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BSI Standards Publication

Chemical disinfectants and antiseptics — Hygienic handrub — Test method and requirements (phase 2/step 2)

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National foreword

This British Standard is the UK implementation of EN 1500:2013. It supersedes BS EN 1500:1997 which is withdrawn.

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

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

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Chemische Desinfektionsmittel und Antiseptika - Hygienische Händedesinfektion - Prüfverfahren und Anforderungen (Phase 2/Stufe 2)

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Foreword

This document (EN 1500:2013) has been prepared by Technical Committee CEN/TC 216 “Chemical disinfectants and antiseptics”, the secretariat of which is held by AFNOR.

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of an identical text or by endorsement, at the latest by October 2013, and conflicting national standards shall be withdrawn at the latest by October 2013.

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

This document supersedes EN 1500:1997.

This document was revised to adapt it to the latest state of science, to correct errors and ambiguities, to harmonise the structure and wording with other tests of CEN/TC 216 existing or in preparation and to improve the readability of the standard and thereby make it more understandable.

The following technical changes have been made:

— Neutralization (5.5.1.2).

— The number of volunteers (5.5.1.4).

— The statistical evaluation (5.8).

— The annexes have been completely revised.

Data obtained using the former version of EN 1500 may still be used, if it is supplemented by data on neutralization, additional results from more volunteers and the new statistical evaluation of the “mixed” (old and new) set of data. The additional results will be obtained preferably in the same laboratory and with volunteers not having participated in the previous (“old”) study. If the neutralizer used in the test using the former version is not sufficiently neutralizing, a complete new test will be run. The changed procedure in Annex A is regarded as having no (or negligible) influence on the results.

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

1 Scope

This European Standard specifies a test method simulating practical conditions for establishing whether a product for hygienic handrub reduces the release of transient microbial flora on hands when rubbed onto the artificially contaminated hands of volunteers.

NOTE 1 Attention is drawn to the fact that tests on human volunteers are the subject of legal provisions in certain European countries/regions.

This European Standard applies to products for hygienic handrub for use in 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, of kindergartens 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 patient.

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

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

2 Normative references

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

EN 12353*, Chemical disinfectants and antiseptics — Preservation of test organisms used for the determination of bactericidal (including Legionella), mycobactericidal, sporicidal, fungicidal and virucidal (including bacteriophages) activity*

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

3 Terms and definitions

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

4 Requirements

When tested in accordance with Clause 5, the mean reduction of the release of the test organism *Escherichia coli K12* achieved by the hygienic handrub with the product under test shall be at least not inferior to that achieved by a specified reference hygienic handrub (60 % volume concentration of propan-2-ol).

5 Test method

5.1 Principle

Hands of volunteers are artificially contaminated with test organisms. The number of test organisms released from their fingertips into sampling fluids is assessed before and after the hygienic handrub. The ratio of the

two resulting values represents a measure for the antimicrobial activity of the product tested. The necessary precision is achieved by repeating the test on 18 to 22 volunteers. To compensate for extraneous influences it is compared with the reduction obtained by a reference handrub, which is performed with the same volunteers, on the same day and under comparable environmental conditions.

Prior to the test, a suitable neutralizer is validated. The neutralizer is used as a sampling fluid for recovering the test organisms after the hygienic handrub to ensure that the bactericidal and/or bacteriostatic activity in the sampling fluids is neutralized or suppressed.

5.2 Materials and reagents

5.2.1 Test organism

Escherichia coli K12 NCTC 10538; CIP 54.117; NCIMB 10083[1)]

NOTE This test organism has been specifically chosen to meet health and safety guidance and ethical committee considerations. It is a K12 strain of E. coli of normal flora origin internationally recognised as being non-pathogenic. According to the UK catalogue of the National Collections of Industrial & Marine Bacteria (see [2]), NCIMB strain 10083 is classified as a risk group 1 organism. The German Safety Ordinance on Gene Technology [3] also assigns the K12 strain to group 1. Directive 93/88/EEC [4] (Annex III to Directive 90/679/EEC [5]) explicitly states that non-pathogenic strains of Escherichia coli are excluded from the group 2 assignment.

5.2.2 Culture media and reagents

5.2.2.1 General

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

The reagents shall be of analytical grade and/or appropriate for microbiological purposes. They shall be free from substances that are toxic or inhibitory to the test organisms. 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. For each culture medium and reagent, a time limitation for use should be fixed.

5.2.2.2 Water

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

Sterilise in the autoclave [5.3.2.1 a)]. Sterilisation is not necessary if the water is used e.g. for preparation of culture media and subsequently sterilised.

NOTE See 5.2.2.7 for the procedure to prepare hard water.

5.2.2.3 Tryptone soya agar and tryptone soya selective agar

a) Tryptone Soya Agar (TSA)

Tryptone soya agar, consisting of:

[1)] The NCTC, CIP and NCIMB numbers are the collection numbers of this strain supplied by these cultures collections. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named.

Tryptone, pancreatic digest of casein	15,0 g
Soya peptone, papaic digest of soybean meal	5,0 g
Sodium chloride (NaCl)	5,0 g
Agar	15,0 g
Water (5.2.2.2)	to 1 000,0 ml

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

NOTE 1 TSA is used for preparing and counting N, N_v and N_{vB} (5.4.1.4, 5.4.1.5).

b) Tryptone Soya Selective Agar (TSSA)

Tryptone soya selective agar, consisting of:

Tryptone, pancreatic digest of casein	15,0 g
Soya peptone, papaic digest of soybean meal	5,0 g
Sodium chloride (NaCl)	5,0 g
Sodium-desoxycholate	0,5 g
Agar	15,0 g
Water (5.2.2.2)	to 1 000,0 ml

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

NOTE 2 TSSA is used for quantitative cultures of the sampling fluids and their dilutions (5.5.3.2, 5.5.3.3.4).

5.2.2.4 Tryptone Soya Broth (TSB)

Tryptone soya broth, consisting of:

Tryptone, pancreatic digest of casein	15,0 g
Soya peptone, papaic digest of soybean meal	5,0 g
Sodium chloride (NaCl)	5,0 g
Water (5.2.2.2)	to 1 000,0 ml

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

5.2.2.5 Neutralizer

The neutralizer shall be chosen, controlled and validated for the product under test in accordance with 5.5.1.2, 5.5.2.1 and 5.5.2.2. Only neutralizers using TSB (5.2.2.4) as diluent are allowed. It shall be sterile. The reference product is neutralized by dilution only.

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 Diluted soft soap

Linseed oil	50,0 parts by weight
Potassium hydroxide [1]	9,5 parts by weight
Ethanol (min. 95 %) [1]	7,0 parts by weight
Hot distilled water (75 ± 5) °C	as needed

Prepare a solution of 9,5 parts potassium hydroxide in 15 parts water (5.2.2.2) and add 50 parts linseed oil. Heat up to approximately 70 °C while constantly stirring. Add the ethanol and continue heating while stirring until the saponification process is completed and a sample dissolves clearly in water and almost clearly in alcohol. The weight of the soft soap is then brought up to 100 parts by addition of water (5.2.2.2), and heated up to (75 ± 5) °C to dilute the soft soap. Take 200 g of the soft soap, fill up to 1 000 g with water (5.2.2.2) and sterilise in the autoclave (5.5.2.1). The pH of the final diluted soft soap shall range between 10,0 and 11,0.

For quality control of the soft soap, see Annex D.

5.2.2.7 Hard water for dilution of products

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

— prepare solution A: dissolve 19,84 g magnesium chloride ($MgCl_2$) and 46,24 g calcium chloride ($CaCl_2$) in water (5.2.2.2) and dilute to 1 000 ml. Sterilise by membrane filtration (5.3.2.7) or in the autoclave [5.3.2.1 a)]. Autoclaving – if used - may cause a loss of liquid. In this case make up to 1000 ml with water (5.2.2.2) under aseptic conditions. Store the solution in the refrigerator (5.3.2.8) for no longer than one month;

— prepare solution B: dissolve 35,02 g sodium bicarbonate ($NaHCO_3$) in water (5.2.2.2) and dilute to 1000 ml. Sterilise by membrane filtration (5.3.2.7). Store the solution in the refrigerator (5.3.2.8) for no longer than one week;

— place 600 ml to 700 ml of water (5.2.2.2) in a 1 000 ml volumetric flask (5.3.2.12) and add 6,0 ml (5.3.2.9) of solution A, then 8,0 ml of solution B. Mix and dilute to 1000 ml with water (5.2.2.2). The pH of the hard water shall be 7,0 ± 0,2, when measured at 20 °C ± 1 °C (5.3.2.4). If necessary, adjust the pH by using a solution of approximately 40 g/l (about 1 mol/l) of sodium hydroxide (NaOH) or approximately 36,5 g/l (about 1 mol/l) of hydrochloric acid (HCl).

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

NOTE When preparing the product test solutions (5.4.2), the addition of the product to the hard water produces a different final water hardness in each test tube. In any case, the final hardness, expressed as calcium carbonate ($CaCO_3$) in the test tube, is lower than 375 mg/l.

5.2.2.8 Propan-2-ol as reference handrub [52,3 % (weight concentration) corresponding to 60 % (volume concentration) at 20 °C]

Fill 471 g propan-2-ol [1] with a purity of min. 99,5 % *V/V* (determined by gas chromatography; density 0,785) in a 1000 ml flask equipped with a glass stopper on the weighing platform of a scale (precision 0,1 g). Add 429 g water (5.2.2.2). This will give a volume of approximately 1 000 ml. Close the flask with the matching glass stopper and shake the contents of the flask thoroughly.

NOTE This solution can be kept indefinitely at approximately room temperature if protected from light.

5.3 Apparatus and glassware

5.3.1 General

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

a) by moist heat, in the autoclave [5.3.2.1 a)];

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

5.3.2 Usual microbiological laboratory equipment[2)] and, in particular, the following:

5.3.2.1 Apparatus for sterilisation

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

b) for dry heat sterilisation, a hot air oven capable of being maintained at ($180\,^{+5}_{0}$) °C for a minimum holding time of 30 min, at ($170\,^{+5}_{0}$) °C for a minimum holding time of 1 h or at ($160\,^{+5}_{0}$) °C for a minimum holding time of 2 h.

5.3.2.2 Water baths, capable of being controlled at 20 °C ± 1 °C and at 45 °C ± 1 °C (to maintain melted TSA and TSSA in case of pour plate technique)

5.3.2.3 Incubator, capable of being controlled either at 36 °C ± 1 °C or 37 °C ± 1 °C (5.2.1). The same temperature shall be used for incubations performed during a test and its control and validation**.**

5.3.2.4 pH-meter, having an inaccuracy of calibration of no more than ± 0,1 pH units at 20 °C ± 1 °C. A puncture electrode or a flat membrane electrode should be used for measuring the pH of the agar media (5.2.2.3).

5.3.2.5 Stopwatch

5.3.2.6 Shakers

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

b) mechanical shaker.

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

The apparatus shall have a filter holder of at least 50 ml volume. It shall be suitable for use with filters of diameter 47 mm to 50 mm and 0,45 µm pore size for sterilisation of hard water (5.2.2.7).

The vacuum source used shall give an even filtration flow rate. 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.

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

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

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

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

5.3.2.10 **Petri dishes, (plates)** 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**

5.3.2.13 **Spreader**, made of glass or other material

5.3.2.14 **Container** of sufficient capacity to immerse two hands vertically up to the mid-metacarpals simultaneously in 2 l of contamination fluid

5.3.2.15 **Two bottles of at least 1 l capacity**

5.4 Preparation of test organism suspensions and product test solutions

5.4.1 Test organism suspensions (test and validation suspension)

5.4.1.1 General

For the test organism, two different suspensions have to be prepared: the “test suspension”, i.e. contamination fluid to perform the test, and the “validation suspension” to perform the controls and method validation.

5.4.1.2 Preservation and stock cultures of test organisms

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

5.4.1.3 Working culture of test organisms

In order to prepare the working culture of the test organism (5.2.1), prepare a first subculture from the stock culture (5.4.1.2) by streaking onto TSA [5.2.2.3 a)] slopes or plates and incubate (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. The second subculture is used to prepare the test suspension.

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 (5.3.2.3) during the 48 h period.

5.4.1.4 Test suspension (“*N*”)/ contamination fluid

a) Take loopfuls of the cells from the working culture (5.4.1.3) in two tubes, each containing 5 ml of TSB (5.2.2.4), and incubate (5.3.2.3) for 18 h to 24 h. Inoculate these cultures into two bottles (5.3.2.15) with maximum 1 l TSB (5.2.2.4) each and incubate again (5.3.2.3) for 18 h to 24 h. Pool the resulting bacterial suspensions in a container (5.3.2.14).

b) Adjust the number of cells in the suspension to 1,5 x 10^8 cfu/ml[4)] to 5,0 x 10^8 cfu/ml using TSB (5.2.2.4), estimating the number of cfu by any suitable means. Maintain this test suspension in the water bath at 20 °C and use within 4 h as contamination fluid and to prepare the validation suspensions (5.4.1.5). The use of a spectrophotometer for adjusting the number of cells is highly recommended (about

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

620 nm wavelength - cuvette 10 mm path length). Each laboratory should therefore produce calibration data for each test organism knowing that suitable values of optical density are generally found between 0,150 and 0,460. To achieve reproducible results of this measurement, it may be necessary to dilute the test suspension, e.g. 1+9.

NOTE A colorimeter is a suitable alternative.

c) For counting, prepare 10-6 and 10-7 dilutions of the test suspension using TSB (5.2.2.4). Mix [5.3.2.6 a)]. Take a sample of 1,0 ml of each dilution in duplicate and inoculate using the pour plate or the spread plate technique.

   1) When using the pour plate technique, transfer each 1 ml sample into separate Petri dishes and add 15 ml to 20 ml melted TSA [5.2.2.3 a)], cooled to 45 °C ± 1 °C.

   2) When using the spread plate technique, spread each 1,0 ml sample – divided into portions of approximately equal size – on an appropriate number (at least two) of surface dried plates containing TSA [5.2.2.3 a)].

For incubation and counting, see 5.4.1.6.

5.4.1.5 Validation suspension ("*N*v","*N*vB")

a) To prepare the validation suspension (*N*v), dilute the test suspension (5.4.1.4) with TSB (5.2.2.4) to obtain 3,0 x 102 cfu/ml to 1,6 x 10^3 cfu/ml [about one-fourth (1+3) of the 10-5 dilution].

b) To prepare the validation suspension (*N*vB) for the neutralizer control (5.5.2.1), dilute the test suspension (5.4.1.4) with TSB (5.2.2.4) to obtain 3,0 x 104 cfu/ml to 1,6 x 105 cfu/ml [about one-fourth (1+3) of the 10-3 dilution] (*N*vB).

c) Maintain and use these validation suspensions (*N*v and *N*vB) the same way as the test suspension [5.4.1.4 b)].

d) For counting, prepare a 10-1 dilution with TSB (5.2.2.4), but prepare a 10-3 dilution of the validation suspension to count the neutralizer control [see b)].

   Mix [5.3.2.6 a)]. Take a sample of 1,0 ml in duplicate and inoculate using the pour plate or the spread plate technique [5.4.1.4 c)].

For incubation and counting, see 5.4.1.6.

5.4.1.6 Incubation and counting of the test and the validation suspensions

a) Incubate (5.3.2.3) the plates for 20 h to 24 h. Discard any plates that are not countable for any reason. Count the plates and determine the number of cfu. Incubate the plates for a further 20 h to 24 h. Do not recount plates that no longer show well-separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.

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

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

5.4.2 Product test solutions

The product as received shall be used as the product test solution if recommended by the manufacturer. Product test solutions of products recommended by the manufacturer to be diluted shall be prepared in hard water (5.2.2.7).

For solid products, dissolve the product as received by weighing at least 1,0 g ± 10 mg of the product in a volumetric flask and filling up with hard water (5.2.2.7). Subsequent dilutions (= lower concentrations) shall be prepared in volumetric flasks (5.3.2.12) on a volume/volume basis in hard water (5.2.2.7).

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

The product test solutions shall be prepared freshly and used in the test within 3 h. They shall give a physically homogenous preparation, stable during the whole procedure. If during the procedure a visible inhomogeneity appears due to the formation of a precipitate or flocculant, it shall be recorded in the test report.

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

Record the test concentration in terms of mass per volume or volume per volume and details of the product sample as received.

5.5 Procedure for assessing the bactericidal activity of the product on volunteers' hands

5.5.1 General

5.5.1.1 Experimental conditions

a) temperature:

The temperature for the control and validation of the neutralizer and the test suspension (contamination fluid) is 20 °C ± 1 °C.

b) contact time t (in s):

The contact time to be tested is to be chosen according to the manufacturer’s recommendation, but not shorter than 30 s and not longer than 60 s. Contact time is the total rubbing time. For the reference hygienic handrub [propan-2-ol (5.2.2.8)] it is 60 s.

The allowed deviation for each chosen contact time is ± 5 s.

NOTE Due to the standardised rub procedure (Annex A), a contact time shorter than 30 s is not feasible and cannot be verified.

c) test organism:

The test organism is *Escherichia coli* K12 (5.2.1).

5.5.1.2 Neutralization

The product under test has to be neutralized during the test. A suitable neutralizer (5.2.2.5) has to be found before the test procedure (5.5.3) is performed. For that purpose carry out the validation of the neutralization (5.5.2.1 and 5.5.2.2) in connection with 5.5.4 using a neutralizer, chosen according to laboratory experience and published data. For certain products, TSB (5.2.2.4) may act as a suitable neutralizer.

If this neutralizer is not valid, repeat the validation test using an alternative neutralizer taking into account the information given in Annex B.

The neutralizer control and the method validation shall be performed with the same neutralizer that will be used in the test procedure (5.5.3).

5.5.1.3 Equilibration of temperature

Prior to testing, equilibrate all reagents [product test solutions (5.4.2), propan-2-ol (5.2.2.8), diluted soft soap (5.2.2.6), test suspension (5.4.1.4), validation suspension (5.4.1.5), TSB (5.2.2.4), the neutralizer (5.2.2.5) and – if necessary - hard water (5.2.2.7)] to the test temperature of 20 °C using the water bath (5.3.2.2) controlled at 20 °C. Check that the temperature of the reagents is stabilised at 20 °C.

5.5.1.4 Selection of volunteers

The test shall be performed on 18 to 22 healthy persons who have hands with healthy skin, without cuts or abrasions, and with short and clean fingernails. Although, in general, age is not a limiting factor, volunteers should be at least 18 years of age. As it may happen that values of volunteers cannot be used for calculation, it is recommended to do the test rather with a higher number than 18 volunteers. On the day of test, the volunteers should not wear any jewellery or other items on the hands and wrists.

5.5.1.5 Experimental design

For testing a single product, a cross-over design is used. The volunteers are randomly divided into two groups of approximately the same size. Group 1 uses the reference hygienic handrub (RP, 5.5.3.3.2), group 2 the product under test (PP, 5.5.3.3.3).

The test is then repeated on the same day with group 1 using the handrub procedure with the test product and group 2 using the reference handrub procedure. Before every reference handrub procedure and every handrub procedure with the product under test, the procedures described in 5.5.3.1 and 5.5.3.2 shall be carried out.

For testing more than one product at a time, a Latin square design is used with as many groups of volunteers and as many experimental runs as there are products to be tested (including the reference propan-2-ol). In each run, all handrub procedures are employed in parallel. At the end of the whole series, every subject shall have used each product under test once, including the reference propan-2-ol. For each product under test, the hands have to be cleaned for 1 min between the testing of the different products with tap water and diluted soft soap (5.2.2.6) because residual neutralizer or product on the volunteers' hands may influence the performance of the subsequently tested product. Finally, the hands shall be thoroughly dried with a clean (paper) towel. After the reference hand rub a rinsing with tap water is enough.

NOTE 1 In a Latin square design, only products can be simultaneously tested for which the same neutralizer can be used.

NOTE 2 Due to the sample size (too small for Latin Square design) it may happen that a product is evaluated as not passing the standard (5.9) although it might pass in a cross over design.

5.5.2 Neutralization – control and validation[5)]

5.5.2.1 Neutralizer control "*B*" – verification of the absence of toxicity of the neutralizer

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

a) Pipette 9,0 ml of the neutralizer – used in the test (5.5.3) – into a tube. Add 1,0 ml of the validation suspension (N_{VB}) [5.4.1.5 b)] containing 3,0 x 10^4 cfu/ml to 1,6 x 10^5 cfu/ml. Start the stopwatch at the beginning of the addition, mix [5.3.2.6 a)]. Transfer 0,5 ml of this mixture into a tube containing 4,5 ml of neutralizer to obtain 10^{-1} dilution of N_{VB}; repeat this procedure to obtain 10^{-2} dilution of N_{VB}.

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

Place the tubes of the 10^{-2} dilution of N_{VB} in a water bath controlled at (20 ± 1) °C for the neutralization time of 10 s ± 1 s. Just before the end of this time, mix [5.3.2.6a)].

NOTE The high amount of neutralizer in relation to the test organisms reflects the additional dilutions with neutralizer.

b) At the end of this time, take a sample of 1,0 ml of this mixture "*B*" (10^{-2} dilution of N_{VB}) in duplicate and inoculate TSA plates [5.2.2.3 a)] using the pour plate or the spread plate technique [5.4.1.4 c)].

For incubation and counting, see 5.5.4.

5.5.2.2 Method validation "*C*"

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

a) Pipette 2,0 ml of TSB (5.2.2.4) into a tube. Starting a stopwatch, add 8,0 ml of the product test solution. Mix [5.3.2.6 a)] and place the tube in a water bath controlled at 20 °C for *t*. Just before the end of *t*, mix [5.3.2.6a)] again.

b) At the end of *t,* transfer 1,0 ml of the mixture into a tube containing 8,0 ml of neutralizer (used in 5.5.3). Restart the stopwatch at the beginning of the addition. Mix [5.3.2.6 a)] and place the tube in a water bath controlled at (20 ± 1) °C for 10 s ± 1 s (neutralization time). Add 1,0 ml of the validation suspension (5.4.1.5). Start a stopwatch at the beginning of the addition and mix [5.3.2.6 a)]. Place the tube in a water bath controlled at (20 ± 1) °C for 30 min ± 1 min. Just before the end of this time, mix [5.3.2.6 a)] again. At the end of this time, take a sample of 1,0 ml of the mixture "*C*" in duplicate and inoculate TSA plates [5.2.2.3 a)] using the pour plate or the spread plate technique [5.4.1.4 c)].

For incubation and counting, see 5.5.4.

5.5.3 Test procedure with volunteers

5.5.3.1 Application of the contamination fluid

Volunteers' hands are prepared by washing for 1 min with 5 ml diluted soft soap (5.2.2.6) without use of a brush. After being rinsed with running tap water, they are thoroughly dried with paper towels for at least 30 s. The contamination fluid [5.4.1.4 b)] is poured into a container (5.3.2.14) and both hands are immersed up to the mid-metacarpals for 5 s with fingers spread apart. Carefully allow surplus liquid to drain back into the container for a maximum of 30 s.

Allow the hands to dry in the air for 3 min, holding them in a horizontal position with the fingers spread out and rotating them to and fro to avoid the formation of droplets. During this procedure care should be taken to avoid contamination of the surrounding work area.

One batch of contamination fluid shall be used no longer than 3 h after the first volunteer's hands have been contaminated. Additionally, it shall be ensured that, in a test, all subjects' hands shall be treated with the same batch of contamination fluid, even if various products are tested against the reference handrub. The container with the contamination fluid should be used for all volunteers.

5.5.3.2 Sampling of the test organisms before treatment ("Prevalue")

Immediately after drying, rub the fingertips (including that of the thumb) for 1 min on the base of a Petri dish (5.3.2.10) containing 10 ml of TSB (5.2.2.4) as sampling fluid in order to assess the release of test organisms before treatment of the hands (prevalues).

A separate Petri dish is used for each hand.

Dilutions of 10^{-2}, 10^{-3} and 10^{-4} of these sampling fluids are prepared with the sampling fluid, i.e. TSB (5.2.2.4). From each dilution, 0,1 ml is spread on surface dried plates containing TSSA [5.2.2.3 b)] using spreaders

(5.3.2.13) to obtain 10^{-3}, 10^{-4} and 10^{-5} dilutions. The interval between sampling and plating shall not exceed 30 min. As an alternative technique to the spread plate technique the pour plate technique may be used by transferring each 0,1 ml sample into separate Petri dishes and adding 15 ml to 20 ml melted TSSA [5.2.2.3 b)], cooled to 45 °C ± 1 °C. See the procedure in 5.4.1.4 c) 1).

NOTE 1 The sampling fluid for the prevalues does not contain neutralizer (5.2.2.5) as this may influence the performance of the product under test. The different sampling procedures for pre- and postvalues will not influence the evaluation of the product since the reference handrub is treated the same way.

NOTE 2 Sodium-desoxycholate in TSSA is used to inhibit the growth of skin staphylococci. As TSSA may influence the growth in the contamination fluid (5.4.1.4) and the susceptibility of the test organisms against the products, it is not used for the preparation of the prevalue sampling fluid.

For incubation and counting, see 5.5.4.

5.5.3.3 Hygienic handrub procedure

5.5.3.3.1 General

After sampling for the prevalues (5.5.3.2) let the hands dry. Immediately after drying and without recontaminating the hands, perform the handrub in accordance with either 5.5.3.3.2 or 5.5.3.3.3, as applicable (5.5.1.5).

5.5.3.3.2 Reference hygienic handrub procedure (RP)

Pour 3 ml of propan-2-ol (5.2.2.8) into the cupped dry hands and rub vigorously for 30 s onto the skin up to the wrists in accordance with the standard handrub procedure shown in Annex A, to ensure total coverage of the hands.

As a first step, distribute the propan-2-ol (5.2.2.8) all over the hands including the wrists palm to palm; continue with five times right palm over left dorsum and left palm over right dorsum, then continue with five strokes backwards and forwards, palm to palm with fingers interlaced; continue with five times rubbing the backs of fingers to opposing palms with fingers interlocked, then five times rotational rubbing of right thumb clasped in left palm and left thumb clasped in right palm; then rub five times rotationally with clasped fingers of the right hand in the wet palm of the left hand and clasped fingers of the left hand in the wet palm of the right hand. Repeat the whole procedure with a further 3 ml propan-2-ol (5.2.2.8), to give a total rubbing time of 60 s.

For sampling, see 5.5.3.3.4.

5.5.3.3.3 Hygienic handrub procedure with product under test (PP)

This procedure shall be performed according to the recommendation provided by the manufacturer, which shall include volume of product, frequency of application and the contact time [between 30 s and 60 s, 5.5.1.1 b)]. In any case, the steps of the standard handrub procedure as described in Annex A shall be followed.

NOTE Due to the standardised rub procedure (Annex A) a contact time shorter than 30 s is not feasible and cannot be verified.

For sampling, see 5.5.3.3.4.

5.5.3.3.4 Sampling of the test organisms after treatment (“Postvalue”)

Immediately after treatment (5.5.3.3.2 and 5.5.3.3.3) a similar sampling procedure is used as described for the prevalues (5.5.3.2), but volumes of 1,0 ml and 0,1 ml of undiluted sampling fluid and 0,1 ml from its 10^{-1} dilution are plated out for quantitative culture on surface dried plates containing TSSA [5.2.2.3 b)] to obtain 10^{0}, 10^{-1} and 10^{-2} dilutions. The interval between sampling and plating shall not exceed 30 min. As an alternative technique to the spread plate technique the pour plate technique may be used by transferring each

0,1 ml sample into separate Petri dishes and adding 15 ml to 20 ml melted TSSA [5.2.2.3 b)], cooled to 45 °C ± 1 °C. See the procedure in 5.4.1.4 c) 1).

In contrast to the prevalue sampling (5.5.3.2), the neutralizer chosen according to 5.5.1.2 (and the results of 5.5.2.1 and 5.5.2.2) is used as the sampling fluids and as diluent for the 10^{-1} dilution (5.2.2.5). The regular time for rubbing on the base of the Petri dish is 1 min.

For incubation and counting, see 5.5.4.

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

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

a) Incubate (5.3.2.3) the plates for 20 h to 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of cfu. Incubate the plates for a further 20 h to 24 h. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.

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

c) Calculate the numbers of cfu/ml in the test mixtures of prevalue and postvalue (5.6.2.6) and in the validation mixtures *B* and *C* using the method given in 5.6.2.4 and 5.6.2.5. Verify according to 5.7.

5.6 Experimental data and calculation

5.6.1 Explanation of terms and abbreviations

5.6.1.1 Overview of the different suspensions and test mixtures

N, *N*v and Nv_B represent the bacterial suspensions, *B* (neutralizer control) and *C* (method validation) represent the different control test mixtures.

5.6.1.2 V_C-values

All experimental data are reported as V_C-values. A V_C-value is the number of cfu counted per 1,0 ml sample.

5.6.2 Calculation

5.6.2.1 General

The first step in the calculation is the determination of the V_C-values, the second the calculation of *N*, *N*v, $Nv_{0,}$, Nv_B, *B* and *C*. The third step is the calculation of the reduction *R* (5.6.2.6).

5.6.2.2 Determination of V_C-values

The V_C-values are determined as follows.

a) The usual limits for counting bacteria on agar plates are between 15 and 300. In this European Standard a deviation of 10 % is accepted, so the limits are 14 and 330.

NOTE The lower limit (14) is based on the fact that the variability increases the smaller the number counted in the sample (1 ml or 0,1 ml) is and therefore subsequent calculations may lead to wrong results. The lower limit refers only to the sample (and not necessarily to the counting on one plate), e.g. three plates per 1 ml sample with 3 cfu, 8 cfu and 5 cfu give a V_C -value of 16.

The upper limit (330) reflects the imprecision of counting confluent colonies and growth inhibition due to nutriment depletion. It refers only to the counting on one plate and not necessarily to the sample.

b) For all countings (5.4.1.6 and 5.5.4), determine and record the V_C-values according to the number of plates used per 1 ml sample (5.6.1.2). If more than one plate per 1 ml sample has been used to determine the V_C-value, the countings per plate should be noted.

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

If a V_C-value is lower than 14, report the number.

c) Only V_C-values within the counting limits are taken into account for further calculation, except in the case of prevalues and postvalues (5.6.2.6).

5.6.2.3 Calculation of *N*

N is the number of cells per ml in the test suspension / contamination fluid (5.4.1.4; 5.6.1.1).

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

$$N = \frac{c}{(n_1 + 0{,}1\, n_2)\ \mathrm{d}} \qquad (1)$$

where

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

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

n_2 is the number of V_C-values taken into account in the higher dilution, e.g. 10^{-7};

d is the dilution factor corresponding to the lower dilution, in this example 10^{-6}.

Round off the results calculated to two significant figures. For this, if the last figure is below 5, the preceding figure is not modified; if the last figure is more than 5, the preceding figure is increased by one unit; if the last figure is equal to 5, round off the preceding figure to the next nearest even figure. Proceed stepwise until two significant figures are obtained. As a result, the number of cfu/ml is expressed by a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

EXAMPLE $N = \frac{168 + 213 + 20 + 25}{(2 + 0{,}1 \times 2)10^{-6}} = \frac{426}{2{,}2 \times 10^{-6}} = 1{,}9363 \times 10^{8} = 1{,}9 \text{ x } 10^{8}$ (cfu/ml)

5.6.2.4 Calculation of *N*v, *N*vo and *N*v$_B$

*N*v is the number of cells per ml in the validation suspension [5.4.1.5 b)]. It is tenfold higher than the counts in terms of V_C-values due to the dilution step of 10^{-1} [5.4.1.5 b)].

*N*vo is the number of cells per ml in the mixtures *B* and *C* at the beginning of the contact time (time 0) (5.6.1.1). In the case of neutralizer control *B* (5.5.2.1) it is the number of cells per ml after 100 fold dilution. *N*vo is one-tenth of the mean of the V_C-values of *N*v [5.4.1.5 b)] taken into account; in the case of *N*v$_B$ it is one-thousandth.

Calculate *N*v, *N*vB and *N*vo using the following formulae:

$$Nv = 10\ c/n \quad (2)$$

$$N_{vB} = 1\ 000\ c/n \quad (3)$$

$$Nvo = c/n \quad (4)$$

where

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

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

5.6.2.5 Calculation of *B* and *C*

B and *C* are the numbers of survivors in the neutralizer control *B* (5.5.2.1) and method validation *C* (5.5.2.2) at the end of the neutralization time (in case of *B*) and 30 min (in case of *C*). They correspond to the mean of the V_C-values of the mixtures *B* and *C* taken into account.

Calculate *B* and *C* using the following formula:

$$B, C = c/n \quad (5)$$

where

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

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

5.6.2.6 Calculation of the lg reduction R (lg prevalue minus lg postvalue)

Record the number of cfu per plate for each dilution step of the test procedure with volunteers (prevalues and postvalues) and note if the volunteer belonged to group 1 (RP→PP) or group 2 (PP→RP) (5.5.1.5). Calculate the dilution factor by multiplying the sample dilution and the sample volume (ml). Calculate the number of cfu per ml of sampling fluid; multiply the plate count (cfu) by the dilution factor.

Whenever possible, the counts should be obtained from plates showing 14 to 330 colonies. With very efficient handrubs some counting plates for postvalues may show fewer than 14 colonies or no growth at all even if inoculated with 1 ml of undiluted sampling fluid (5.5.3.3.4). These values are used for calculation.

If suitable counts are obtained from two subsequent dilution steps, calculate the weighted arithmetic mean from these counts using the following formula:

$$Z = \frac{\sum C}{v_1 \times d_1 + v_2 \times d_2} \quad (6)$$

where

Z is the weighted mean cfu per ml sampling fluid of a prevalue or postvalue count;

ΣC is the sum of the cfus counted on plates retained for calculation;

v_1 is the volume of inoculum on the plate retained at the lower dilution in ml;

v_2 is the volume of inoculum on the plate retained at the higher dilution in ml;

d_1 is the dilution factor corresponding to the lower dilution of sampling fluid retained;

d_2 is the dilution factor corresponding to the higher dilution of sampling fluid retained.

EXAMPLE

$$Z = \frac{(299 + 31)\,\text{cfu}}{0{,}1\,\text{x}\,10^{\circ} + 0{,}1x10^{-1}} = \frac{330}{0{,}11} = 3000\,\text{cfu/ ml sampling fluid}$$

If colony counts of different dilution steps are grossly disproportional (e.g. countable results in each of three dilution steps), insufficient neutralization of the product should be taken into consideration. See also 5.7.2.

All viable counts per ml sampling fluid are transformed to decimal logarithms (lg). For computational reasons values of "0" (lg 0 = -∞) have to be set "1" (lg 1 = 0).

NOTE 1 Since 0-values should be found only among postvalues and should occur only with the most active products, this adjustment can, at worst, introduce a conservative bias of underestimating the antimicrobial efficiency of a product.

For both the reference and product test procedure, the lg counts from right and left hands of each subject shall be averaged separately for prevalues and postvalues.

NOTE 2 This double weighting increases the precision of the measurement.

From the difference between this individual combined lg prevalue and the lg postvalue, a lg reduction (lgR) is established for each volunteer.

Then, the arithmetic means of all individual lg reductions are calculated for both the reference and the product test procedure.

If the data conforms to 5.7.1 a) to e), compare the results of both procedures, PP and RP, with each other.

5.7 Verification of the methodology - Test validation

5.7.1 Acceptance criteria for test results

Only if the results of the test procedure fulfil the following requirements, shall they be accepted for further evaluation, otherwise the test shall be repeated.

a) A complete set of results from at least 18 volunteers shall be available. All complete sets of results shall be used for further evaluation.

b) The overall means of the lg prevalues for RP and PP shall be both at least 5,00.

c) Not more than three individual lg reductions less than 3,00 shall occur in RP.

d) The absolute difference of mean differences between lg reductions of RP and PP of group RP → PP and group PP → RP shall be less than 2,00.

e) The criteria of 5.7.2 and 5.7.3 shall be fulfilled.

5.7.2 Control of weighted mean counts

For results calculated by weighted mean of two subsequent dilutions (e.g. "*N*"), the quotient of the two means shall be not higher than 15 and not lower than 5. Results below the lower limit are taken as the lower limit number (14). Results above the respective upper limit [5.6.2.2 b)] are taken as the upper limit number.

EXAMPLE:

For *N*: 10^{-6} dilution: 168 + 215 cfu/ml, 10^{-7} dilution: 20 +< 14 cfu/ml;

(168 + 215) / (20 + 14) = 383/34 = 11, 26 = between 5 and 15.

5.7.3 Basic limits

Check that:

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

b) Nv is between $3,0 \times 10^2$ and $1,6 \times 10^3$
Nv_0 is between 30 and 160 ($3,0 \times 10^1$ and $1,6 \times 10^2$)
Nv_B is between $3,0 \times 10^4$ and $1,6 \times 10^5$

c) *B* is equal to or greater than 0,000 5 x Nv_B (half of one-thousandth)
C is equal to or greater than $0,5 \times Nv_0$

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

5.8 Statistical evaluation (significance testing), expression of results and precision

If the quality of the data has been found to be acceptable (5.7.1), they shall be used for the evaluation of the product(s) under test by applying the following pass criterion:

PP (procedure with product) shall not be inferior to RP (procedure with reference product propan-2-ol).

For testing the performance of PP against that of RP, a test for non-inferiority shall be applied to the lg Rs in each evaluation. The statistical method as described in Annex F shall be used. Other methods are acceptable if they have the same or superior power and their applicability can be demonstrated by suitable statistical methods, for example if there is no significant deviation from a normal distribution of the intraindividual differences of RP – PP parametric methods may be used.

For testing the data obtained in a Latin square design experiment, the test for non-inferiority shall be used, comparing the results of more than one test procedure with that of the reference in a pairwise manner. The statistical method E.4 b) should be used. Other methods are acceptable, if they have the same or superior power and their applicability can be demonstrated by suitable statistical methods, for example, in case of normal distribution, parametric tests for non-inferiority may be used.

The level of significance is set at p = 0,025. The test is to be used one-sided.

The margin of inferiority is 0,6 lg units.

5.9 Conclusion

A product which has fulfilled the requirements (Clause 4 and 5.8) is deemed suitable to be used as medical hygienic handrub.

5.10 Test report

The test report shall refer to this European Standard (EN 1500).

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

a) identification of the testing laboratory;

b) identification of the sample:

   1) name of the product;
   2) batch number and – if available – expiry date;
   3) manufacturer;
   4) date of delivery;
   5) storage conditions;
   6) product diluent recommended by the manufacturer for use;
   7) active substance(s) and its/their concentration(s) (optional);
   8) appearance of the product;

c) validation of the neutralizer:

   full details of the test for validation of the neutralizer (5.7.3) shall be given (including non-toxicity testing);

d) experimental conditions:

   1) date of test;
   2) diluent used for product test solution (hard water or water);
   3) product test concentrations;
   4) appearance product dilutions;
   5) contact time(s);
   6) temperature of incubation;
   7) neutralizer;
   8) identification of the test organisms used;

e) test results:

   1) a viable count of the contamination fluid;
   2) an exact description of how PP was performed (5.5.3.3.3): volume, contact time, frequency of application;
   3) lists of experimental results for RP and PP (Tables E.1 and E.2) containing the colony counts found on the plates in relation to the respective dilution of the sampling fluid together with labels indicating which of the colony counts have been used for further calculation;
   4) a list of the processed lg values, i.e. decimal logarithms (Table E.3) of left-right averaged and, when applicable, weighted viable counts per ml sampling fluid as derived from the marked/underlined colony counts. This list contains the individual lg prevalues and lg postvalues and the lg reduction for each test person separately for the RP and the PP, as well as the overall means and standard deviations and the chronological sequence of the handrub procedures [PP before RP (PP->RP) or vice versa (RP->PP)];

5) a list demonstrating the computation of Hodges-Lehmann upper one-sided 97,25 % confidence limits [Table E.4 a)] and a table showing the sorting and computation for Hodges-Lehmann upper confidence limits [Table E.4 b)];

f) special remarks;

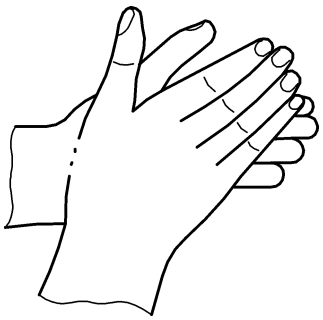
g) conclusion;

h) locality, date and identified signature.

Annex A
(normative)

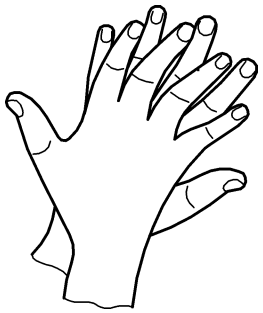
Standard handrub procedure

Follow steps 1 to 6 (apply 3 ml of the reference handrub to the cupped hands; for the product under test, apply the volume indicated by the manufacturer).



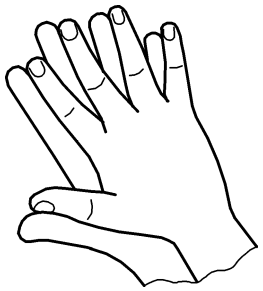
Step 1

Palm to palm



Step 2

Right palm over left dorsum and left palm over right dorsum (five times)

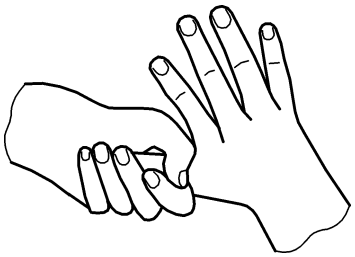


Step 3

Palm to palm with fingers interlaced (five times)

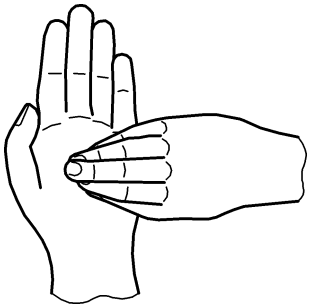
Step 4

Backs of fingers to opposing palms with fingers interlocked (five times)



Step 5

Rotational rubbing of right thumb clasped in left palm and vice versa (five times)



Step 6

Rotational rubbing, backwards and forwards with clasped fingers of right hand in left palm and vice versa (five times)

For the reference handrub, continue rubbing hands for a contact time of 30 s and repeat the whole procedure for another 30 s. For the product under test, follow the manufacturer's instructions regarding the contact time and eventual repeats!

Figure A.1 — Standard handrub procedure

Annex B
(informative)

Neutralizers and rinsing liquids

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

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

Antimicrobial agent	**Chemical compounds able to neutralize residual antimicrobial activity**	**Examples of suitable neutralizers and of rinsing liquids (for membrane filtration methods)** [a]
Quaternary ammonium compounds and fatty amines Amphoteric compounds	Lecithin, Saponin, Polysorbate 80, Sodium dodecyl sulphate, Ethylene oxide condensate of fatty alcohol (non-ionic surfactants) [b]	— Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. Polysorbate 80, 30 g/l + sodium dodecyl sulphate, 4 g/l + lecithin, 3 g/l. — Ethylene oxide condensate of fatty alcohol, 3 g/l + lecithin, 20 g/l + polysorbate 80, 5 g/l. — Rinsing liquid : tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.
Biguanides and similar compounds	Lecithin[c], Saponin, Polysorbate 80	— Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. *Rinsing liquid : tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*
Oxidizing compounds (Chlorine, iodine, hydrogen peroxide, peracetic acid, hypochlorites, etc…)	Sodium thiosulphate [d] Catalase [for hydrogen peroxide or products releasing hydrogen peroxide]	— Sodium thiosulphate, 3 g/l to 20 g/l + polysorbate 80, 30 g/l + lecithin, 3 g/l. — Polysorbate 80, 50 g/l + catalase 0,25 g/l + lecithin 10 g/l. *Rinsing liquid : sodium thiosulphate, 3 g/l.*
Aldehydes	L – histidine Glycine	— Polysorbate 80, 30 g/l + lecithin, 3 g/l + L-histidine, 1 g/l (or + glycine, 1 g/l). — Polysorbate 80, 30 g/l + saponin, 30 g/l + L-histidine, 1 g/l (or + glycine, 1 g/l). *Rinsing liquid : polysorbate 80, 5 g/l + L-histidine, 0,5 g/l (or + glycine, 1 g/l).*
Phenolic and related compounds: orthophenylphenol, phenoxyethanol, triclosan, phenylethanol, etc…. Anilides	Lecithin Polysorbate 80 Ethylene oxide condensate of fatty alcohol [b]	— Polysorbate 80, 30 g/l + lecithin, 3 g/l. — Ethylene oxide condensate of fatty alcohol, 7 g/l + lecithin, 20 g/l, + polysorbate 80, 4 g/l. *Rinsing liquid : tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*

"to be continued"

Antimicrobial agent	Chemical compounds able to neutralize residual antimicrobial activity	Examples of suitable neutralizers and of rinsing liquids (for membrane filtration methods) [a]
Alcohols	Lecithin, Saponin, Polysorbate 80 [e]	— Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. *Rinsing liquid : tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*

[a] According to the pH of the tested product, the pH of the neutralizer or the rinsing liquid may be adjusted at a suitable value or prepared in phosphate buffer [ex: phosphate buffer 0,25 mol/l: potassium dihydrogen phosphate (KH_2PO_4) 34 g; distilled water (500 ml); adjusted to pH 7,2 ± 0,2 with sodium hydroxide (NaOH) 1 mol/l; distilled water up to 1 000 ml].

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

[c] Egg and soya; egg is preferable.

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

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

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

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

Annex C
(informative)

Control and validation of neutralization

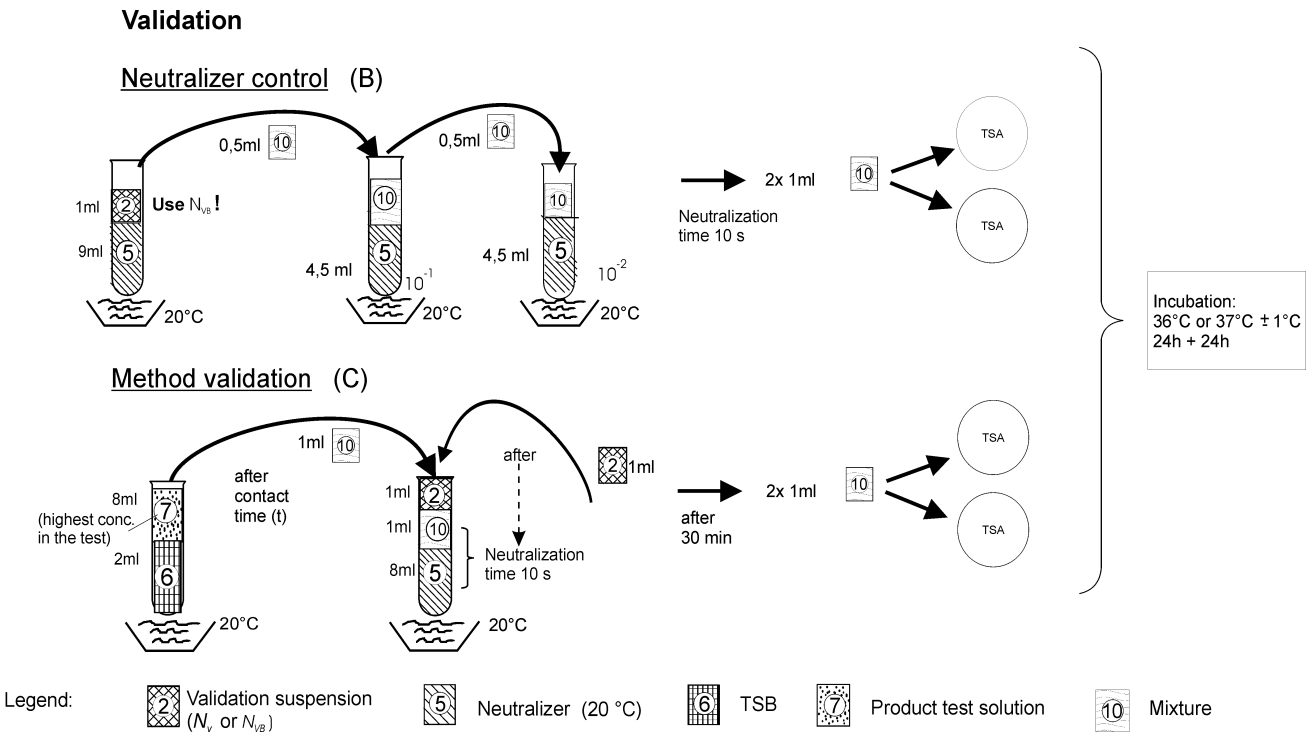


Figure C.1 — Control and validation of neutralizer

Annex D
(informative)

Quality control of soft soap[6)]

The following tests have to be performed with the **undiluted** soft soap (5.2.2.6).

Identity: If sulphuric acid (H_2SO_4) 10 % [1] is added to an undiluted soft soap solution the free fatty acids will separate out as a dense white precipitate which, when gently heated, melts into oily droplets collecting on the surface of the liquid.

Purity: 1 g of soft soap shall dissolve in 2,0 ml of warm water (5.2.2.2) into a clear liquid.

Alcohol-insoluble substances: Dissolve 2,5 g of soft soap in 10 ml of ethanol 96 % [1] while gently heating. Filter the warm solution through a filtering crucible that had been dried to constant weight, and carefully rewash with ethanol 96 % [1]. The weight of the undissolved residue accumulated in the crucible shall not exceed 5 mg after having dried.

Free alkali, free acid: A solution of 2,5 g of soft soap in 10 ml of ethanol 96 % [1] for neutralizing (phenolphtalein solution [1] shall not consume more than 0,1 ml of hydrochloric acid [1] or 0,1 ml of sodium hydroxide solution [1].

Loss on drying: Maximum 45,0 %. For determination, first grind the soft soap with an equal quantity of washed and glowed seashore sand and then dry conforming to specification.

Determination of content: Dissolve 2,5 g of soft soap in 50 ml of hot water (5.2.2.2) in an Erlenmeyer flask; mix the solution with 5 ml of sulphuric acid 10 % [1] and heat gently until the fatty acids have separated out as an oily film on top of the aqueous liquid. After cooling, add 10 ml of petroleum ether [1] and swirl carefully until the fatty acids have dissolved.

Then put the entire liquid into a 250 ml separating funnel, re-rinse twice, each time with 10 ml of petroleum ether [1] and shake vigorously. After separating the layers, allow the aqueous phase to run off, wash the petroleum ether solution with 25 ml of water (5.2.2.2) and again allow the aqueous liquid to run off as complete as possible.

Then shake well with anhydrous sodium sulphate [1]. Filter through wadding in a tarred flask holding 200 ml, rewash twice, each time with 5 ml of petroleum ether [1] and distil the solvent off on the water bath. Allow the residue to dry at a temperature not exceeding 75 °C.

The residue shall weigh 1,125 g to 1,25 g, corresponding to a content of 45,0 % to 50,0 % of fatty acids.

---

6) Not "diluted soft soap".

Annex E
(informative)

Examples of reporting of results and significance testing

Table E.1 — Reference hygienic handrub – experimental results

Product: "RP" (propan-2-ol 60 % V/V) **Handrub procedure**: rub-in 3 ml/30 s, repeat once
Test organism: E. coli K 12 NCTC 10538 **Number in contamination fluid (N):** 2,1 x 10^8 cfu/ml
Date of experiment: 17 February 2008

Volunteer		Number of cfu per plate from dilution 10^x					
No.	Hand	Prevalues			Postvalues		
	left or right	10^{-3}	10^{-4}	10^{-5}	10^0	10^{-1}	10^{-2}
1	l	>330	121*	15*	8	1	0
	r	>330	135*	15*	9	0	0
2	l	267*	27*	3	60	7	0
	r	300*	53*	3	113	11	1
3	l	>330	59	5	>330	43	7
	r	>330	48	7	>330	42	2
4	l	211*	24*	3	19	3	0
	r	>330	56	1	6	2	0
5	l	>330	83	11	8	2	0
	r	>330	119	10	30	3	1
6	l	>330	71	8	2	0	0
	r	>330	96	10	12	3	0
7	l	>330	41	7	2	0	0
	r	>330	47	5	53	6	2
8	l	224*	25*	1	43	4	0
	r	300*	34*	5	167*	21*	4
		>330	225*	30*	41	1	0
9	l	>330	>330	52	49	5	1
	r	300*	34*	5	0	0	0

Volunteer		Number of cfu per plate from dilution 10^x					
No.	Hand	Prevalues			Postvalues		
	left or right	10^{-3}	10^{-4}	10^{-5}	10^{0}	10^{-1}	10^{-2}
10	l	>330	45	7	21	1	0
	r	59	8	0	6	0	0
11	l	130	12	1	39	4	0
	r	>330	91	12	>330	54	6
12	l	>330	187*	22*	169*	20*	1
	r	>330	46	5	4	0	0
13	l	>330	47	4	0	0	0
	r	>330	86*	18*	224*	23*	2
14	l	>330	131	11	>330	48	1
	r	>330	89	8	>330	213*	24*
15	l	>330	118	11	>330	56	7
	r	>330	72	7	30	6	0
16	l	>330	91*	15*	32	4	1
	r	>330	55	3	66	3	0
17	l	>330	82	2	22	1	0
	r	171*	18*	2	9	1	0
18	l	156*	25*	2	43	4	0
	r	>330	118*	14*	3	0	0
19	l	>330	60	6	9	1	0
	r	>330	103*	15*	251*	30*	1
20	l	>330	69	8	39	7	2
	r	>330	121*	15*	8	1	0

underlined = count used for further computation ; * indicate adjacent dilutions used for computation
> 330 = not countable

Table E.2 — Hygienic handrub procedure with the product under test – experimental results

Product: "PP"
Test organism: E. coli K 12 NCTC 10538
Date of experiment: 17 February 2008

Hand rub procedure: rub-in 3 ml/30 s,
Number in contamination fluid (N): 2,1 x 10^8 cfu/ml

Volunteer		Number of cfu per plate from dilution 10^x					
No.	Hand	Prevalues			Postvalues		
	left or right	10^{-3}	10^{-4}	10^{-5}	10^0	10^{-1}	10^{-2}
1	l	>330	90	9	1	0	0
	r	>330	69	6	2	0	0
2	l	>330	52	3	0	0	0
	r	155*	16*	0	3	0	0
3	l	246*	25*	3	9	1	0
	r	186*	18*	2	35	3	0
4	l	>330	110	11	7	1	0
	r	>330	87	12	5	1	0
5	l	>330	105	8	20	3	0
	r	>330	148	13	10	0	0
6	l	>330	126*	19*	4	0	0
	r	>330	131*	17*	45	5	1
7	l	184	11	1	4	0	0
	r	164*	27*	4	69	7	0
8	l	300*	33*	4	>330	45	5
	r	>330	49	7	32	3	1
9	l	>330	145*	18*	11	2	0
	r	>330	176*	21*	20	1	0
		>330	72	3	8	1	0
10	l	224*	25*	2	9	1	0
	r	14	2	0	1	0	0
11	l	47	6	0	19	2	0
	r	>330	47	2	65	3	1
12	l	>330	105	10	81	9	0
	r	>330	45	5	7	1	0

Volunteer		Number of cfu per plate from dilution 10^x					
No.	Hand	Prevalues			Postvalues		
	left or right	10^{-3}	10^{-4}	10^{-5}	10^{0}	10^{-1}	10^{-2}
13	l	>330	72	11	3	0	0
	r	137	12	2	>330	63	5
14	l	161*	25*	2	>330	127*	19*
	r	>330	67	12	>330	64	3
15	l	>330	126*	16*	154	13	3
	r	>330	51	8	13	1	0
16	l	>330	78	9	22	2	0
	r	>330	113	12	159*	16*	1
17	l	>330	133	11	13	0	0
	r	>330	107	12	178*	21*	1
18	l	>330	106	5	>330	94	8
	r	>330	126	11	1	1	0
19	l	>330	77	12	3	0	0
	r	>330	>330	37	>330	173	12
20	l	>330	320*	32*	75	6	1
	r	>330	90	9	1	0	0

underlined = count used for further computation ; * indicate adjacent dilutions used for computation
> 330 = not countable

Table E.3 — List of computed lg values (means of left and right hands) and lg reductions

Volun-teers	Chrono-logical Sequence (5.5.1.5, 5.6.2.6)	Reference handrub (RP) (Propan-2-ol 60 % V/V)			Handrub with product under test (PP)		
		lg prevalues	lg postvalues	lg R	lg prevalues	lg postvalues	lg R
1	RP->PP	7,11	1,93	5,18	6,90	1,15	5,75
2	PP->RP	6,47	2,92	3,55	6,45	1,24	5,22
3	RP->PP	6,73	3,63	3,10	6,33	2,25	4,08
4	PP->RP	6,54	2,03	4,51	6,99	1,77	5,22
5	PP->RP	7,00	2,19	4,81	7,10	2,15	4,95
6	PP->RP	6,92	1,69	5,23	7,12	2,13	5,00
7	RP->PP	6,64	2,01	4,63	6,25	2,22	4,03
8	RP->PP	6,42	2,93	3,49	6,59	3,08	3,51
9	PP->RP	7,54	2,65	4,89	7,21	2,17	5,04
10	RP->PP	6,57	1,66	4,91	6,61	1,93	4,68
11	PP->RP	5,94	2,18	3,76	5,41	1,64	3,77
12	PP->RP	7,12	3,48	3,64	6,85	2,86	3,99
13	PP->RP	6,67	1,30	5,37	6,76	1,66	5,09
14	PP->RP	7,05	3,52	3,53	6,18	3,96	2,22
15	PP->RP	7,01	4,04	2,97	6,97	3,50	3,47
16	RP->PP	6,92	2,49	4,43	6,80	2,23	4,57
17	RP->PP	6,83	2,58	4,25	7,09	2,66	4,43
18	RP->PP	6,23	2,29	3,93	7,03	3,62	3,41
19	RP->PP	6,93	1,72	5,21	6,99	1,24	5,75
20	RP->PP	6,93	3,00	3,94	7,54	3,56	3,98
X	Overall	6,78	2,51	4,27	6,76	2,35	4,41
s		0,36	0,75	0,75	0,47	0,84	0,89
NN		20	20	20	20	20	20
X	RP->PP	6,73	2,42	4,31	6,81	2,39	4,42
s		0,27	0,63	0,70	0,38	0,85	0,81
NN		10	10	10	10	10	10
X	PP->RP	6,83	2,60	4,23	6,70	2,31	4,40
s		0,44	0,88	0,83	0,56	0,87	1,00
NN		10	10	10	10	10	10

lg R = decimal log reduction
RP->PP Sequence: first RP, second PP
PP->RP Sequence: first PP, second RP
X = Mean
s = Standard deviation
NN = Number of values (=volunteers)

Difference of mean Rs (RP->PP): 4,31 - 4,42 = -0,11 Difference of mean Rs (PP->RP): 4,23 - 4,40 = -0,17
Absolute difference of differences: Abs [(-0,11) - (-0,17)] = 0,06

Check of acceptance criteria according to 5.7.1 a) to e)

— Complete set of results from 20 volunteers available (hence, more than the minimum of 18).

— Mean of lg prevalues for RP = 6,78 and for PP = 6,76 (hence both greater than 5,00).

— Individual lg reductions less than 3,00: with RP = 1, with PP = 1 [hence each less than 3,00 reduction (R) values].

— For group with sequence RP->PP difference of lg R: 4,31 - 4,42 = -0,11; for group with sequence PP->RP difference of lg R: 4,23-4,40 = -0,17; absolute difference of mean differences: abs [-0,11 - (-0,17)] = 0,06 (hence = less than 2,00).

— All quotients of weighted mean counts between 5 and 15 (results which were used for weighted mean counts in Tables E.1 and E.2 and in the validation of neutralizer) (5.7.2). N, N_{V0}, N_{VB}, B and C see "validation of neutralizer": Neutralizer validated (5.7.3).

All acceptance criteria are fulfilled.

Table E.4 a) — Computation of individual differences of lg Rs of RP – PP

Volun-teer	lg reduction (R)		Difference RP-PP
	Reference procedure(RP)	Product procedure (PP)	
1	5,18	5,75	-0,56
2	3,55	5,22	-1,66
3	3,10	4,08	-0,98
4	4,51	5,22	-0,71
5	4,81	4,95	-0,14
6	5,23	5,00	0,23
7	4,63	4,03	0,60
8	3,49	3,51	-0,02
9	4,89	5,04	-0,15
10	4,91	4,68	0,23
11	3,76	3,77	-0,01
12	3,64	3,99	-0,35
13	5,37	5,09	0,27
14	3,53	2,22	1,31
15	2,97	3,47	-0,50
16	4,43	4,57	-0,14
17	4,25	4,43	-0,18
18	3,93	3,41	0,52
19	5,21	5,75	-0,54
20	3,94	3,98	-0,04

Table E.4b) — Sorting of individual differences and computation for Hodges-Lehmann 97,5 % upper confidence limits

	Sorted	Mean pairwise differences $(d_i+d_{ii})/2$										
	differences	1,31	0,60	0,52	0,27	0,23	0,23	-0,01	-0,02	-0,04	-0,14	-0,14
1	1,31	1,31[1]										
2	0,60	0,95[2]	0,60[10]									
3	0,52	0,91[3]	0,56[15]	0,52[16]								
4	0,27	0,79[4]	0,44[18]	0,40[22]	0,27[31]							
5	0,23	0,77[5]	0,41[19]	0,37[24]	0,25[33]	0,23[38]						
6	0,23	0,77[6]	0,41[20]	0,37[25]	0,25[34]	0,23[39]	0,23[40]					
7	-0,01	0,65[7]	0,29[28]	0,25[32]	0,13[49]	**0,11[53]**	0,11	-0,01				
8	-0,02	0,64[8]	0,29[29]	0,25[35]	0,13[50]	0,10	0,10	-0,02	-0,02			
9	-0,04	0,63[9]	0,28[30]	0,24[36]	0,11[52]	0,09	0,09	-0,03	-0,03	-0,04		
10	**-0,14**	0,59[11]	0,23[37]	0,19[44]	0,07	0,05	0,05	-0,07	-0,08	-0,09	-0,14	
11	**-0,14**	0,58[12]	0,23[41]	0,19[45]	0,07	0,04	0,04	-0,08	-0,08	-0,09	-0,14	
12	-0,15	0,58[13]	0,22[42]	0,18[46]	0,06	0,04	0,04	-0,08	-0,09	-0,10		
13	-0,18	0,56[14]	0,21[43]	0,17[47]	0,04	0,02	0,02	-0,10	-0,10	-0,11		
14	-0,35	0,48[17]	0,12[51]	0,08	-0,04	-0,06	-0,06					
15	-0,50	0,40[21]	0,05	0,01	-0,11	-0,14	-0,14					
16	-0,54	0,38[23]	0,03	-0,01	-0,13							
17	-0,56	0,37[26]	0,02	-0,02								
18	-0,71	0,30[27]	-0,05	-0,09								
19	-0,98	0,16[48]										
20	-1,66											

The differences of the individual lg Rs of RP – PP from Table E.4a) are sorted in the second column and in the headline according to their size in descending order.

The median is between the 10th and 11th value: [- 0,14 + (- 0,14)]/2 = - 0,14. The small exponents represent the ranks.

The mean pairwise differences that do not exceed the median (here: -0,14) are computed. From Table E.5 of critical values for Wilcoxon's matched-pairs signed-ranks test the entry for n=20 and a one-sided 0,025 level of significance, the critical value of 52 is found. **Hence c=52+1=53**. The pairwise differences are sorted in descending order. **The 53rd value is 0,11**. Hence the Hodges-Lehmann upper one-sided 97,5 % confidence limit for the difference in lg Rs between RP and PP is 0,11, which is less than the agreed inferiority margin of 0,6. Therefore, the hypothesis of inferiority of PP is rejected and it can be concluded that the test preparation PP is non-inferior to RP.

Table E.5 — WILCOXON'S matched-pairs signed-ranks test:

Np (number of pairs)	**One-sided level of significance (directional test)**		
	0,05	**0,025**	**0,01**
18	47	**40**	32
19	53	**46**	37
20	60	**52**	43
21	68	**59**	49
22	75	**66**	56

Critical values of the lower of both sums of ranks with (+) or (-) sign at different significance levels used for calculation of the Hodges-Lehmann confidence limits. In this case, zero differences are included.

Annex F
(normative)

Test for non-inferiority [7)]

The zero hypothesis (H_0) for the test of non-inferiority is given by:

$$\mathbf{H_o: \mu_R - \mu_P \geq \delta}$$

where

δ is an agreed inferiority margin (see below);

μ_R is the expected lg R for the reference procedure RP;

μ_P is the respective value for the product procedure PP.

This is the hypothesis of inferiority. It states that the expected difference of lg Rs is greater than or equal to a predefined inferiority margin, hence the average of that RP is greater than that of PP by at least δ lg units (= PP is inferior to RP).

The alternative hypothesis (H_1) is:

$$\mathbf{H_1: \mu_R - \mu_P < \delta}$$

This is the hypothesis of non-inferiority. It states that RP hand rub is not superior by an amount exceeding the inferiority margin δ.

H_0 is tested by computing the one-sided Hodges-Lehmann confidence interval for the differences of lg Rs for RP – PP.

Unless a computer program is used, the procedure is as follows:

1) The inferiority margin is specified to be 0,6 lg.

2) The level of significance shall be p = 0,025; one-sided.

3) Compute the individual differences of lg Rs of RP – PP as shown in Table E.4 a).

4) Sort the differences in descending order as shown in Table E.4 b).

5) Consult Table E.5 to obtain the critical value for the number np of pairs (including zero differences) and add 1 (“one”) to that value. Denote this value “c”.

6) Compute the c most extreme means of pairs of differences by the following algorithm as shown in Table E.4 b):

   — Let d_i and d_{ii} be any two differences: their mean is $(d_i+d_{ii})/2$;

7) See [6].

- Start with the highest value d_1 and compute $(d_1+d_1)/2(=d_1)$. $(d_1+d_2)/2$. $(d_1+d_3)/2$ a.s.o. until this mean is lower than the median of all lg R differences of RP-PP. Then take the next highest difference d_2 and compute $(d_2+d_2)/2$ $(=d_2)$. $(d_2+d_3)/2$ and so on and proceed until the median of differences is reached;

- Sort the computed means in descending order;

- The value at position c is the upper one-sided confidence limit.

7) Compare the obtained upper confidence limit of differences with the inferiority margin. If the upper confidence limit is greater or equal to the inferiority margin then H_0 of inferiority cannot be rejected. Otherwise H_0 is rejected and the product under test is assumed to be non-inferior.

Bibliography

[1] European Pharmacopeia – edition 2002 (monographies): water for injection; 2-propanol (reagents): potassium hydroxide; ethanol 96 %; sulphuric acid 10 %; phenolphthalein; hydrochloric acid; sodium hydroxide solution; petroleum ether; anhydrous sodium sulphate; polysorbate 80

[2] The National Collections of Industrial & Marine Bacteria Ltd Catalogue of Strains (1994); ISBN No. 0 9510269 3 3

[3] Gentechnik-Sicherheitsverordnung (GenTSV) vom 14. März 1995. Anh. II A in Kombination mit § 6. Abs. 4. Nr. 4

[4] Council Directive 93/88/EEC of 12 October 1993 amending Council Directive 90/679/EEC on the protection of workers from risks related to exposure to biological agents at work. OJEC No. L268/71 of 29.10.1993

[5] Council Directive 90/679/EEC of 26 November 1990 on the protection of workers from risks related to exposure to biological agents at work. OJEC No. L374/1 of 31.12.1990

[6] Lehmann E.L. Nonparametrics: Statistical Methods Based on Ranks. San Francisco: Holden-Day. 1975; StatXact™ or SAS™ (with macro)

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