derived from 'bactericide' is 'bactericidal'.

3.3 bactericidal activity (EN 1040)

Capability of a product to produce a reduction in the number of viable bacterial cells of relevant organisms under conditions defined by this European Standard.

4 Requirements

The product, when tested in accordance with clause 5, shall demonstrate at least a 10^5 log reduction in viable counts when the test organism is *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

5 Test method

5.1 Principle

5.1.1 A test suspension of bacteria is added to a prepared sample of the product under test. The mixture is maintained at 20 °C. At a specified contact time chosen from one of the following: 1 min, 5 min, 15 min, 30 min, 45 min or 60 min ± 10 s, an aliquot is taken; the bactericidal action in this portion is immediately neutralized or suppressed by a validated method. The method of choice is dilution-neutralization. If a suitable neutralizer cannot be found, membrane filtration is used. The number of surviving bacteria in each sample is determined and the reduction in viable counts calculated.

5.1.2 The test is performed using *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

5.2 Materials and reagents

5.2.1 ***Test organisms***

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

Pseudomonas aeruginosa	ATCC 15442[2]
Staphylococcus aureus	ATCC 6538[2]

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

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[1] CEN/TC 216 will develop relevant tests.

[2] ATCC 15442 and ATCC 6538 are the collection numbers of strains supplied by the American Type Culture Collection. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of the products named. Corresponding strains supplied by other culture collections may be used if they can be shown to lead to the same results.

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5.2.2 ***Culture media and reagents***

5.2.2.1 *General*

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

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

5.2.2.2 *Water*

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

Sterilize in the autoclave (see **5.3.1**).

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

NOTE 2. If distilled water of adequate quality is not available, water for injectable preparations (see European Pharmacopoeia) can be used.

5.2.2.3 *Tryptone soya agar (TSA)*

For maintenance of bacterial strains and performance of viable counts.

Tryptone, pancreatic digest of casein	15,0 g
Soya peptone, papaic digest of Soybean meal	5,0 g
NaCl	5,0 g
Agar	15,0 g
Water (see **5.2.2.2**)	to 1000,0 ml

Sterilize in the autoclave (see **5.3.1**). After sterilization the pH of the medium shall be equivalent to 7,2 ± 0,2 when measured at 20 °C.

5.2.2.4 *Diluent*

Tryptone sodium chloride solution:

Tryptone, pancreatic digest of casein	1,0 g
NaCl	8,5 g
Water (see **5.2.2.2**)	to 1000,0 ml

Sterilize in the autoclave (see **5.3.1**). After sterilization the pH of the medium shall be equivalent to 7,0 ± 0,2 when measured at 20 °C.

5.2.2.5 *Neutralizer*

The neutralizer shall be validated for the product under test in accordance with annex A. The neutralizer shall be sterile.

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

5.2.2.6 *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.

5.3 Apparatus and glassware

5.3.1 ***General***

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

a) in the autoclave (see **5.3.2.1**) by maintaining it at 121 $^{+3}_{0}$ °C for a minimum holding time of 15 min;

b) in the dry heat sterilizer (see **5.3.2.1**) by maintaining it at 180 °C for a minimum holding time of 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***[3] and, in particular, the following:

5.3.2.1 *Apparatus for sterilization.*

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

b) For dry heat sterilization, a hot air oven capable of being maintained at a temperature of 180 °C for a minimum holding time of 30 min, at 170 °C for a minimum holding time of 1 h or at 160 °C for a minimum holding time of 2 h.

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 either 36 °C ± 1 °C or 37 °C ± 1 °C. An incubator at 37 °C ± 1 °C may be used if an incubator at 36 °C ± 1 °C is not available.

5.3.2.4 *pH-meter*, having an accuracy of calibration of ± 0,1 pH units at 25 °C.

5.3.2.5 *Stopwatch.*

5.3.2.6 *Agitator*, (electromechanical agitator, i.e. Vortex® mixer[4]).

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[3] Disposable equipment is an acceptable alternative to reusable glassware.

[4] 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.

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5.3.2.7 *Membrane filtration apparatus*, (if this method is used) constructed of a material compatible with the product under test, with a filter holder which shall have a usable volume of 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 flow rate. In order to obtain a uniform distribution of the microorganisms over the membrane and in order to prevent overlong filtration, the device shall be set so as to obtain the filtration of 100 ml of rinsing liquid in 20 s to 40 s.

5.3.2.8 *Containers*, test tubes or flasks of suitable capacity.

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

5.3.2.10 *Petri dishes*, of size 90 mm to 100 mm.

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

5.3.2.12 *Volumetric flasks*, calibrated at 20 °C.

5.3.2.13 *Mechanical shaker*.

5.4 Preparation of bacterial suspensions and test solutions

5.4.1 *Bacterial suspensions*

5.4.1.1 *Stock cultures of test organisms*

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

5.4.1.2 *Working culture of test organisms*

In order to prepare the working culture of *Pseudomonas aeruginosa*, subculture from the stock culture (see **5.4.1.1**) by streaking onto TSA slopes and incubate (see **5.3.2.3**). After 18 h to 24 h prepare a second subculture from the first subculture in the same way and incubate for 18 h to 24 h. From this second subculture, a third subculture may be produced in the same way.

NOTE. The second and/or third subculture are the working culture(s).

If it is not possible to prepare the second subculture on a particular day, a 48 h subculture may be used for subsequent subculturing, provided that the subculture has been kept in the incubator during the 48 h period. In these circumstances, prepare a further 24 h subculture after proceeding. Do not take a fourth subculture.

Use the same procedure for *Staphylococcus aureus*.

5.4.1.3 *Bacterial test suspensions*

Take 10 ml of diluent (see **5.2.2.4**) and place in a 100 ml flask with 5 g of glass beads (see **5.3.2.11**). Take the working cultures (see **5.4.1.2**) and transfer loopfuls of the cells into the diluent. The cells should be suspended in the diluent by immersing the loop in the diluent and rubbing it against the side of the flask to dislodge the cells. Shake the flask for 3 min using a mechanical shaker (see **5.3.2.13**). Aspirate the suspension from the glass beads and transfer to another tube. Adjust the number of cells in the suspension to $1,5 \times 10^8$ cfu/ml[5] to 5×10^8 cfu/ml using the diluent, estimating the numbers of units by any suitable means. Maintain this suspension in the water bath at 20 °C ± 1 °C and use within 2 h.

For counting of the bacterial test suspension prepare 10^{-6} and 10^{-7} dilutions of the test suspension (see **5.4.1.3**) using diluent (see **5.2.2.4**). Mix (see **5.3.2.6**). Take a sample of 1,0 ml of each dilution in duplicate and transfer each 1,0 ml sample into separate Petri dishes (see **5.3.2.10**) and add 12 ml to 15 ml melted TSA (see **5.2.2.3**), cooled to 45 °C ± 1 °C.

5.4.1.4 *Counting of bacterial test suspensions*

Incubate the plates at 36 °C ± 1 °C (or 37 °C ± 1 °C) (see **5.3.2.3**) for 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units for each plate. Incubate the plates for a further 24 h. Do not recount plates which no longer show well-separated colonies. Recount the remaining plates. Determine the higher number of colonies for each 1,0 ml sample. Calculate the number of cfu/ml in the test suspension (*N*) using the method given in **5.6.1.1**.

5.4.2 *Product test solution*

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

Product test solutions shall be prepared at three different concentrations to include at least two concentrations in the active range. The concentrations shall decrease in a geometrical progression with a factor of at least 2 (i.e. at least doubling the dilutions). The concentration of the product test solution shall be 1,25 times the required test concentration.

NOTE. The product as received may be used as one of the product test solutions.

For solid products, dissolve the product as received by weighing at least 1 g ± 10 mg of the product in a volumetric flask and filling up with water (see **5.2.2.2**). Subsequent dilutions shall be prepared in volumetric flasks (see **5.3.2.12**) on a volume/volume basis.

For liquid products, dilutions of the product shall be prepared in water (see **5.2.2.2**) on a volume/volume basis using volumetric flasks (see **5.3.2.12**).

The concentration of the product stated in the test report shall be the test concentration. Record the test concentration in terms of volume per volume or mass per volume.

The product test solutions shall be prepared freshly and used for not more than one working day (or less if of low stability).

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[5] cfu/ml: colony forming units per ml.

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5.5 Procedure

5.5.1 *General*

The method of choice is the dilution-neutralization method. To determine a suitable neutralizer the following procedure shall be adopted. The validation of the dilution-neutralization method (see **A.4.1**) shall be carried out using a suitable neutralizer, chosen according to laboratory experience and published data.

If this neutralizer is not valid, repeat the validation test using an alternative neutralizer containing a combination of polysorbate 80 30 g/l, saponin 30 g/l, L-histidine 1 g/l, lecithin 3 g/l, sodium thiosulphate 5 g/l in either diluent (see **5.2.2.4**) or in phosphate buffer 0,0025 mol/l. If both neutralizers are found to be invalid, the membrane filtration method may be used in place of the dilution-neutralization method.

5.5.2 *Dilution-neutralization method*

5.5.2.1 *General*

Prior to testing, equilibrate all reagents (product test solution, water, bacterial test suspension, neutralizer) to the test temperature of 20 °C ± 1 °C using the water bath (see **5.3.2.2**). Check that the temperature of the reagents is stabilized at 20 °C ± 1 °C.

5.5.2.2 *Test procedure for bactericidal activity of products*

Pipette 8,0 ml of one of the product test solutions into a container of suitable capacity and add 1,0 ml of water (see **5.2.2.2**). Add 1,0 ml of one of the bacterial test suspensions containing $1,5 \times 10^8$ to 5×10^8 cfu/ml (see **5.4.1.3**). Immediately start the stopwatch (see **5.3.2.5**), mix (see **5.3.2.6**) and place the container in a water bath controlled at 20 °C ± 1 °C.

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

The activity of the product shall be determined for a contact time chosen from one of the following: 1 min, 5 min, 15 min, 30 min, 45 min or 60 min ± 10 s.

Just before the end of the chosen contact time, mix (see **5.3.2.6**). At the chosen contact time, pipette 1,0 ml of the test mixture into a tube containing 8,0 ml neutralizer (see **5.2.2.5**) and 1,0 ml water (see **5.2.2.2**). Mix (see **5.3.2.6**) and place in a water bath controlled at 20 °C ± 1 °C.

After a neutralization time of 5 min ± 10 s immediately take a sample of 1,0 ml of neutralized mixture in duplicate and transfer each 1,0 ml sample into separate Petri dishes (see **5.3.2.10**) and immediately add 12 ml to 15 ml melted TSA (see **5.2.2.3**), cooled to 45 °C ± 1 °C.

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

5.5.2.3 *Counting of the test mixture*

Incubate the plates at 36 °C ± 1 °C (or 37 °C ± 1 °C) (see **5.3.2.3**) for 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units for each plate. Incubate the plates for a further 24 h. Do not recount plates which no longer show well-separated colonies. Recount the remaining plates. Determine the higher number of colonies for each plate. Calculate the number of cfu/ml in the test mixture (N_a) using the method given in **5.6.1.2**.

For calculating the viable count of the test mixture, the dilution factor is 10^{-1}.

5.5.3 *Membrane filtration method*

5.5.3.1 *General*

Prior to testing, equilibrate all reagents (product test solution, water, bacterial test suspension, rinsing liquid) to the test temperature of 20 °C ± 1 °C using the water bath (see **5.3.2.2**) controlled at 20 °C ± 1 °C. Check that the temperature of the reagents is stabilized at 20 °C ± 1 °C.

5.5.3.2 *Test procedure for bactericidal activity of products*

Pipette 8,0 ml of one of the product test solutions into a container of suitable capacity and add 1,0 ml of water (see **5.2.2.2**). Add 1,0 ml of one of the bacterial test suspensions containing $1,5 \times 10^8$ to 5×10^8 cfu/ml (see **5.4.1.3**). Start immediately a stopwatch, mix (see **5.3.2.6**) and place the container in a water bath controlled at 20 °C ± 1 °C.

NOTE 1. When adding bacterial suspensions to containers, care should be taken to avoid touching the upper part of the container sides.

The activity of the product shall be determined for a contact time chosen from one of the following: 1 min, 5 min, 15 min, 30 min, 45 min or 60 min ± 10 s.

Just before the end of the chosen contact time, mix (see **5.3.2.6**). At the chosen contact time pipette two samples of 0,1 ml of the test mixture and transfer each sample into a separate membrane filtration apparatus equipped with a membrane and containing 50 ml of the rinsing liquid (see **5.2.2.6**). Filter immediately. The time required for transfer and filtration should not exceed 1 min. If greater than 1 min, this time shall be recorded in the test report. Rinse with at least 150 ml but not more than 500 ml of rinsing liquid (see **5.2.2.6**). Then transfer the membranes to the surface of two separate TSA plates.

NOTE 2. When transferring, care should be taken to ensure that the bacteria are on the upper side of the membrane when placed on the TSA and to avoid trapping air between the membrane and agar surface.

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

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5.5.3.3 *Counting of test mixture*

Incubate the plates at 36 °C ± 1 °C (or 37 °C ± 1 °C) (see **5.3.2.3**) for 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units for each plate. Incubate the plates for a further 24 h. Do not recount plates which no longer show well-separated colonies. Recount the remaining plates. Determine the higher number of colonies for each plate. Calculate the number of cfu/ml in the test mixture (N_a) using the method given in **5.6.1.2**.

5.5.4 ***Validation of dilution-neutralization method and membrane filtration method***

The dilution-neutralization and membrane filtration methods shall be validated for each of the test organisms according to annex A.

The validation test (see annex A) shall also be carried out at the same time as the test procedure (see **5.5**) using only the highest concentration and the same conditions (bacterial test suspension, product test solution and neutralizer or rinsing liquid) as used in the test (see **5.5.2** or **5.5.3**).

5.6 Calculation and expression of results

5.6.1 *Calculation of viable counts (cfu/ml)*

5.6.1.1 *Bacterial test suspension*

Viable counts of the bacterial test suspension (see **5.4.1.4**) should be calculated according to the principles described in ISO 7218 : 1985 (see note) as follows.

a) Only colony counts which are less than 300 cfu/plate should be used for calculation of viable counts. For a result to be valid at least one plate shall contain 15 or more colonies; viable counts should be calculated using at least one pair of plates, where one or both plates contain more than 15 colonies and both plates contain less than 300 colonies. If plates from 2 dilutions fall within this range calculate the number of cfu/ml as the weighted mean count. If plates from only one dilution fall within this range calculate the arithmetic mean.

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

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

$$\frac{c}{(n_1 + 0{,}1 n_2)d}$$

where:

- c is the sum of the colonies counted on all the plates taken into account;
- n_1 is the number of plates taken into account at the first dilution;
- n_2 is the number of plates taken into account at the second dilution;
- d is the dilution factor corresponding to the first dilution taken into account.

Round off the results calculated to two significant figures. For this, if the last figure is below 5, the preceding figure is not modified; if the last figure is above 5 the preceding figure is increased by one unit; if the last figure is equal to 5, round off the preceding figure to the next nearest even figure. Proceed stepwise until two significant figures are obtained.

b) As a result the number of cfu/ml is expressed by a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

Example:

$$\frac{168 + 215 + 14 + 25}{(2 + 0{,}1 \times 2)\ 10^{-6}} = \frac{422}{2{,}2 \times 10^{-6}} = 1{,}9182 \times 10^8$$
$$= 1{,}9 \times 10^8 \text{ (in cfu/ml)}$$

5.6.1.2 *Test procedure and test for validation*

For the test procedure (see **5.5.2.2** and **5.5.3.2**) and test for validation (see **A.2**, **A.4.1** and **A.4.2**) the viable count should be calculated using the following method. Only colony counts which are less than 300 cfu per plate should be used for calculation of viable counts. Viable counts should be calculated using colony counts from both plates. When at least one plate contains 15 or more colonies use the following formula for calculation of viable counts (in cfu/ml).

$$\frac{c}{n \times d \times V}$$

where:

- c is the sum of the colonies counted on both plates;
- n is the number of plates taken into account;
- d is the dilution factor corresponding to the dilution taken into account. For the dilution-neutralization test procedure (see **5.5.2.3**) and the bacterial suspension (see **A.2**) the dilution factor is 10^{-1};
- V is the sample volume. For the dilution-neutralization and validation procedures (see **5.5.2.2** and **A.4.1.2**) and the bacterial suspension (see **A.2**) the sample volume is 1,0 ml. For the membrane filtration test and validation procedure (see **5.5.3.2** and **A.4.2.2**) the sample volume is 0,1 ml.

For the test procedure (see **5.5**) where the number of cfu on all plates counted is lower than 15, record the viable count of the test mixture as lower than $1{,}5 \times 10^2$ cfu/ml (< $1{,}5 \times 10^2$ cfu/ml). Where the number of cfu on all plates counted is higher than 300 record the viable count of the test mixture as higher than 3×10^3 cfu/ml (> 3×10^3 cfu/ml).

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5.6.2 ***Verification of methodology***

For each test organism check that:

a) N is between $1,5 \times 10^8$ cfu/ml and 5×10^8 cfu/ml;

b) N_v is between 6×10^2 and 3×10^3 cfu/ml;

c) N_x is equal to or greater than 0,05 times N_v;

d) N_y is equal to or greater than 0,05 times N_v;

where:

N is the number of cfu/ml of the bacterial test suspension (see **5.4.1.4**);

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

N_x is the number of cfu/ml of the neutralizer toxicity control (see **A.4.1.3**) or of the filtration control (see **A.4.2.3**);

N_y is the number of cfu/ml of the dilution-neutralization control (see **A.4.1.3**) or of the filtration test control (see **A.4.2.3**);

5.6.3 ***Expression of results***

For each test organism record the number of cfu/ml in the bacterial test suspension (N) (see **5.4.1.4**) and after the test procedure for bactericidal activity of the product (N_a) (see **5.5.2.3** or **5.5.3.3**).

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

For validation of the dilution-neutralization method (see **A.4.1**) record the number of cfu/ml in the neutralizer toxicity control (N_x) and the dilution neutralization control (N_y).

For validation of the membrane filtration method (see **A.4.2**), record the number of cfu/ml in the filtration control (N_x) and the filtration test control (N_y).

For each test organism and product test concentration calculate and record the reduction in viability (in cfu/ml) as follows:

$$\text{reduction in viability} = \frac{N \times 10^{-1}}{N_a}$$

5.7 Conclusion

The product shall be deemed to have passed the test if it demonstrates a 10^5 or more reduction in viability within 60 min or less at 20 °C under the conditions defined by this test when the test organisms are *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

The precision of the test result is indicated in annex F. This precision of the test result is increased by increasing the number of replicate tests performed (for information see annex F).

The number of replicate tests shall be decided on the basis of the test level of precision which is required, taking into account the intended use of the test results.

The highest concentration shall produce a reduction in viability of 10^5 or more. The intermediate concentration may produce a reduction in viability which is greater or less than 10^5.

A product which passes the test is characterized as possessing bactericidal activity. In order to qualify the product as an antiseptic and/or chemical disinfectant for a defined purpose it shall be evaluated using additional standard tests which are appropriate to its intended use.

Information regarding the additional standard tests which shall be used to qualify a product as antiseptic and/or disinfectant for a defined purpose is given in annex G.

NOTE. Although the replication of this test is not obligatory, this does not constitute a precedent for the phase 2 test methods for establishing the activity of chemical disinfectant and/or antiseptic.

5.8 Test report

The report shall refer to this European Standard (i.e EN 1040).

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

a) identification of the laboratory;

b) identification of the sample:

1) name of the product;

2) batch number;

3) manufacturer;

4) date of delivery;

5) storage conditions;

6) active substance(s) and its (their) concentration(s) (optional);

c) test method and its validation:

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

If the membrane filtration method is used full details of the tests for validation of the method shall be given. Full details of the procedure which was carried out to justify the use of the membrane filtration method shall also be given;

d) experimental conditions:

1) period of analysis;

2) product test concentrations;

3) test temperature;

4) contact times;

5) temperature of incubation;

e) test results:

Validation tests:

Evaluation of bactericidal activity (see tables D.1, D.2, D.3 and D.4):

f) conclusion:

g) locality, date and identified signature:

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

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Annex A (normative)

Validation of dilution-neutralization and membrane filtration methods

A.1 Principle

A neutralizer is chosen for each product in accordance with **A.4.1** (see **5.5**). If a suitable neutralizer 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 (see **5.4.1.3**) with the diluent (see **5.2.2.4**) to obtain the bacterial suspension 6×10^2 cfu/ml to 3×10^3 cfu/ml.

For counting of the suspension prepare a 10^{-1} dilution with the diluent (see **5.2.2.4**). Mix (see **5.3.2.6**). Take a sample of 1,0 ml of the 10^{-1} dilution in duplicate and transfer each 1,0 ml sample into separate Petri dishes (see **5.3.2.10**) and add 12 ml to 15 ml melted TSA (see **5.2.2.3**), cooled to 45 °C ± 1 °C.

Incubate the plates at 36 °C ± 1 °C (or 37 °C ± 1 °C) (see **5.3.2.3**) for 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units for each plate. Incubate the plates for a further 24 h. Do not recount plates which no longer show well-separated colonies. Recount the remaining plates. Determine the higher number of colonies for each plate. Calculate the number of cfu/ml in the bacterial suspension (N_v) using the method given in **5.6.1.2**.

A.3 Preparation of product test solution

See **5.4.2**. Prepare a product test solution of the highest strength used in the test. Add 2 parts of water (see **5.2.2.2**) to 8 parts of this solution.

A.4 Test for validation

A.4.1 *Dilution-neutralization method*

A.4.1.1 *General*

Prior to testing, equilibrate all reagents (product test solutions, water, bacterial test suspension, neutralizer) to the test temperature of 20 °C ± 1 °C using the water bath (see **5.3.2.2**) controlled at 20 °C ± 1 °C. Check that the temperature of the reagents is stabilized at 20 °C ± 1 °C.

A.4.1.2 *Procedure*

Pipette 8,0 ml neutralizer (see **5.2.2.5**) into each of two containers of suitable capacity. Place the containers in a water bath (see **5.3.2.2**) controlled at 20 °C ± 1 °C:

- for the neutralizer toxicity control, add 1,0 ml of water (see **5.2.2.2**) to the first container;
- for the dilution-neutralization control, add 1,0 ml of product test solution prepared as described in **A.3** to the second container.

Mix (see **5.3.2.6**) and leave in contact for 5 min ± 10 s in a water bath controlled at 20 °C ± 1 °C.

Then add 1,0 ml of the bacterial suspension containing 6×10^2 to 3×10^3 cfu/ml (see **A.2**) to each of the containers, mix (see **5.3.2.6**) and leave at 20 °C ± 1 °C for 30 min ± 1 min.

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

Mix again. Sample 1,0 ml from each of the containers in duplicate and transfer each 1,0 ml sample into separate Petri dishes (see **5.3.2.10**) and add 12 ml to 15 ml melted TSA (see **5.2.2.3**), cooled to 45 °C ± 1 °C.

Perform this procedure for the other test organism.

A.4.1.3 *Counting of the neutralizer toxicity and dilution-neutralization control*

Incubate the plates at 36 °C ± 1 °C (or 37 °C ± 1 °C) (see **5.3.2.3**) for 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units for each plate. Incubate the plates for a further 24 h. Do not recount plates which no longer show well-separated colonies. Recount the remaining plates. Determine the higher number of colonies for each plate. Calculate the number of cfu/ml in the neutralizer toxicity control (N_x) and the dilution neutralization control (N_y) using the method given in **5.6.1.2**.

A.4.2 *Membrane filtration method*

A.4.2.1 *General*

Prior to testing equilibrate all reagents (product test solutions, water, bacterial suspension, rinsing liquid) to the test temperature of 20 °C ± 1 °C using the water bath (see **5.3.2.2**) controlled at 20 °C ± 1 °C. Check that the temperature of the reagents is stabilized at 20 °C ± 1 °C.

A.4.2.2 *Procedure*

Perform this procedure for each of the test organisms.

For the filtration control, sample (in duplicate) 0,1 ml of the bacterial suspension containing 6×10^2 to 3×10^3 cfu/ml (see **A.2**) and transfer into two separate membrane filtration apparatus equipped with a membrane and containing 50 ml of the rinsing liquid (see **5.2.2.6**). Filter and rinse with 50 ml of rinsing liquid (see **5.2.2.6**) and then transfer the membranes to the surface of two separate TSA plates (see **5.2.2.3**).

For the filtration test control, sample (in duplicate) 0,1 ml of the product test solution (see **A.3**). Transfer into two separate membrane filtration apparatus equipped with a membrane and containing 50 ml of the rinsing liquid (see **5.2.2.6**). Filter and rinse the membrane with at least 150 ml and not more than 500 ml of rinsing liquid. Then cover the membrane with 50 ml of rinsing liquid (see **5.2.2.6**). To each of the membranes add 0,1 ml of the bacterial suspension containing 6×10^2 to 3×10^3 cfu/ml (see **A.2**).

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Filter and rinse with 50 ml of water (see **5.2.2.2**). Transfer to the surface of two separate TSA plates (see **5.2.2.3**). The time required for transfer and filtration should not exceed 1 min. If greater than one minute this time shall be recorded in the test report.

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

A.4.2.3 *Counting of filtration and filtration test controls*

Incubate the plates at 36 °C ± 1 °C (or 37 °C ± 1 °C) (see **5.3.2.3**) for 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units for each plate. Incubate the plates for a further 24 h. Do not recount plates which no longer show well-separated colonies. Recount the remaining plates. Determine the higher number of colonies for each plate. Calculate the number of cfu/ml in the filtration control (N_x) and the filtration test control (N_y) using the method given in **5.6.1.2**.

A.5 Validation

Check that the test results conform to the relevant requirements of **5.6.1.2** or **5.6.2**.

Annex B (informative)
Neutralizers

Any of the following neutralizers may be used:

- lecithin 3 g/l; polysorbate 80[6] 30g/l; sodium thiosulphate 5 g/l; L-histidine 1 g/l; saponine 30 g/l in diluent (see **5.2.2.4**) or in phosphate buffer 0,25 mol/l at 1 % (*V/V*);
- phosphate buffer 0,25 mol/l:
  KH_2PO_4 34 g
  Water (see **5.2.2.2**) 500 ml
  adjusted to pH 7,2 ± 0,2 with NaOH 1 mol/l
  Water (see **5.2.2.2**) up to 1000 ml
  sterilized in an autoclave (see **5.3.1**);
- fresh egg yolk diluted to 5 % (*V/V*) or 0,5 % (*V/V*);
- 30 g/l polysorbate 80 [6]; 4 g/l sodium lauryl sulphate; lecithin 3 g/l;
- 5 % (*V/V*) fresh egg yolk; 40 g/l polysorbate 80 [6];
- 7 % (*V/V*) ethylene oxide condensate of fatty alcohol; 20 g/l lecithin 4 % (*V/V*) polysorbate 80 [6];
- 4 % (*V/V*) ethylene oxide condensate of fatty alcohol; 4 g/l lecithin;
- 30 g/l polysorbate 80 [6]; lecithin 3 g/l; L-histidine 1 g/l;
- glycine as a function of concentration of product;
- 30 g/l polysorbate 80 [6]; lecithin 3 g/l;
- phospholipid emulsion (commercial) at 50 mg/ml (diluted 1 to 10);
- sodium thioglycollate at 0,5 g/l or 5 g/l;
- L-cysteine at 0,8 g/l or 1,5 g/l;
- thiomalic acid at 0,075 % (*V/V*) (adjusted to pH 7 with NaOH);
- sodium thiosulphate at 5 g/l;
- catalase or peroxidase: One unit of these enzymes catalyzes the decomposition of 1 µmol of hydrogen peroxide per minute at 25 °C and at pH 7;
- polysorbate 80 [6] 30 g/l; saponin 30 g/l; L-histidine 1 g/l; L-cysteine 1 g/l.

NOTE. The above list is not exhaustive and other neutralizers may be tried.

Annex C (informative)
Rinsing liquids

Any of the following rinsing liquids may be used:

- water (see **5.2.2.2**);
- diluent (see **5.2.2.4**);
- 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 0,7 g/l lecithin;
- neutralizer (see **5.2.2.5**);
- buffer solutions.

NOTE. The above list is not exhaustive and other rinsing liquids may be tried.

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[6] Analytical quality, non-hydrolized in accordance with European Pharmacopaeia volume 1.TWEEN 80® is an example of a suitable product available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of this product.

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Annex D (informative)

Example of a typical test report

Determination of the basic bactericidal activity

a) Identification of the test laboratory	Besson test house
b) Identification of the sample	
Name of the product	C 216
Batch number	94-71-51
Manufacturer	Centipede Formulations Inc.
Date of delivery	1994-02-11
Storage conditions	room temperature and darkness
Active substance(s) and its/their concentration(s) (optional)	Not indicated
c) Test method and its validation	
Method	Dilution-neutralization
Neutralizer	30 g/l, lecithin, sterilized in the autoclave
d) Experimental conditions	
Period of analysis	1994-02-20 to 1994-03-12
Product test concentrations	2 g/l, 4 g/l and 8 g/l
Test temperature	20 °C ± 1 °C
Contact times	5 min
Temperature of incubation	37 °C ± 1 °C

e) Test results

See tables D.1, D.2, D.3 and D.4.

f) Conclusion

According to EN 1040 (date of edition) the batch 94.71.51 of the product C 216 possesses bacterial activity for the referenced strains of *Pseudomonas aeruginosa* ATCC 15 442 and *Staphylococcus aureus* ATCC 6538. In order to qualify the product as an antiseptic and or chemical disinfectant for a defined purpose, it will be evaluated using additional standard tests which are appropriate to its intended use.

g) Locality, date and identified signature

NOTE. The test product, batch No. and manufacturer are given as imaginary examples only.

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Table D.1 Verification of the methodology and validation of dilution-neutralization method for the test concentration of 8 g/l of the test product as received

Test organism	Viable count (cfu/ml)			
	Bacterial test suspension (see 5.4.1.4) (N)	Bacterial suspension (N_v) (see A.2)	Neutralizer toxicity control (N_x) (see A.4.1.2)	Dilution-neutralization control (N_y) (see A.4.1.2)
Pseudomonas aeruginosa	$3{,}2 \times 10^8$	$2{,}8 \times 10^3$	$2{,}6 \times 10^2$	$1{,}5 \times 10^2$
Staphylococcus aureus	$3{,}0 \times 10^8$	$3{,}0 \times 10^3$	$2{,}8 \times 10^2$	$2{,}2 \times 10^2$

For the 2 strains tested:

N is between $1{,}5 \times 10^8$ cfu/ml and 5×10^8 cfu/ml;

N_v is between 6×10^2 cfu/ml and 3×10^3 cfu/ml;

N_x is equal to or greater than $0{,}05\ N_v$;

N_y is equal to or greater than $0{,}05\ N_v$

The neutralization is validated with the neutralizer tested for the test concentration of 8 g/l of product as received and for the two strains tested.

Table D.2 Verification of the methodology and validation of membrane filtration method for the test concentration of 8 g/l of the test product as received

Test organism	Viable count (cfu/ml)			
	Bacterial test suspension (see 5.4.1.4) (N)	Bacterial suspension (N_v) (see A.2)	Filtration control (N_x) (see A.4.2.2)	Filtration test control (N_y) (see A.4.2.2)
Pseudomonas aeruginosa	$3{,}2 \times 10^8$	$2{,}8 \times 10^3$	$2{,}6 \times 10^2$	$1{,}5 \times 10^2$
Staphylococcus aureus	$3{,}0 \times 10^8$	$3{,}0 \times 10^3$	$2{,}8 \times 10^2$	$2{,}2 \times 10^2$

For the 2 strains tested:

N is between $1{,}5 \times 10^8$ cfu/ml and 5×10^8 cfu/ml;

N_v is between 6×10^2 cfu/ml and 3×10^3 cfu/ml;

N_x is equal to or greater than $0{,}05 \times N_v$;

N_y is equal to or greater than $0{,}05 \times N_v$;

The filtration is validated with the rinsing liquid tested for the test concentration of 8 g/l of product as received and for the two strains tested.

Table D.3 Test results: Example for dilution-neutralization method

Test organism	Viable counts (cfu/ml) for test mixture (see 5.5.2.3)		
	(N_a) at concentrations :		
	2 g/l	4 g/l	8 g/l
Pseudomonas aeruginosa	$1{,}6 \times 10^3$	$2{,}0 \times 10^2$	$< 1{,}5 \times 10^2$
Staphylococcus aureus	$2{,}0 \times 10^6$	$1{,}5 \times 10^3$	$< 1{,}5 \times 10^2$
Reduction in viability at test concentration			
	2 g/l	4 g/l	8 g/l
Pseudomonas aeruginosa	2×10^4	$1{,}6 \times 10^5$	$> 2{,}1 \times 10^5$
Staphylococcus aureus	$< 1{,}0 \times 10^4$	$2{,}0 \times 10^4$	$> 2{,}0 \times 10^5$

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Table D.4 Test results: Example for membrane filtration method			
Test organism	**Viable counts (cfu/ml) for test mixture (see 5.5.2.2) (N_a) at concentrations :**		
	2 g/l	**4 g/l**	**8 g/l**
Pseudomonas aeruginosa	$1{,}3 \times 10^3$	$2{,}0 \times 10^2$	$< 1{,}5 \times 10^2$
Staphylococcus aureus	$2{,}0 \times 10^6$	$1{,}5 \times 10^3$	$< 1{,}5 \times 10^2$
Reduction in viability at test concentration			
	2 g/l	**4 g/l**	**8 g/l**
Pseudomonas aeruginosa	$2{,}5 \times 10^4$	$1{,}6 \times 10^5$	$> 2{,}1 \times 10^5$
Staphylococcus aureus	$< 1{,}0 \times 10^4$	$2{,}0 \times 10^4$	$> 2{,}0 \times 10^5$

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Annex E (informative)
Referenced strains in national collections

Pseudomonas aeruginosa:	ATCC	15442
	CIP	103467
	DSM	939
	NCIMB	10421
Staphylococcus aureus:	ATCC	6538
	CIP	483
	DSM	799
	NCTC	10788
	NCIMB	9518

Annex F (informative)
Precision of the test result

A collaborative study was carried out to determine the precision of the test method between different laboratories. The study was carried out in 24 laboratories in different European countries. In each laboratory the test was carried out up to 10 times on separate days.

The tests were performed using the dilution-neutralization and membrane filtration methods using *Pseudomonas aeruginosa* as the test organism. The products used for the study were benzalkonium chloride and phenol.

The agreement between laboratories expressed in terms of the bactericidal effect (reduction in viable count $\geq 10^5$) is very good at low and high concentrations but less good at intermediate levels (see tables F.1 and F.2).

Table F.1 Percentage of laboratories having more than half of their valid replicates with at least a 10^5 reduction at a given phenol concentration

Test method	Concentration of phenol %		
	0,5	1,0	2,0
Membrane filtration	0	30	100
Dilution neutralization	0	5	94

Table F.2 Percentage of laboratories having more than half their valid replicates with at least a 10^5 reduction at a given benzalkonium chloride concentration

Test method	Concentration of benzalkonium chloride %		
	0,001	0,004	0,008
Membrane filtration	10	75	90
Dilution neutralization	5	50	80

Based on the results of the collaborative study, the standard deviation for each method and each product was calculated (see table F.3).

Table F.3 Standard deviation (σ) of $\log_{10}$ reduction — by method and product

Product	Test method	
	Membrane filtration	Dilution neutralization
Phenol	0,50	0,78
Benzalkonium chloride	1,15	1,34

The maximum error on the test result (d) can be estimated using the following equation:

$$d = \sqrt{\frac{t^2(1-\rho/2)\cdot\sigma^2}{n}} \qquad \text{(F.1)}$$

where:

- d is the maximum error of the test result;
- t is the Student's t value (t is equal to 1,96 when n is large);
- n is the number of replicate tests performed;
- σ is the standard deviation of the log reduction;
- ρ is the type 1 error.

It was decided that the test should provide a 95 % confidence level, which sets σ at 5 %.

n can be deduced as a function of σ and d using following equation:

$$n = \frac{t^2(1-\rho/2)\cdot\sigma^2}{d^2} \qquad \text{(F.2)}$$

An extrapolation based on this assumption based on the above equation (F.2), where n is now expressed as a function of σ and d is given in table F.4.

Application of table F.4

According to table F.4 the conclusions which can be drawn from the results of replicate tests are as follows.

If the average log reduction is the required log reduction (5 in this case) and if the mean is the observed log reduction (6 if the observed reduction is 10^6), it is possible to assume, as observed in the interlaboratory study, that the standard deviation (σ) is not greater than 1,3.

- In order to guarantee that the average log reduction is equal to 5 with a confidence level of 95 % (which implies a mean of 6), approximately 7 replicates (n) will need to be performed (see table F.4, standard deviation = 1,3, maximum absolute error on the log reduction is 6 − 5 = 1).

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With the same assumption as in the previous paragraph, it can be said that:

- If a consistent pass value of 5 or more log reduction in viable count is obtained at the highest concentration, it can be concluded (at the 95 % confidence level) that the average log reduction is equal to or greater than:

3,9 if 5 replicates have been performed; where

$$3{,}9 = 5 - \sqrt{\frac{(1{,}96)^2 \times (1{,}3)^2}{5}}$$

3,5 if 3 replicates have been performed; and

2,5 if only 1 replicate has been performed.

NOTE. All replicate tests should be carried out using separate subcultures taken from the stock culture as defined in 5.4.1.2. The value of 1,3 for the standard deviation is only valid for replicate test results obtained from separate subcultures taken from the stock culture as defined in 5.4.1.2. It is not valid for replicate tests carried out using sequential second and third subcultures taken from the stock culture.

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Table F.4

Standard deviation	Maximum absolute error on log reduction																
	0,125	0,25	0,375	0,5	0,625	0,75	0,875	1	1,125	1,25	1,5	1,75	2	2,25	2,5	2,75	3
0,5	62	16	7	4	3	2	2	1	1	1	1	1	1	1	1	1	1
0,6	89	23	10	6	4	3	2	2	2	1	1	1	1	1	1	1	1
0,7	121	31	14	8	5	4	3	2	2	2	1	1	1	1	1	1	1
0,8	158	40	18	10	7	5	4	3	2	2	2	1	1	1	1	1	1
0,9	200	50	23	13	8	6	5	4	3	2	2	2	1	1	1	1	1
1	246	62	28	16	10	7	6	4	4	3	2	2	1	1	1	1	1
1,1	298	75	34	19	12	9	7	5	4	3	3	2	2	1	1	1	1
1,2	355	89	40	23	15	10	8	6	5	4	3	2	2	2	1	1	1
1,3	416	104	47	26	17	12	9	7	6	5	3	3	2	2	2	1	1
1,4	482	121	54	31	20	14	10	8	6	5	4	3	2	2	2	1	1
1,5	554	139	62	35	23	16	12	9	7	6	4	3	3	2	2	2	1
1,6	630	158	70	40	26	18	13	10	8	7	5	4	3	2	2	2	2

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Annex G (informative)

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

CEN/TC 216 would like to draw the attention of the reader of this standard to the agreements which were reached concerning the relationship between this standard and future standards.

This information should be followed when using the European Standards on chemical disinfectants and antiseptics.

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

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

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

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

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

– phase 1	suspension tests for the basic activity of the product;
– phase 2 step 1	suspension tests under conditions representative of practical use;
– phase 2 step 2	other laboratory tests e.g. handwash, handrub, and surface tests simulating practical conditions;
– phase 3	field tests under practical conditions.

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

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

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

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

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

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

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

G.2 Guide to interpretation of tests for chemical disinfectants and antiseptics

A separate standard (or standards) which will be used as a 'Guide to interpretation of tests for chemical disinfectants and antiseptics' will be prepared after the standard test methods have been agreed; the purpose of this standard will be to specify in detail the relationship of the various tests to one another and to use recommendations.

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London
W4 4AL

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ENCKISER
PHARMACEUTICALS INC.

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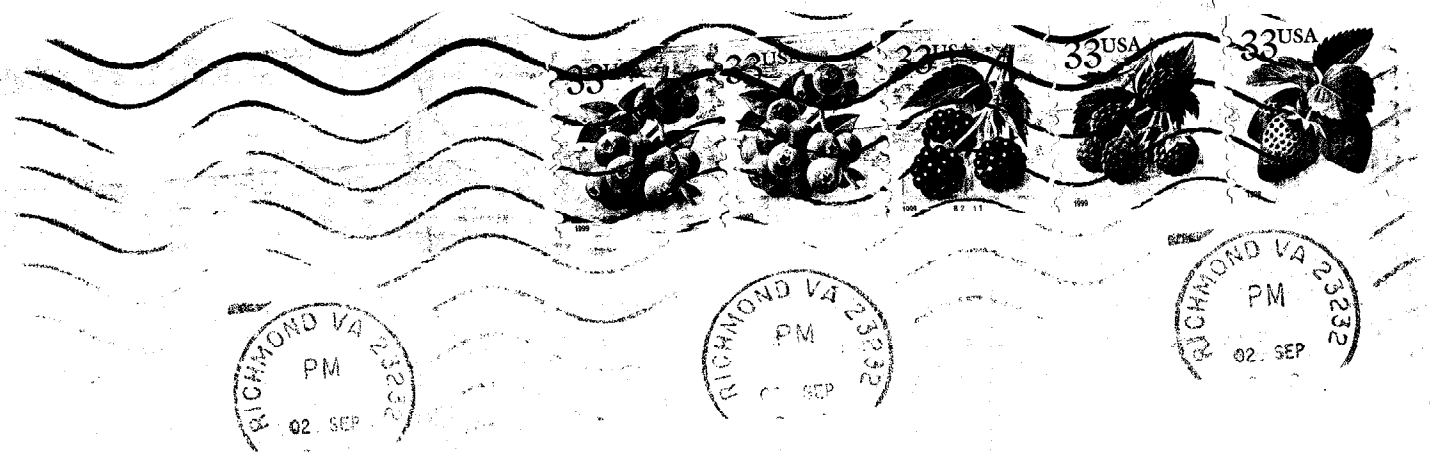
CHMOND, VIRGINIA 23235



Dockets Management Branch (HFA-305)
Food & Drug Administration
5600 Fishers Lane
Rockville, MD 20857