SLOVENSKI STANDARD
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Kemična razkužila in antiseptiki - Higiensko virucidno razkuževanje rok - Preskusna metoda in zahteve (faza 2, stopnja 2)

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

Chemische Desinfektionsmittel und Antiseptika - Viruzide hygienische Händedesinfektion - Prüfverfahren und Anforderungen (Phase 2, Stufe 2)

Désinfectants chimiques et antiseptiques - Traitement hygiénique virucide de mains par frictions - Méthode d'essai et prescriptions (phase 2, étape 2)

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11.080.20	Dezinfektanti in antiseptiki	Disinfectants and antiseptics

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

Désinfectants chimiques et antiseptiques ¿ Traitement hygiénique virucide de mains par frictions ¿ Méthode d'essai et prescriptions (phase 2, étape 2)

Chemische Desinfektionsmittel und Antiseptika - Viruzide hygienische Händedesinfektion - Prüfverfahren und Anforderungen (Phase 2, Stufe 2)

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EUROPÄISCHES KOMITEE FÜR NORMUNG

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European foreword

This document (prEN 17430:2019) has been prepared by Technical Committee CEN/TC 216 “Chemical disinfectants and antiseptics”, the secretariat of which is held by AFNOR.

This document is currently submitted to the CEN Enquiry.

1 Scope

This document specifies a test method simulating practical conditions for establishing whether a product for hygienic handrub reduces the release of virus contamination 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 document 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 kindergardens and of nursing homes;

and can occur in the workplace and in the home. It can 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 are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 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.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- IEC Electropedia: available at http://www.electropedia.org/
- ISO Online browsing platform: available at http://www.iso.org/obp

4 Requirements

When tested in accordance with Clause 5, the mean reduction of the release of the test organism *Murine Norovirus* strain S99 Berlin 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 (a volume fraction of 70 % concentration of ethanol).

5 Test methods

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 (virus titres) 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.

5.2 Materials and reagents

5.2.1 Test organism

Murine Norovirus strain S99 Berlin[1]

This test organism has been specifically chosen to meet health and safety guidance and ethical committee considerations. According to the German Safety Ordinance on Gene Technology [4], the *Murine Norovirus* is classified as a risk group 1 organism.

5.2.2 Culture media and reagents

5.2.2.1 General

All weights of chemical substances given in this document 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 demineralized water. If distilled water of adequate quality is not available, water for injections (see bibliographic reference [3]) may be used.

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

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

5.2.2.3 Phosphate buffered saline (PBS)

Sodium chloride (NaCl)	8,00 g
Potassium chloride (KCl)	0,20 g
Disodium hydrogen phosphate, 12-hydrate	2,89 g

[1] *Murine Norovirus* can be obtained from Friedrich-Loeffler-Institut Bundesinstitut für Tiergesundheit, Hauptsitz Insel Riems Südufer 10, 17493 Greifswald-Insel Riems; phone: +49 38351 7-0; fax: +49 38351 7-121. http://www.fli.de/. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of the product named.

(Na2HPO4 × 12H2O)	
Potassium phosphate, monobasic (KH2PO4)	0,20 g
Water (5.2.2.2)	to 1 000,0 ml

5.2.2.4 Neutral Red (1:1000 solution)

Prepare neutral red (Sigma N7005) stock solution at 0,1 mg/ml in water (5.2.2.2). Filter through a 0,40 µm pore size filter and store 4 °C in the dark.

5.2.2.5 Foetal calf serum (FCS)

FCS has to be certified free of viruses and mycoplasma. Extraneous viruses and mycoplasma may interfere with cell and virus growth resulting in false results.

For RAW 264.7 cells, special FCS has to be used due to the cells' high sensitivity to endotoxins.

5.2.2.6 Trichloroacetic acid (10 % solution) (TCA)

Dissolve 10 g of TCA crystals in 80 ml of water (5.2.2.2), then adjust the volume to 100 ml with water. Stir to complete solution.

5.2.2.7 Hard water

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. Sterilize by membrane filtration (5.3.2.12) or in the autoclave [5.3.2.1 a)]. Autoclaving – if used – may cause a loss of liquid. In this case make up to 1 000 ml with water (5.2.2.2) under aseptic conditions. Store the solution in the refrigerator (5.3.2.16) 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 (5.3.2.12). Store the solution in the refrigerator (5.3.2.16) 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 (5.3.2.10) and add 6,0 ml (5.3.2.9) of solution A, then 8,0 ml of solution B. Mix and dilute to 1 000 ml with water (5.2.2.2). The pH (5.3.2.4) of the hard water shall be 7,0 ± 0,2. 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 (5.6.3), the addition of the product to the hard water produces different final water hardness in each test tube. In any case, the final hardness in the test tube expressed as calcium carbonate ($CaCO_3$) is lower than 375 mg/l.

5.2.2.8 Growth and maintenance media

Dulbecco's Modified Eagle's Medium (DMEM) or equivalent, supplemented with appropriate concentration of heat inactivated (e.g. 56 °C for 30 min) and mycoplasma-free fetal calf serum FCS (5.2.2.5), antibiotics, and other growth factors as needed shall be used.

a) A *growth medium* for cell multiplication is supplemented with 10 % FCS (5.2.2.5). Add 10 parts of FCS to 90 parts of MEM.

b) A *maintenance medium* to maintain the cell culture metabolism without stimulation of cell proliferation is supplemented with 2 % FCS. Add 2 parts of FCS (5.2.2.5) to 98 parts of MEM.

Other media may be used if appropriate for certain cell lines.

See also bibliographic reference [5]. See EN 12353 for a detailed description.

NOTE Materials and reagents for cell culture can be purchased from biological supply companies.

5.2.2.9 Diluted soft soap

Linseed oil	50 parts by weight
Potassium hydroxide [3]	9,5 parts by weight
Ethanol (min. 95 %) [3]	7 parts by weight
Hot distilled water (75 °C ± 5 °C)	as needed

Prepare a solution of 9,5 parts potassium hydroxyde 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 °C ± 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 sterilize in the autoclave [5.3.2.1 a)]. 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 C.

5.2.2.10 Ethanol **as reference handrub [volume fraction of 70 % at 20°C]**

Fill 547,2 g ethanol with a purity of min volume fraction of 99,5 % (determined by gas chromatography; density 0,790) in a 1 000 ml flask equipped with a glass stopper on the weighing platform of a scale (precision 0,1 g). Add 322,7 g water (5.2.2.2). Close the flask with the matching glass stopper and shake the contents of the flask thoroughly. During mixing the solution warms up about to 30 °C. After cooling down to 20 °C, it will give a volume of approximately 980 ml.

NOTE 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 equipment[2] and, in particular, the following:

5.3.2.1 Apparatus for sterilization (moist and dry heat).

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

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

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

5.3.2.2 **Water baths**, capable of being controlled at 20 °C ± 1 °C.

5.3.2.3 **Inverted microscope** for reading cell cultures microscopically.

5.3.2.4 **pH meter**, having an inaccuracy of calibration of 0,1 pH units at 20 °C ± 1 °C.

5.3.2.5 **Stopwatch**

5.3.2.6 **Deep freezer** (−20°C and −70°C or less).

5.3.2.7 **Electromechanical agitator** e.g. Vortex ® mixer[3].

5.3.2.8 **Containers:** sterile test tubes, culture bottles or flasks of suitable capacity.

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

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

5.3.2.11 **Sterile microtitre plates**, six well plates for cell cultures, and flasks for cell cultures.

5.3.2.12 **Membrane filtration apparatus** for filtration of media, 0,2 µm pore size.

5.3.2.13 **CO_2 incubator** (95 % air, 5 % CO_2), capable of being controlled at either 36 °C ± 1 °C, or at 37°C ± 1 °C for incubation of cell cultures.

5.3.2.14 **Biological safety cabinet**, class II.

5.3.2.15 **Centrifuge** (400 g_N to 1000 g_N).

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

5.3.2.17 **Cylindrical polypropylene screw cap tubes**, volume 25 ml, length 54 mm, diameter 27 mm (for the stainless steel discs), e.g. Sarstedt AG & Co. D-51582 Nümbrecht, Art.No.: 60.9922.115 PS[4].

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

[4] These "Art. No." from Sarstedt AG & Co. D-51582 Nümbrecht are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of these products.

5.3.2.18 Cylindrical polypropylene screw cap tubes, volume 10 ml, length 79 mm, diameter 16 mm (for the glass carrier), e.g. Sarstedt AG & Co., D-51582 Nümbrecht, Art.No.: 60.551.001)[4].

5.4 Preparation of test organism suspensions and product test solutions

5.4.1 Test virus suspension (test organisms suspensions, contamination fluid)

The test organisms and their stock cultures shall be prepared and kept in accordance with EN 12353.

The stock virus suspension is multiplied in an appropriate cell line that produces high titres of infectious viruses. The cell debris is separated by centrifugation (400 g_N for 15 min). This preparation is called “test virus suspension”.

It is suggested that the minimum titre of the virus suspension - determined by a quantal test (5.6.1) or by plaque test (5.6.2) - is at least 10^8 $TCID_{50}$/ml. In any case, it shall be sufficiently high to at least 10^4 $TCID_{50}$/ml may be recovered from the hands.

In exceptional cases the test virus suspension may be concentrated by appropriate methods (e.g. ultracentrifugation).

The test virus suspension is kept in small volumes below −70 °C or preferably at −196 °C under nitrogen. Due to safety reasons, and – in some cases – to limit the possibility of genetic mutations, only 10 passages from the original seed (e.g. virus from culture collection) are allowed.

The test virus suspension is used undiluted for the test procedure (5.7).

The titre of the virus suspension is determined as described (see 6.2 and 6.3).

5.4.2 Cell lines

Murine Norovirus is multiplied in RAW 264.7 cells (ATCC TIB-71) or other cell lines of appropriate sensitivity.

5.5 Cell culture preparation for virucidal testing

Cells used as suspension in quantal tests shall be added to the dilutions of the test mixture in such a density to enable the formation of a monolayer in at least two days in the cell control (see 5.6.1.1).

Cell monolayers shall be > 90 % confluent before inoculation. Cell lines and culture media will be selected in accordance with the test virus (see 5.2.1).

5.6 Infectivity assay

5.6.1 Quantal tests (end point titration)

5.6.1.1 Virus titration on cells in suspension

Dilute the test virus suspension (5.4.1) by tenfold dilution steps with a volume of 0,5 ml or 0,1 ml of test virus suspension plus an appropriate volume of maintenance medium (see 5.2.2.8). Pipettes shall be changed after each dilution step.

Transfer 0,1 ml of each dilution into six or eight wells of a microtitre plate, beginning with the highest dilution. Add 0,1 ml of cell culture in such a density to enable the formation of a monolayer in at least 2 days in the cell control. The last row of six or eight wells shall not receive any test virus suspension and serve as the cell control.

After the appropriate incubation time, according to the virus type, the viral cytopathic effect is read using an inverted microscope (see 5.3.2.3).

5.6.1.2 Virus titration on cell monolayers

Transfer 0,1 ml of each dilution (see 5.6.1.1) into six or eight wells of a microtitre plate containing a confluent (>90 %) cell monolayer. The last row of six or eight wells will receive 0,2 ml of maintenance medium (see 5.2.2.8) and will serve as cell control. If the cells were inoculated without any medium after 1 h of incubation at 37 °C, 0,1 ml of maintenance medium is added to each well.

The plates are incubated at 37 °C for the appropriate incubation time. The viral cytopathic effect is read using an inverted microscope (see 5.3.2.3).

5.6.2 Plaque assay

Plastic tray wells (surface diameter 30 mm to 35 mm) with confluent cell monolayers are washed once with phosphate buffered saline (PBS) (5.2.2.3) and inoculated with 0,2 ml of serial dilutions of virus in DMEM + 2 % FCS (see 5.2.2.8). Three wells are generally used per dilution. After absorption period of 1 h at 37 °C, during which the cell monolayers are kept moist by tilting the dishes every 8 min to 10 min, the inoculum is removed and the cell monolayers are washed once with PBS. Subsequently, the wells are overlaid with 3 ml of a mixture consisting of 2 % melted agarose or another appropriate semisolid medium and 2times concentrated DMEM with 4 % FCS. The cultures are incubated for 5 days to 6 days at 37 °C in a CO_2 incubator (see 5.3.2.13). Plaques can be counted after addition of 2 ml of a second overlay with the same composition of the first and also containing 5 % of a 1:1 000 solution of neutral red and further incubation (in the dark) at 37 °C for 24 h to 48 h in a CO_2 incubator (see 5.3.2.13). Counting can be performed also after addition of crystal violet. The cell monolayers are fixed by adding 2 ml of 10 % trichloroacetic acid (TCA) (5.2.2.6) over the agar overlay for 10 min to 15 min at room temperature. The agar overlay is then removed and 2 ml of 0,1 % crystal violet in 20 % ethanol are added. After 10 min to 15 min at room temperature, the wells are extensively washed with water and the plaques (white spots) are counted.

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).

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 (5.3.2.10) 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 (5.3.2.10) 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 microorganisms 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.