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Chemiczne środki dezynfekcyjne i antyseptyczne
Chirurgiczna dezynfekcja rąk
Metoda badania i wymagania (faza 2/etap 2)

Norma Europejska EN 12791:2016+A1:2017 *Chemical disinfectants and antiseptics — Surgical hand disinfection — Test method and requirements (phase 2, step 2)* ma status Polskiej Normy

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NORME EUROPÉENNE
EUROPÄISCHE NORM

EN 12791:2016+A1

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English Version

**Chemical disinfectants and antiseptics - Surgical hand
disinfection - Test method and requirements (phase 2,
step 2)**

Antiseptiques et désinfectants chimiques - Désinfection
chirurgicale des mains - Méthodes d'essai et
prescriptions (phase 2/étape 2)

Chemische Desinfektionsmittel und Antiseptika -
Chirurgische Händedesinfektionsmittel - Prüfverfahren
und Anforderungen (Phase 2, Stufe 2)

This European Standard was approved by CEN on 13 December 2015 and includes Amendment 1 approved by CEN on 20 July 2017.

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Contents	Page
European foreword.....	3
1 Scope	4
2 Normative references	4
3 Terms and definitions	4
4 Requirements	4
5 Test methods	4
5.1 Principle	4
5.2 Materials and reagents.....	5
5.2.1 Test organism.....	5
5.2.2 Culture media and reagents	5
5.3 Apparatus and glassware	7
5.3.1 General.....	7
5.3.2 Usual microbiological laboratory equipment.....	7
5.4 Product test solutions.....	8
5.5 Procedure for assessing the microbicidal activity of the product on volunteers' hands	9
5.5.1 General.....	9
5.5.2 Preparatory handwash	10
5.5.3 Test procedure with volunteers	10
5.5.4 Incubation and counting of the test mixture.....	12
5.6 Experimental data and calculation.....	12
5.6.1 Determination of V_c -values.....	12
5.6.2 Calculation of the individual lg reduction (lg R; lg prevalue minus lg postvalue).....	13
5.7 Verification of the methodology	14
5.7.1 Acceptance criteria for test results.....	14
5.7.2 Control of weighted mean counts.....	14
5.8 Statistical evaluation (significance testing), expression of results and precision	14
5.9 Conclusion.....	15
5.10 Test report.....	15
Annex A (informative) Standard surgical handrub/-wash procedure	17
Annex B (informative) Quality control of soft soap	18
Annex C (informative) Examples of reporting of results and significance testing	19
Annex D (informative) WILCOXON'S matched-pairs signed-ranks test	30
Bibliography.....	31

European foreword

This document (EN 12791:2016+A1:2017) 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 May 2018, and conflicting national standards shall be withdrawn at the latest by May 2018.

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 includes Amendment 1 approved by CEN on 2017-07-20.

This document supersedes A1 EN 12791:2016 A1.

The start and finish of text introduced or altered by amendment is indicated in the text by tags A1 A1.

A1 *deleted text* A1

Data obtained using the former version of EN 12791 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 should 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 should be run.

According to the CEN/CENELEC Internal Regulations, the national standards organizations 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, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

EN 12791:2016+A1:2017 (E)

1 Scope

This European Standard specifies a test method simulating practical conditions for establishing whether a product for surgical handrub and handwash reduces the release of resident and eventually present transient microbial flora on hands when used for the treatment of clean hands of volunteers.

This European Standard applies to products for surgical handrub or handwash 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 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 13624, *Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of fungicidal or yeasticidal activity in the medical area — Test method and requirements (phase 2, step 1)*

EN 13727:2012+A2:2015, *Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of bactericidal activity in the medical area — Test method and requirements (phase 2, step 1)*

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

The mean reduction for immediate effect and 3 h effect of a product shall - when tested in accordance with Clause 5 - at least be not inferior to that achieved by a specified reference product (60 % volume concentration of propan-1-ol).

To demonstrate additionally a “sustained effect”, the mean reduction for the 3 h effect of a product shall be superior to that achieved by the reference product.

5 Test methods

5.1 Principle

A specified preparatory handwash (pre-wash) is carried-out on volunteers in order to remove most of the transient flora and foreign material, which could otherwise influence the “prevalues”, i.e. the number of microorganisms on the hands before treatment. The following samples from the hands are taken for bacterial counts:

- immediately after the pre-wash (before treatment with a product or the reference handrub);
- immediately after the surgical handrub or –wash procedure;
- 3 h after the surgical handrub or –wash procedure.

The ratio of the resulting values before and after treatment (reduction in numbers) represents a measure for the antimicrobial activity of the product under test. The immediate effect is characterized by the reduction achieved immediately after the procedure with the product. The 3 h effect is characterized by the reduction achieved three hours after the procedure with a product. To compensate for extraneous influences, these reductions from surgical handrub or surgical handwash procedures are compared individually with the corresponding reductions of a reference surgical handrub performed in parallel on the same volunteers.

Prior to the test a suitable neutralizer is validated. The neutralizer is used as sampling fluids for recovering the test organisms (5.2.1) after the surgical handrub or handwash procedure 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

The test is performed on the resident microbial flora of volunteers' hands and not on specified test organisms.

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.

All specified pH values are measured at $(20 \pm 1) ^\circ \text{C}$.

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 [1]) may be used.

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

NOTE See 5.2.2.7 for the procedure to prepare hard water.

5.2.2.3 Tryptone Soya Agar (TSA)

Tryptone soya agar, consisting of:

Tryptone, pancreatic digest of casein	15,0 g
Soya peptone, papaic digest of soybean meal	5,0 g

EN 12791:2016+A1:2017 (E)

Sodium chloride (NaCl)	5,0 g
Agar	15,0 g
Water (5.2.2.2)	to 1000,0 ml

Sterilize in the autoclave [5.3.2.1a)]. After sterilization the pH of the medium shall be equivalent to $7,2 \pm 0,2$.

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 1000,0 ml

Sterilize in the autoclave [5.3.2.1a)]. After sterilization the pH of the medium shall be equivalent to $7,0 \pm 0,2$.

5.2.2.5 Neutralizer

The neutralizer shall be chosen, controlled and validated for the product under test in accordance with EN 13727 and EN 13624 (only yeasticidal activity). See 5.5.1.2 for more details.

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 ($75\text{ °C} \pm 5\text{ °C}$)	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), heated up to $75\text{ °C} \pm 5\text{ °C}$ to dilute the soft soap. Take 200 g of the soft soap, fill up to 1000 g with water (5.2.2.2) and sterilize in the autoclave [5.3.2.1a)]. 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 B.

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 1000 ml. Sterilize by membrane filtration (5.3.2.7) or in the autoclave [5.3.2.1a)]. 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. Sterilize 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 1000 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 $7,0 \pm 0,2$ (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), 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), is in the test tube lower than 375 mg/l.

5.2.2.8 Propan-1-ol as reference product [48,3 % w/w (weight concentration) corresponding to 60 % v/v (volume concentration) at 20 °C]

Fill 483 g propan-1-ol with a purity of min 99,5 % V/V (determined by gas chromatography; density 0,804) in a 1000 ml graduated flask equipped with a glass stopper on the weighing platform of a scale (precision 0,1 g). Add 420 g water (5.2.2.2). This will give a volume of approximately 1000 ml. Close the flask with the matching glass stopper and shake the contents of the flask thoroughly.

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

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) 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 equipment¹⁾

and, in particular, the following:

5.3.2.1 Apparatus for sterilization (moist and dry heat):

- a) For moist heat sterilization, an autoclave capable of being maintained at (121_0^{+3}) °C for a minimum holding time of 15 min;
- b) for dry heat sterilization, a hot air oven capable of being maintained at (180_0^{+5}) °C for a minimum holding time of 30 min, at (170_0^{+5}) °C for a minimum holding time of 1 h or at (160_0^{+5}) °C for a minimum holding time of 2 h.

5.3.2.2 Water baths, capable of being controlled at $20\text{ °C} \pm 1\text{ °C}$, and at additional test temperatures $\pm 1\text{ °C}$.

5.3.2.3 Incubator, capable of being controlled either at $36\text{ °C} \pm 1\text{ °C}$ or $37\text{ °C} \pm 1\text{ °C}$. The same temperature shall be used for incubations performed during a test and its control and validation.

¹⁾ Disposable sterile equipment is an acceptable alternative to reusable glassware.

EN 12791:2016+A1:2017 (E)

5.3.2.4 pH-meter, having an inaccuracy of calibration of no more than $\pm 0,1$ pH units at $(20 \pm 0,1)$ °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 Electromechanical agitator, e.g. Vortex® mixer²⁾

5.3.2.7 Membrane filtration apparatus, constructed of a material compatible with the substances to be filtered, with a filter holder of at least 50 ml volume, and suitable for use of filters of diameter 47 mm to 50 mm and 0,22 µm pore size for sterilization 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.

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 Surgical gloves, unpowdered glove for use in invasive surgery, sterile and free of antimicrobial activity.

If the manufacturer does not certify the freedom of any antimicrobial activity the following agar diffusion test may be used:

Prepare a suspension of 10^8 per ml of *Bacillus subtilis* spores (commercially available e.g. as strain ATCC 6633). This suspension can be stored at 4 °C for months.

Mueller-Hinton agar commercially available as powered medium is prepared and autoclaved according to the manufacturer's instructions and – while still liquid – temperature-equilibrated at 50 °C in a water bath. A quantity of the spore suspension equalling 1 % of the medium's volume is added, carefully mixed with the agar, and poured into Petri dishes (5.3.2.10) (20 ml per plate). After the agar has solidified these plates can be stored at 4 °C for 2 to 4 weeks.

A round test piece of 6 mm to 8 mm diameter is punched out with the cork borer (diameter 6 mm to 8 mm) from the surgical glove under test and placed onto the surface of a seeded Mueller-Hinton-plate. After incubation (5.3.2.3) for 48 h the plate is inspected for the presence of any inhibition zone around the test piece.

5.3.2.15 Towels, fresh and clean, e.g. made of paper or sterile cotton.

5.4 Product test solutions

The product as received shall be used as 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).

²⁾ 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.

For solid products, dissolve the product as received by weighing at least $1,0 \text{ g} \pm 10 \text{ mg}$ 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 flocculate, it shall be recorded in the test report.

Counting microorganisms embedded in a precipitate or flocculate 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 microbicidal activity of the product on volunteers' hands

5.5.1 General

5.5.1.1 Experimental conditions

Contact time t (in min):

The contact time to be tested is to be chosen according to the manufacturer's recommendation, but not shorter than 60 s and not longer than 5 min. The contact time for the reference surgical handrub [propan-1-ol (5.2.2.8)] is 3 min.

The allowed deviation for each chosen contact time is $\pm 5 \text{ s}$.

NOTE The minimum contact time of 60 s takes into account the time needed to treat the hands the prescribed way, see 5.5.3.2.2.

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 according to EN 13727 and EN 13624 (only yeasticidal activity). Pay special attention to the following points of these norms: 5.2.2.5 ("Neutralizer"), 5.5.1.2 ("Choice of test method"), 5.5.1.3 ("General instructions for validation and control procedures"), 5.5.2.3 ("Control A..."), 5.5.2.4 ("Neutralizer control B"), 5.5.2.5 ("Method validation 'C' ...") in connection with 5.5.2.6 ("Incubation and counting ..."). If the membrane filtration method is used follow 5.5.3, 5.5.3.1, 5.5.3.3, 5.5.3.4, 5.5.3.5 in connection with 5.5.3.6). Calculation and verification shall be performed according to 5.6.2.4, 5.6.2.6 and 5.7.

The selected neutralizer shall work on all test-organisms and with the relevant interfering substances to be tested according to EN 13727 and EN 13624 (only yeasticidal activity) for surgical handrub and surgical handwash (EN 13727:2012+A2:2015, Clause 4 and EN 13624).

5.5.1.3 Equilibration of temperature

Prior to testing, equilibrate all reagents [product test solutions (5.4), propan-1-ol (5.2.2.8), diluted soft soap (5.2.2.6), 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 stabilized at 20°C .

5.5.1.4 Selection of volunteers

The test shall be performed on 23 to 28 healthy persons who have hands with healthy skin, without cuts or abrasions, and with short and clean fingernails. Starting from three days prior to test, they should not

EN 12791:2016+A1:2017 (E)

use substances with antimicrobial activity, e.g. medicated soaps or hand creams. During a period of 10 days prior to the test they should not take antibiotics and products with similar efficacy. Furthermore they should not wear any jewellery or other items on the hands including the wrists on the actual test day.

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 (for example volunteers drop out after the first test round, 5.5.1.5), it is recommended to do the test rather with more than 23 volunteers.

5.5.1.5 Experimental design

For testing a single product a crossover design is used. The volunteers are randomly divided into two groups of approximately the same size in a first run. Group 1 uses the reference surgical handrub ("RP", 5.5.3.2.2), group 2 the product under test ("PP", 5.5.3.2.3 or 5.5.3.2.4). After at least one week, allowing reconstitution of the normal skin flora, the test is repeated with changed roles in a second run.

Half of the volunteers are randomly chosen that their right hand is used for the "immediate postvalue" and the left hand for the "3-hour postvalue". The other half of the volunteers is treated the other way round.

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 (including the reference propan-1-ol). Only products can be simultaneously tested for which either no neutralization is necessary or for which the same neutralizer can be used for the assessment of post-values. In each run all disinfection procedures are employed in parallel. At least one week is required between the individual experimental runs, allowing reconstitution of the normal skin flora. At the end of the whole series every volunteer shall have used each product, including propan-1-ol, once.

5.5.2 Preparatory handwash

The volunteers' hands are prepared by washing for 1 min with 5 ml diluted soft soap (5.2.2.6). After having been rinsed with running tap water, they are thoroughly dried with towels (5.3.2.15).

5.5.3 Test procedure with volunteers**5.5.3.1 Sampling of the resident skin flora before treatment ("Prevalue")**

Immediately after drying (5.5.2), rub all 10 fingertips for 1 min on the base of two Petri dishes (5.3.2.10) – one for each hand – each containing 10 ml of TSB (5.2.2.4) as sampling fluids in order to assess the release of the skin microorganisms before treatment of the hands (prevalues).

Dilutions of 10^{-1} and 10^{-2} of these sampling fluids are prepared with the sampling fluid, i.e. TSB (5.2.2.4). For each dilution, 0,1 ml is spread on surface dried plates containing TSA (5.2.2.3) using spreaders (5.3.2.13). 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 TSA (5.2.2.3), cooled to $45\text{ °C} \pm 1\text{ °C}$.

The sampling fluid for the prevalues should 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 product is treated the same way.

For incubation and counting, see 5.5.4.

5.5.3.2 Surgical handrub / handwash procedure

5.5.3.2.1 General

After sampling for the prevalues (5.5.3.1) rub the finger pads against each other and let the hands dry. Immediately after drying, perform the handrub or handwash in accordance with either 5.5.3.2.2, 5.5.3.2.3 or 5.5.3.2.4, as applicable (5.5.1.5).

5.5.3.2.2 Reference hand disinfection procedure (RP)

Pour 3 ml of propan-1-ol (5.2.2.8) into the cupped dry hands and rub vigorously 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-1-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, at last 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. When almost dry, additional aliquots of 3 ml of propan-1-ol (5.2.2.8) are applied as it evaporates. Keep the hands wet for the contact time of 3 min. Sample immediately afterwards the fingertips of the appropriate hand described in 5.5.3.2.5.

For sampling see 5.5.3.2.5.

5.5.3.2.3 Surgical 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 60 s and 5 min, 5.5.1.1) In any case, the steps of the standard handrub procedure as described in Annex A shall be followed. Sample immediately afterwards the fingertips of the appropriate hand described in 5.5.3.2.5.

The total volume used per volunteer shall be recorded.

For sampling see 5.5.3.2.5.

5.5.3.2.4 Surgical handWASH procedure with product under test (PP)

This procedure shall be performed according to the recommendation provided by the manufacturer, which shall include need for prewetting the hands, volume of product, frequency of application of the product, additional application of tap water to produce a lather (the minimum amount of tap water is 1 ml), need for and duration of a final rinse and the contact time (between 60 s and 5 min, 5.5.1.1). In any case, the steps of the standard handrub procedure as described in Annex A shall be followed. The procedure is completed by a 10 s rinse of the fingers under running tap water – even if the manufacturer recommends a shorter, longer or no final rinse. Excess water is shaken off and the hands are dried with a towel (5.3.2.15). The total rinsing and drying time is 15 s ± 1 s. Sample immediately afterwards the fingertips of the appropriate hand described in 5.5.3.2.5.

However, if the manufacturer's instructions require wearing of gloves on hands still covered by the surgical handwash product, only the hand allotted for assessment of the immediate effect ("immediate postvalue" - see 5.5.1.5) is rinsed and dried.

The total volume used per volunteer shall be recorded.

For sampling see 5.5.3.2.5.

5.5.3.2.5 Sampling of the resident flora after treatment ("Postvalue")

Sampling for the immediate effect ("immediate postvalue")

EN 12791:2016+A1:2017 (E)

Immediately after treatment (5.5.3.2.2, 5.5.3.2.3 and 5.5.3.2.4) the same sampling procedure is used as described for the prevalues (5.5.3.1) only on the one randomly allotted hand (5.5.1.5) except that the chosen neutralizer (5.2.2.5; 5.5.1.2) is used instead of TSB as sampling fluid and as diluent for the dilutions. 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 TSA (5.2.2.3). The 1 ml sample may be spread on more than one plate. The regular duration of rubbing on the base of the Petri dish is 1 min. 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 to 20 ml melted TSA (5.2.2.3), cooled to $45\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$.

Sampling for the 3 h effect ("3-hours postvalue")

Immediately after drying the other hand is protected from extraneous contamination by donning a surgical glove (5.3.2.14) to be removed after 3 h. Then the same sampling procedure is applied to this hand as described for the immediate postvalue.

For incubation and counting see 5.5.4.

5.5.4 Incubation and counting of the test mixture

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

- 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 by determining the number of colony forming units (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.
- 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 5.6.1.
- Calculate the numbers of cfu/ml in the sampling fluids from the prevalue and postvalue assessments using the method given in 5.6.2. Verify according to 5.7.

5.6 Experimental data and calculation

Examples for the recording, calculation, verification and statistical evaluation are given in Annex C.

5.6.1 Determination of 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.

The V_C -values are determined as follows.

- 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. They refer only to the counting on one plate and not necessarily to the sample.

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

5.6.2 Calculation of the individual lg reduction (lg R; lg prevalue minus lg postvalue)

Record the number of cfus per plate for each dilution step of the test procedure with volunteers (prevalues and postvalues) and note if the volunteer belonged to the group "Reference Procedure tested before Procedure with Product = RP→PP" or to the other group ("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 by multiplying the plate count (cfu) by the dilution factor.

Record the results per volunteer for the immediate postvalue separate from those for the 3-h postvalue.

Whenever possible, the counts should be obtained from plates showing 14 to 330 colonies [5.6.1a)]. 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 (5.5.3.2.4). 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:

$$Z = \frac{\sum C}{v_1 \times d_1 + v_2 \times d_2} \quad (1)$$

Where

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

$\sum C$ 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

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

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 = $-\infty$) have to be set "1" (lg 1 = 0).

NOTE 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 efficacy of a product.

From the difference between the lg prevalue and the lg postvalue assessed per hand (one hand: immediate, the other hand: 3 h effect), a lg reduction (lg R) is established for each volunteer's hand.

EN 12791:2016+A1:2017 (E)

Then, the arithmetic means and standard deviations of all individual lg reductions (lg Rs) are calculated for both the reference procedure (RP) and the product test procedure (PP).

5.7 Verification of the methodology**5.7.1 Acceptance criteria for test results**

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

- a) A complete set of results from at least 23, but maximum 28 volunteers shall be available. All complete sets of results shall be used for further evaluation, but it is allowed to remove up to three volunteers from further evaluation if only this manipulation enables meeting the requirements of 5.7.1 b) (see below) and if still at least 23 volunteers will be evaluated. It has to be documented that the manipulation is the smallest to achieve the goal.
- b) The overall means of the lg prevalues for RP and PP shall be both at least 3,5.
- c) The absolute difference of mean differences between lg reductions of RP and PP of group RP→PP (= RP tested before PP) and group PP→RP (= PP tested before RP) shall be less than 2,0 – for both immediate and 3 h effect.
- d) The criteria of 5.7.2 shall be fulfilled.

5.7.2 Control of weighted mean counts

For results calculated by the weighted mean of two subsequent dilutions, the quotient shall not be higher than 15 and not lower than 5. Colony counts below the lower limit are taken as the lower limit number (= 14). Colony counts above the respective upper limit [5.6.1b)] are taken as the upper limit number (=330).

EXAMPLE:

10⁻¹ dilution: 161 cfu/ml;

10⁻² dilution: 28 cfu/ml;

(161) / (28) = 5,75, i.e. between 5 and 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 under test by applying the following pass criterion:

- a) Neither the immediate effect nor the 3 h effect of the product (PP) shall be inferior to the respective effects of the reference product, propan-1-ol (RP).
- b) For testing the performance of PP against that of RP, a non-parametric test for non-inferiority such as that of Hodges and Lehmann [2] shall be applied to the lg reductions in each evaluation. The statistical method described in Annex C should be used.

NOTE Computer programs exist for this method, for instance: StatXact™ or SAS™ (with macro).

Other methods are acceptable if they have the same or superior power and their applicability can be demonstrated by suitable statistical methods (e.g. given there is no significant deviation from a normal distribution of the differences of RP – PP parametric methods may be used).

- c) The level of significance is set at $P = 0,025$ (one-sided) for the statistical evaluation of the immediate effect and the 3h effect of PP.

- d) For the non-inferiority tests a safety margin of 0,75 lg (for the 3h effect 0,85 lg) is agreed upon.
- e) For claiming an additional “sustained effect” the following pass criterion shall be applied:

The mean lg reduction obtained with PP for the 3 h effect shall be significantly larger than that with RP. For the statistical evaluation of a sustained effect of PP the Wilcoxon matched-pairs signed-ranks test is used at $P = 0,01$. A one-sided test is to be used (Annex D).

5.9 Conclusion

A product which has fulfilled the requirements (4 and 5.8) is deemed suitable to be used as surgical handrub or handwash.

If – in addition - a product fulfils the requirements for the sustained effect (4 and 5.8) this effect may be claimed.

5.10 Test report

The test report shall refer to this standard (EN 12791).

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 and controls:
 - 1) validation of the neutralizer: full details of the test for validation of the neutralizer shall be given (including non-toxicity testing);
 - 2) quality control of the soap (in accordance with Annex B) the results of the checks for identity, purity and content of the soft soap shall be given;
 - 3) quality control of the surgical gloves (see 5.3.2.14) the results of the check for freedom of any antimicrobial activity shall be given;
- d) Experimental conditions:
 - 1) dates of test;
 - 2) diluent used for product test solution (hard water or water);

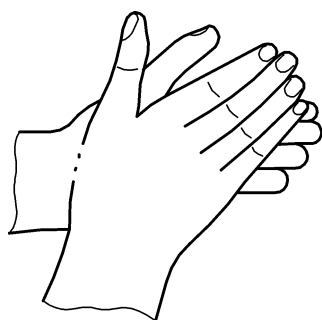
EN 12791:2016+A1:2017 (E)

- 3) product test concentrations;
- 4) appearance product dilutions;
- 5) contact time(s);
- 6) temperature of incubation;
- 7) neutralizer;
- e) Test results:
 - 1) an exact description of how PP was performed (5.5.3.2.3 or 5.5.3.2.4): volume (e.g. 2x5 ml or 3x3 ml); contact time, other instructions for use (e.g. "Keep hands wet for ... min"); if PP is a surgical handwash, prewetting of hands or not; frequency of application of PP and – if applicable – of the addition of tap water as well as if the handwash product was left on the hand under the surgical glove;
 - 2) lists of experimental results for RP and PP (Tables C.1 and C.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 and the chronological sequence of the handrub procedures [PP before RP (PP- > RP) or vice versa (RP- > PP)];
 - 3) a list of the processed lg values, i.e. decimal logarithms (Table C.3) 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;
 - 4) unless the computation of Hodges-Lehmann was performed by computer a list demonstrating upper one-sided 97,25 % confidence limits and a table showing the sorting and computation for Hodges-Lehmann upper confidence limits (Annex C);
 - 5) in case a claim for sustained effect is made also the results of the statistical evaluation according to Wilcoxon matched-pairs signed ranks test at $P = 0,01$ shall be provided.
- f) Special remarks;
- g) Conclusion;
- h) Locality, date and identified signature.

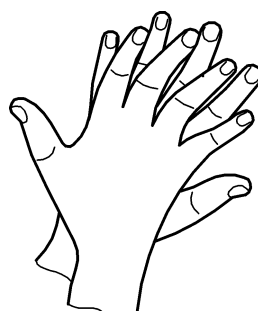
Annex A (informative)

Standard surgical handrub/-wash procedure

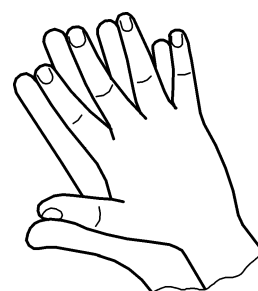
Follow steps 1 to 6. For the reference handrub apply 3 ml to the cupped hands and rub in. For the product under test follow the manufacturer's instructions regarding prewetting (handwash), volume of product, frequency of application and volume and time of addition of lukewarm tap water (handwash). Perform each step five times.



Step 1
Palm to palm



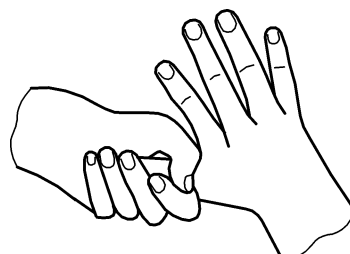
Step 2
Right palm over left dorsum and
left palm over right dorsum (five
times)



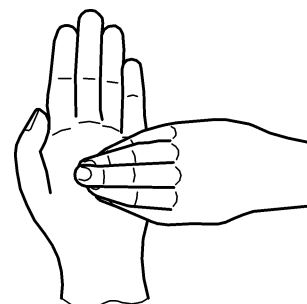
Step 3
Palm to palm with fingers
interlaced (five times)



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 until they are almost dry and repeat the whole procedure as often as necessary to keep the hands wet until 3 min contact time have elapsed. For the product under test follow the manufacturer's instructions regarding the contact time and eventual repeats.

Figure A.1 — Standard handrub procedure

EN 12791:2016+A1:2017 (E)

Annex B (informative)

Quality control of soft soap³⁾

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 neutralising (phenolphthalein solution [1] shall not consume more than 0,1 ml of 1 mol/l 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 glow-dried 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 % - 50,0 % of fatty acids.

³⁾ (not "Diluted soft soap")

Annex C (informative)

Examples of reporting of results and significance testing



Table C.1 — Reference surgical hand disinfection procedure – Experimental results

Preparation: "RP" (propan-1-ol 60 % v/v)

Dates of experiment: 15 July 2011 / 22 July 2011

Application: rubbing hands during 3 min

Volunteer		Number of cfu per plate from dilution 10 ^x									
		Hand	Prevalues			Immediate postvalues			3 h postvalues		
No	Sequence	left or right	-1	-2	-3	0	-1	-2	0	-1	-2
1	RP- > PP	l	> 330	> 330	<u>188</u>	> 330	<u>39</u>	1			
		r	> 330	> 330	<u>226</u>				> 330	<u>184</u>	11
2	RP- > PP	l	> 330	> 330	<u>71</u>				> 330	<u>184</u>	11
		r	> 330	> 330	<u>43</u>	<u>47</u>	4	1			
3	PP- > RP	l	> 330	> 330	<u>121</u>				> 330	<u>215*</u>	<u>25*</u>
		r	> 330	> 330	<u>147</u>	<u>105*</u>	<u>17*</u>	3			
4	RP- > PP	l	> 330	> 330	<u>48</u>				> 330	> 330	<u>232</u>
		r	> 330	> 330	<u>45</u>	> 330	<u>124</u>	9			
5	PP- > RP	l	> 330	> 330	<u>56</u>	> 330	<u>36</u>	8			
		r	> 330	> 330	<u>38</u>				> 330	> 330	<u>43</u>
6	PP- > RP	l	> 330	<u>67</u>	4				> 330	> 330	<u>58</u>
		r	> 330	<u>46</u>	2	<u>20**</u>	<u>2**</u>	<u>0**</u>			
7	RP- > PP	l	> 330	<u>175</u>	12				<u>214*</u>	<u>19*</u>	3
		r	> 330	<u>310*</u>	<u>31*</u>	<u>143*</u>	<u>14*</u>	0			
8	PP- > RP	l	> 330	> 330	<u>73</u>	> 330	<u>134*</u>	<u>17*</u>			
		r	> 330	<u>131*</u>	<u>16*</u>				> 330	<u>84</u>	5
9	RP- > PP	l	> 330	> 330	<u>81</u>				> 330	<u>188*</u>	<u>18*</u>
		r	> 330	> 330	<u>54</u>	> 330	<u>151*</u>	<u>16*</u>			
10	RP- > PP	l	> 330	<u>181*</u>	<u>18*</u>	<u>193*</u>	<u>22*</u>	2			
		r	> 330	<u>243*</u>	<u>26*</u>				<u>96</u>	8	1
11	RP- > PP	l	> 330	<u>272*</u>	<u>34*</u>				> 330	<u>42</u>	3
		r	> 330	<u>231*</u>	<u>33*</u>	<u>42</u>	4	0			

EN 12791:2016+A1:2017 (E)

Volunteer		Number of cfu per plate from dilution 10 ^x									
		Hand	Prevalues			Immediate postvalues			3 h postvalues		
No	Sequence	left or right	-1	-2	-3	0	-1	-2	0	-1	-2
12	PP- > RP	l	> 330	> 330	<u>86</u>	<u>102</u>	12	0			
		r	> 330	> 330	<u>181</u>				> 330	> 330	<u>53</u>
13	PP- > RP	l	> 330	> 330	<u>106</u>				> 330	> 330	<u>37</u>
		r	> 330	<u>201*</u>	<u>17*</u>	<u>68</u>	9	0			
14	PP- > RP	l	> 330	<u>161*</u>	<u>28*</u>	<u>122</u>	12	1			
		r	> 330	<u>208*</u>	<u>23*</u>				> 330	> 330	<u>76</u>
15	RP- > PP	l	> 330	<u>141*</u>	<u>16*</u>	> 330	> 330	<u>102</u>			
		r	> 330	<u>122</u>	12				> 330	> 330	<u>87</u>
16	PP- > RP	l	<u>320*</u>	<u>32*</u>	5				<u>44</u>	4	0
		r	> 330	<u>35</u>	1	<u>0</u>	0	0			
17	PP- > RP	l	> 330	> 330	<u>148</u>	<u>6</u>	0	0			
		r	> 330	> 330	<u>71</u>				> 330	<u>69</u>	9
18	RP- > PP	l	> 330	> 330	<u>145</u>				> 330	<u>243*</u>	<u>32*</u>
		r	> 330	> 330	<u>126</u>	> 330	<u>80</u>	12			
19	PP- > RP	l	> 330	<u>195*</u>	<u>25*</u>	> 330	<u>52</u>	7			
		r	> 330	<u>116</u>	12				<u>242*</u>	<u>26*</u>	1
20	PP- > RP	l	> 330	> 330	<u>71</u>				> 330	<u>92</u>	6
		r	> 330	> 330	<u>37</u>	<u>28</u>	3	0			
21	RP- > PP	l	> 330	> 330	<u>61</u>	> 330	<u>62</u>	8			
		r	> 330	> 330	<u>153</u>				<u>32</u>	3	0
22	RP- > PP	l	> 330	> 330	<u>131</u>	<u>32</u>	7	0			
		r	> 330	> 330	<u>320</u>				> 330	<u>93</u>	5
23	PP- > RP	l	> 330	> 330	<u>97</u>				<u>192*</u>	<u>26*</u>	1
		r	> 330	> 330	<u>31</u>	<u>119</u>	13	2			
24	RP- > PP	l	> 330	> 330	<u>278</u>	> 330	> 330	<u>156</u>			
		r	> 330	> 330	<u>136</u>				> 330	<u>61</u>	3
Underlined = count used for further computation											
* indicates adjacent dilutions used for computation											
**values cannot be used for further computation (infringement of control of weighted mean counts 5.7.2 for PP, s. table C2)											

Table C.2 — Surgical hand rub procedure with test product – Experimental results

Preparation: "PP"

Dates of experiment: 15 July 2011 / 22 July 2011

Application: rubbing hands with 3 × 3 ml during 2 min

Volunteer		Number of cfu per plate from dilution 10 ^x									
		Hand	Prevalues			Immediate postvalues			3 h postvalues		
No	Sequence	left or right	-1	-2	-3	0	-1	-2	0	-1	-2
1	RP -> PP	l	> 330	> 330	<u>186</u>	> 330	<u>133*</u>	<u>16*</u>			
		r	> 330	> 330	<u>68</u>				> 330	> 330	<u>153</u>
2	RP -> PP	l	<u>80</u>	8	0				> 330	<u>251*</u>	<u>40*</u>
		r	<u>156*</u>	<u>23*</u>	4	<u>201*</u>	<u>27*</u>	0			
3	PP -> RP	l	> 330	> 330	<u>95</u>				> 330	> 330	<u>329</u>
		r	> 330	> 330	<u>73</u>	> 330	<u>69</u>	9			
4	RP -> PP	l	> 330	> 330	<u>121</u>				> 330	> 330	<u>320</u>
		r	> 330	> 330	<u>171</u>	> 330	> 330	<u>81</u>			
5	PP -> RP	l	> 330	> 330	<u>66</u>	> 330	<u>106</u>	12			
		r	> 330	> 330	<u>71</u>				> 330	> 330	<u>143</u>
6	PP -> RP	l	> 330	<u>36</u>	1				> 330	> 330	<u>47</u>
		r	<u>171</u>	12	1	<u>109**</u>	<u>34**</u>	1			
7	RP -> PP	l	> 330	<u>176*</u>	<u>27*</u>				> 330	> 330	<u>124</u>
		r	> 330	> 330	<u>85</u>	> 330	<u>54</u>	5			
8	PP -> RP	l	> 330	> 330	<u>131</u>	> 330	<u>143*</u>	<u>20*</u>			
		r	> 330	> 330	<u>176</u>				> 330	<u>249*</u>	<u>21*</u>
9	RP -> PP	l	> 330	> 330	<u>54</u>				> 330	> 330	<u>74</u>
		r	> 330	> 330	<u>53</u>	> 330	<u>103</u>	8			
10	RP -> PP	l	> 330	> 330	<u>57</u>	> 330	<u>104</u>	7			
		r	> 330	<u>146</u>	12				> 330	<u>128*</u>	<u>15*</u>
11	RP -> PP	l	> 330	<u>191</u>	12				> 330	<u>143*</u>	<u>15*</u>
		r	> 330	<u>118*</u>	<u>14*</u>	> 330	<u>77</u>	13			
12	PP -> RP	l	> 330	> 330	<u>115</u>	> 330	<u>96*</u>	<u>14*</u>			
		r	> 330	> 330	<u>110</u>				> 330	> 330	<u>245</u>
13	PP -> RP	l	> 330	> 330	<u>146</u>				> 330	> 330	<u>101</u>
		r	> 330	> 330	<u>93</u>	> 330	> 330	<u>34</u>			
14	PP -> RP	l	> 330	<u>242*</u>	<u>23*</u>	> 330	<u>103</u>	12			
		r	> 330	<u>310*</u>	<u>31*</u>				> 330	> 330	<u>144</u>
15	RP -> PP	l	> 330	<u>59</u>	7	> 330	> 330	<u>155</u>			
		r	> 330	<u>59</u>	5				> 330	> 330	<u>320</u>

EN 12791:2016+A1:2017 (E)

Volunteer		Number of cfu per plate from dilution 10 ^x									
		Hand	Prevalues			Immediate postvalues			3 h postvalues		
No	Sequence	left or right	-1	-2	-3	0	-1	-2	0	-1	-2
16	PP - > RP	l	> 330	> 330	<u>54</u>				> 330	<u>206*</u>	<u>29*</u>
		r	> 330	<u>41</u>	4	<u>78</u>	12	1			
17	RP - > PP	l	> 330	> 330	<u>43</u>	<u>125</u>	13	1			
		r	> 330	> 330	<u>81</u>				> 330	<u>114*</u>	<u>14*</u>
18	RP - > PP	l	> 330	> 330	<u>126</u>				> 330	> 330	<u>268</u>
		r	> 330	> 330	<u>114</u>	> 330	<u>191*</u>	<u>18*</u>			
19	PP - > RP	l	> 330	> 330	<u>42</u>	> 330	> 330	<u>35</u>			
		r	> 330	> 330	<u>56</u>				> 330	> 330	<u>61</u>
20	PP - > RP	l	> 330	<u>149*</u>	<u>18*</u>				> 330	> 330	<u>216</u>
		r	> 330	<u>174*</u>	<u>21*</u>	> 330	> 330	<u>81</u>			
21	RP - > PP	l	> 330	<u>121*</u>	<u>14*</u>	<u>237*</u>	<u>21*</u>	3			
		r	> 330	> 330	<u>69</u>				> 330	<u>133*</u>	<u>17*</u>
22	RP - > PP	l	> 330	> 330	<u>143</u>	<u>193*</u>	<u>29*</u>	2			
		r	> 330	> 330	<u>298</u>				> 330	> 330	<u>39</u>
23	PP - > RP	l	> 330	> 330	<u>179</u>				> 330	> 330	<u>329</u>
		r	> 330	> 330	<u>128</u>	> 330	<u>237*</u>	<u>24*</u>			
24	RP - > PP	l	> 330	> 330	<u>262</u>	> 330	<u>107</u>	7			
		r	> 330	> 330	<u>321</u>				> 330	> 330	<u>103</u>
Underlined = count used for further computation * indicates adjacent dilutions used for computation **values cannot be used for further computation (infringement of control of weighted mean counts 5.7.2)											

Table C.3 — List of computed lg values and lg reductions

Preparation: "RP" (propan-1-ol 60 % v/v)

Volunteer	Sequence	Immediate effect			3 h effect		
No		lg Prevalues	lg Postvalue s	lg Reduction	lg Prevalues	lg Postvalue s	lg Reduction
1	RP- > PP	5,27	2,59	2,68	5,35	3,26	2,09
2	RP- > PP	4,63	1,67	2,96	4,85	3,26	1,59
3	PP- > RP	5,17	2,04	3,13	5,08	3,34	1,74
4	RP- > PP	4,65	3,09	1,56	4,68	4,37	0,31
5	PP- > RP	4,75	2,56	2,19	4,58	3,63	0,95
6	PP- > RP	3,66	1,30	2,36	3,83	3,76	0,07
7	RP- > PP	4,49	2,15	2,34	4,24	2,33	1,91
8	PP- > RP	4,86	3,14	1,72	4,13	2,92	1,21
9	RP- > PP	4,73	3,18	1,55	4,91	3,27	1,64
10	RP- > PP	4,26	2,29	1,97	4,39	1,98	2,41
11	RP- > PP	4,38	1,62	2,76	4,44	2,62	1,82
12	PP- > RP	4,93	2,01	2,92	5,26	3,72	1,54
13	PP- > RP	4,30	1,83	2,47	5,03	3,57	1,46
14	PP- > RP	4,24	2,09	2,15	4,32	3,88	0,44
15	RP- > PP	4,15	4,01	0,14	4,09	3,94	0,15
16	PP- > RP	3,54	0,00	3,54	3,51	1,64	1,87
17	PP- > RP	5,17	0,78	4,39	4,85	2,84	2,01
18	RP- > PP	5,10	2,90	2,20	5,16	3,40	1,76
19	PP- > RP	4,30	2,72	1,58	4,06	2,39	1,67
20	PP- > RP	4,57	1,45	3,12	4,85	2,96	1,89
21	RP- > PP	4,79	2,79	2,00	5,18	1,51	3,67
22	RP- > PP	5,12	1,51	3,61	5,51	2,97	2,54
23	PP- > RP	4,49	2,08	2,41	4,99	2,30	2,69
24	RP- > PP	5,44	4,19	1,25	5,13	2,79	2,34
Mean		4,67	2,29	2,38	4,68	3,03	1,66
Standard deviation		0,44	0,95	0,91	0,52	0,74	0,84
N		23	23	23	24	24	24

EN 12791:2016+A1:2017 (E)

Table C.4 — List of computed lg values and lg reduction

Preparation: "PP"

Volunteer	Sequence	Immediate effect			3 h effect		
No		lg Prevalues	lg Postvalue s	lg Reduction	lg Prevalues	lg Postvalue s	lg Reduction
1	RP- > PP	5,27	3,13	2,14	4,83	4,18	0,65
2	RP- > PP	3,21	2,32	0,89	2,90	3,42	-0,52
3	PP- > RP	4,86	2,84	2,02	4,98	4,52	0,46
4	RP- > PP	5,23	3,91	1,32	5,08	4,51	0,57
5	PP- > RP	4,82	3,03	1,79	4,85	4,16	0,69
6	PP- > RP	3,23	2,11	1,12	3,56	3,67	-0,11
7	RP- > PP	4,93	2,73	2,20	4,27	4,09	0,18
8	PP- > RP	5,12	3,17	1,95	5,25	3,39	1,86
9	RP- > PP	4,72	3,01	1,71	4,73	3,87	0,86
10	RP- > PP	4,76	3,02	1,74	4,16	3,11	1,05
11	RP- > PP	4,08	2,89	1,19	4,28	3,16	1,12
12	PP- > RP	5,06	3,00	2,06	5,04	4,39	0,65
13	PP- > RP	4,97	3,53	1,44	5,16	4,00	1,16
14	PP- > RP	4,38	3,01	1,37	4,49	4,16	0,33
15	RP- > PP	3,77	4,19	-0,42	3,77	4,51	-0,74
16	PP- > RP	3,61	1,89	1,72	4,73	3,33	1,40
17	PP- > RP	4,63	2,10	2,53	4,91	3,07	1,84
18	RP- > PP	5,06	3,28	1,78	5,10	4,43	0,67
19	PP- > RP	4,62	3,54	1,08	4,75	3,79	0,96
20	PP- > RP	4,25	3,91	0,34	4,18	4,33	-0,15
21	RP- > PP	4,09	2,37	1,72	4,84	3,13	1,71
22	RP- > PP	5,16	2,30	2,86	5,47	3,59	1,88
23	PP- > RP	5,11	3,38	1,73	5,25	4,52	0,73
24	RP- > PP	5,42	3,03	2,39	5,51	4,01	1,50
Mean		4,66	3,03	1,63	4,67	3,89	0,78
Standard deviation		0,58	0,58	0,71	0,63	0,51	0,72
N		23	23	23	24	24	24

Table C.5 — Test for sequence effects

Test of sequence of lg R “Immediate effect”

	Sequence		Absolute Difference
Procedure	RP- > PP	PP- > RP	RP- > PP - PP- > RP
	Mean s.d. N	Mean s.d. N	
RP (Propan-1-ol 60 % v/v)	2,09 0,91 12	2,69 0,83 11	
PP	1,63 0,84 12	1,64 0,58 11	
Difference of Means			
RP-PP	0,46	1,05	0,59
s.d. standard deviation			

Test of sequence of lg R “3-hours effect”

	Sequence		Absolute .Difference
Procedure	RP- > PP	PP- > RP	RP- > PP - PP- > RP
	Mean s.d. N	Mean s.d. N	
RP (Propan-1-ol 60 % v/v)	1,85 0,94 12	1,46 0,71 12	
PP	0,74 0,81 12	0,82 0,66 12	
Difference of Means			
RP-PP	1,11	0,64	0,47
s.d. standard deviation			

“RP->PP” means: RP tested before PP and

“PP->RP” means: PP tested before RP

EN 12791:2016+A1:2017 (E)

Table C.6 — Individual differences of lg Rs between RP and PP for immediate and 3 h effects

Volunteer	lg R immediate effect			lg R 3 h effect		
	RP	PP	Difference RP-PP	RP	PP	Difference RP-PP
1	2,68	2,14	0,54	2,09	0,65	1,44
2	2,96	0,89	2,07	1,59	-0,52	2,11
3	3,13	2,02	1,11	1,74	0,46	1,28
4	1,56	1,32	0,24	0,31	0,57	-0,26
5	2,19	1,79	0,40	0,95	0,69	0,26
6	2,36	1,12	1,24	0,07	-0,11	0,18
7	2,34	2,20	0,14	1,91	0,18	1,73
8	1,72	1,95	-0,23	1,21	1,86	-0,65
9	1,55	1,71	-0,16	1,64	0,86	0,78
10	1,97	1,74	0,23	2,41	1,05	1,36
11	2,76	1,19	1,57	1,82	1,12	0,70
12	2,92	2,06	0,86	1,54	0,65	0,89
13	2,47	1,44	1,03	1,46	1,16	0,30
14	2,15	1,37	0,78	0,44	0,33	0,11
15	0,14	-0,42	0,56	0,15	-0,74	0,89
16	3,54	1,72	1,82	1,87	1,40	0,47
17	4,39	2,53	1,86	2,01	1,84	0,17
18	2,20	1,78	0,42	1,76	0,67	1,09
19	1,58	1,08	0,50	1,67	0,96	0,71
20	3,12	0,34	2,78	1,89	-0,15	2,04
21	2,00	1,72	0,28	3,67	1,71	1,96
22	3,61	2,86	0,75	2,54	1,88	0,66
23	2,41	1,73	0,68	2,69	0,73	1,96
24	1,25	2,39	-1,14	2,34	1,50	0,84

Acceptance criteria for test results:

- a) number of complete sets of test results: 23 for immediate and 24 for 3-h-effect, respectively (required minimum: 23)
- b) overall mean
- of individual lg prevalues RP (immediate/3 h effect): 4,67/4,68 (requ.min:3,5/min: 3,5)
- of individual lg prevalues PP (immediate/3 h effect): 4,66/4,67 (requ.min:3,5/min: 3,5)
- c) absolute difference of mean differences between RP and PP

EN 12791:2016+A1:2017 (E)

c1) between the groups RP- > PP and PP- > RP, immediate effect: 0,59 (<2,0)

c2) between the groups RP- > PP and PP- > RP, 3 h effect: 0,47 (<2,0)

d) control of weighted mean counts according to 5.7.2: all quotients of two adjacent dilutions used for computation, i.e. counts marked by an asterisk in Tables C.1 and C.2, are between 5 and 15.

All acceptance criteria are fulfilled.

Table C.7 — Computation of the Hodges-Lehmann 97,5 upper confidence limit for the immediate effect

Sorted differences of RP-PP (descending order)	Mean pairwise differences $(d_i + d_{ji}) / 2$											
	2,78	2,07	1,86	1,82	1,57	1,11	1,02	0,87	0,78	0,76	0,69	0,58
2,78	2,78 ¹											
2,07	2,43 ²	2,07 ⁶										
1,86	2,32 ³	1,97 ⁷	1,86 ¹¹									
1,82	2,30 ⁴	1,95 ⁸	1,84 ¹²	1,82 ¹³								
1,57	2,18 ⁵	1,82 ¹⁴	1,72 ¹⁹	1,70 ²⁰	1,57 ²⁷							
1,11	1,95 ⁹	1,59 ²⁶	1,49 ³²	1,47 ³³	1,34 ⁴²	1,11 ⁷⁴						
1,03	1,91 ¹⁰	1,55 ²⁸	1,45 ³⁶	1,43 ³⁷	1,30 ⁴⁹	1,07	1,03					
0,86	1,82 ¹⁵	1,47 ³⁴	1,36 ⁴¹	1,34 ⁴³	1,22 ⁵⁸	0,99	0,95	0,86				
0,78	1,78 ¹⁶	1,43 ³⁸	1,32 ⁴⁴	1,30 ⁵⁰	1,18 ⁶²	0,95	0,91	0,82	0,78			
0,75	1,77 ¹⁷	1,41 ³⁹	1,31 ⁴⁶	1,29 ⁵¹	1,16 ⁶⁶	0,93	0,89	0,81	0,77	0,75		
0,68	1,73 ¹⁸	1,38 ⁴⁰	1,27 ⁵⁴	1,25 ⁵⁵	1,13 ⁷¹	0,90	0,86	0,77	0,73	0,72	0,68	
0,56	1,67 ²¹	1,32 ⁴⁵	1,21 ⁵⁹	1,19 ⁶¹	1,07	0,84	0,80	0,71	0,67	0,66	0,62	0,56
0,54	1,66 ²²	1,31 ⁴⁷	1,20 ⁶⁰	1,18 ⁶³	1,06	0,83	0,79	0,70	0,66	0,65		
0,50	1,64 ²³	1,29 ⁵²	1,18 ⁶⁴	1,16 ⁶⁷	1,04	0,80	0,77	0,68	0,64	0,63		
0,42	1,60 ²⁴	1,25 ⁵⁶	1,14 ⁷⁰	1,12 ⁷³	1,00	0,77	0,73	0,64				
0,40	1,59 ²⁵	1,24 ⁵⁷	1,13 ⁷²	1,11	0,99	0,75	0,72	0,63				
0,28	1,53 ²⁹	1,18 ⁶⁵	1,07	1,05	0,93	0,70	0,66					
0,24	1,51 ³⁰	1,16 ⁶⁸	1,05	1,03	0,91	0,68	0,64					
0,23	1,51 ³¹	1,15 ⁶⁹	1,05	1,03	0,90	0,67	0,63					
0,14	1,46 ³⁵	1,11	1,00	0,98	0,86	0,63						
-0,16	1,31 ⁴⁸	0,96	0,85	0,83	0,71							
-0,23	1,28 ⁵³	0,92	0,82	0,80	0,67							
-1,14	0,82											

The median of the differences RP-PP is between the 12th value: = 0,56. The small exponents represent the ranks.

EN 12791:2016+A1:2017 (E)

The mean pairwise differences that do not exceed the median (here: 0,56) are computed. From Table D.1 of critical values for Wilcoxon's matched-pairs signed-ranks test the entry for $n = 23$ and a one-sided $P = 0,025$ level of significance the critical value of 73 is found. Hence $c = 73 + 1 = 74$. The pairwise differences are sorted in descending order. The 74th value is 1,11. Hence, the Hodges-Lehmann upper one-sided 97,5 % confidence limit for the difference in lg Rs between RP and PP is 1,11, which is above the agreed inferiority margin of 0,75.

Therefore, the hypothesis of inferiority of the immediate effect of PP versus RP cannot be rejected.

Table C.8 — Computation of the Hodges-Lehmann 97,5 upper confidence limit for the 3 h effect

Sorted Differences of RP-PP (descending order)	Mean pairwise differences ($d_i + d_{ji}$) / 2											
	2,11	2,04	1,96	1,96	1,73	1,44	1,36	1,28	1,09	0,89	0,89	0,84
2,11	2,11 ¹											
2,04	2,08 ²	2,04 ³										
1,96	2,04 ⁴	2,00 ⁶	1,96 ⁸	1,96 ⁹								
1,96	2,04 ⁵	2,00 ⁷	1,96 ¹⁰	1,96 ¹¹								
1,73	1,92 ¹²	1,89 ¹³	1,85 ¹⁴	1,85 ¹⁵	1,73 ¹⁹							
1,44	1,78 ¹⁶	1,74 ¹⁷	1,70 ²⁰	1,70 ²¹	1,59 ³⁰	1,44 ⁴²						
1,36	1,74 ¹⁸	1,70 ²²	1,66 ²⁴	1,66 ²⁵	1,55 ³²	1,40 ⁵²	1,36 ⁶⁰					
1,28	1,70 ²³	1,66 ²⁶	1,62 ²⁷	1,62 ²⁸	1,51 ³⁵	1,36 ⁶¹	1,32 ⁶⁷	1,28 ⁷⁴				
1,09	1,60 ²⁹	1,57 ³¹	1,53 ³³	1,53 ³⁴	1,41 ⁴⁸	1,27 ⁷⁵	1,23 ⁷⁸	1,19	1,09			
0,89	1,50 ³⁶	1,47 ³⁹	1,43 ⁴⁴	1,43 ⁴⁵	1,31 ⁶⁸	1,17	1,13	1,09	0,99	0,89		
0,89	1,50 ³⁷	1,47 ⁴⁰	1,43 ⁴⁶	1,43 ⁴⁷	1,31 ⁶⁹	1,17	1,13	1,09	0,99	0,89	0,89	
0,84	1,48 ³⁸	1,44 ⁴³	1,40 ⁵³	1,40 ⁵⁴	1,29 ⁷²	1,14	1,10	1,06	0,97	0,87	0,87	0,84
0,78	1,45 ⁴¹	1,41 ⁴⁹	1,37 ⁵⁷	1,37 ⁵⁸	1,26 ⁷⁶	1,11	1,07	1,03	0,94	0,84	0,84	0,81
0,71	1,41 ⁵⁰	1,38 ⁵⁶	1,34 ⁶³	1,34 ⁶⁴	1,22 ⁷⁹	1,08	1,04	0,99	0,90			
0,70	1,41 ⁵¹	1,37 ⁵⁹	1,33 ⁶⁵	1,33 ⁶⁶	1,22 ⁸⁰	1,07	1,03	0,99	0,90			
0,66	1,39 ⁵⁵	1,35 ⁶²	1,31 ⁷⁰	1,31 ⁷¹	1,20	1,05	1,01	0,97	0,88			
0,47	1,29 ⁷³	1,26 ⁷⁷	1,22 ⁸¹	<u>1,22⁸²</u>	1,10	0,96	0,91	0,87				
0,30	1,21	1,17	1,13	1,13	1,02	0,87	0,83					
0,26	1,19	1,15	1,11	1,11	1,00	0,85	0,81					
0,18	1,15	1,11	1,07	1,07	0,96	0,81						
0,17	1,14	1,11	1,07	1,07	0,95							
0,11	1,11	1,08	1,04	1,04	0,92							
-0,26	0,93	0,89	0,85	0,85								
-0,65												

The median of the differences RP-PP is between the 12th and 13th value: $[0,84 + 0,78]/2 = 0,81$. The small exponents represent the ranks.

The mean pairwise differences that do not exceed the median (here: 0,81) are computed. From Table D.1 of critical values for Wilcoxon's matched-pairs signed-ranks test the entry for $n = 24$ and a one-sided $P = 0,025$ level of significance the critical value of 81 is found. Hence $c = 81 + 1 = 82$. In the body of the table, the pairwise differences are sorted in descending order. There the 82nd value is 1,22.

Hence the Hodges-Lehmann upper one-sided 97,5 % confidence limit for the difference in lg Rs between RP and PP is 1,22, which is above the agreed inferiority margin of 0,85.

Therefore, the hypothesis of inferiority of the 3 h effect of PP versus RP cannot be rejected.

Conclusion: As both, the immediate and 3 h effects of PP are significantly inferior to those of RP the product did not fulfill the requirements of EN 12791.

C.8 Test for Non-Inferiority according to Hodges and Lehmann [2]

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

$H_0: \mu_R - \mu_P \geq \delta$, where δ is an agreed inferiority margin (see below).

μ_R is the expected lg reduction (lg R) for the reference procedure, RP, and 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, that RP is on average greater than that of PP by at least δ lg units, meaning that PP is inferior to RP.

The alternative hypothesis (H_1) is:

$H_1: \mu_R - \mu_P < \delta$.

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 individual 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 0,75 lg (for the 3h effect 0,85 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 C.6).
4. Sort the differences in descending order as shown in Tables C.7 and C.8.
5. Consult the table in Annex D to obtain the critical value for the number of pairs, np, 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 Tables C.7 and C.8:
 - Let d_i and d_{ii} be any two differences: their mean is $(d_i + d_{ii})/2$.
 - 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 individual differences with the inferiority margin (here: 0,75; for the 3h effect 0,85 lg)). 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.

Examples for computer programs are: StatXact®, SAS® ⁴⁾ (with a macro). 

4) StatXact® and SAS® are examples of suitable products available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of these products.

EN 12791:2016+A1:2017 (E)

Annex D (informative)

WILCOXON'S matched-pairs signed-ranks test

Table D.1 — WILCOXON'S matched-pairs signed-ranks test: Critical values for the lower of both sums of ranks with (+) or (-) sign at different significance levels

<i>n</i> Number of pairs with difference $\neq 0$	Level of significance		
	$P = 0,025$	$P = 0,01$	$2 P = 0,01$
	one sided	one sided	two sided
23	73	62	54
24	81	69	61
25	89	76	68
26	98	84	75

Bibliography

- [1] European Pharmacopoeia – edition 2007 (monographies): water for injections, 1-propanol (reagents): potassium hydroxide, ethanol 96 %, sulphuric acid 10 %, phenolphthalein, hydrochloric acid, sodium hydroxide solution, petroleum ether, anhydrous sodium sulphate, polysorbate 80
- [2] LEHMANN E.L. *Nonparametrics: Statistical Methods Based on Ranks*. Holden-Day, San Francisco, 1975
- [3] WILCOXON F., WILCOX R.A. (1964). Some rapid approximate statistical procedures. Pearle River, N.Y.: Lederle Laboratories; StatXact™ or SAS™ (with macro)