BS EN 16616:2015

BSI Standards Publication

Chemical disinfectants and antiseptics — Chemical-thermal textile disinfection — Test method and requirements (phase 2, step 2)

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National foreword

This British Standard is the UK implementation of EN 16616:2015.

BSI, as a member of CEN, is obliged to publish EN 16616 as a British Standard. However, attention is drawn to the fact that during the development of this European Standard, the UK committee voted against its approval as a European Standard.

The UK committee believes that this method is limited to washer-extractors. It is also considered that some of the reduction in microbial numbers achieved in the test conditions (in which a very small proportion of the load is contaminated) may be due to redistribution of the contamination amongst the load. Hence the antimicrobial efficacy measured by this test method may be significantly higher, and therefore potentially misleading, than that obtained in practice if a greater proportion of the load is contaminated.

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.

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

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European foreword

This document (EN 16616:2015) 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of an identical text or by endorsement, at the latest by February 2016, and conflicting national standards shall be withdrawn at the latest by February 2016.

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 has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association, and supports essential requirements of EU Directive(s).

For relationship with EU Directive(s), see informative Annex ZA, which is an integral part of this document.

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Introduction

This European Standard specifies a carrier test for establishing whether a single-wash disinfecting product or combination of products for the treatment of contaminated textile has or does not have necessary microbicidal activity. The standard only intends to validate the disinfection part of the laundry process.

This laboratory test takes into account practical conditions of application of the product including contact time, temperature, test organisms and interfering substances, i.e. conditions which may influence its action in practice.

The conditions are intended to cover general purposes and to allow reference between microbiological laboratories and types of detergents and disinfectants. Each effective dosage of the chemical disinfectant found by this test corresponds only to the chosen experimental conditions. Where actual conditions vary additional testing in microbiological laboratories shall be needed to determine the effective dosage. Instructions for use are the responsibility of manufactures of detergents or disinfectants.

1 Scope

This European Standard specifies a test method and the minimum requirements for the microbicidal activity of a defined disinfection process for the treatment of contaminated textile. This procedure is carried out by using a washing machine as defined in 5.3.2.18 and refers to the disinfection step without prewash. This procedure is not limited to certain types of textile. The suppliers instructions shall be sufficient to allow the method in the standard to be carried out fully (e.g. dosing disinfectant in whatever washing phase e.g. rinsing, disinfecting at 40 °C).

This European Standard applies to areas and situations where disinfection is indicated. Such indications occur in patient care, for example:

— in hospitals, in community medical facilities and in dental institutions;

— in schools, kindergartens and nursing homes;

— institutions where patients are accommodated, which could suffer from transmissible diseases;

— other applications where hygienic treatment of textile is necessary (e.g. food processing, hotels, workwear e.g. from the pharmaceutical industry, laboratories, foodstuffs area or similar institutions).

The method described is intended to determine the activity of a product or product combination under the conditions in which they are used. This is a phase 2, step 2 laboratory test that simulates the conditions of application of the product.

NOTE This method corresponds to a phase 2, step 2 test (see EN 14885).

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 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, *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 14348, *Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of mycobactericidal activity of chemical disinfectants in the medical area including instrument disinfectants — Test methods 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 and the following apply.

3.1
liquor ratio
ratio of the weight of dry textile in kilogram and volume of wash liquor in litre (w/v)

3.2
disinfection process
process taking into account practical conditions of application of the product including contact time, temperature, test organisms and interfering substances to disinfect the textile

3.3
treatment of contaminated textile
handling the textile according the disinfection process to obtain disinfected textile

4 Requirements

The test results shall fulfil the basic limits (see 5.7.3).

The following phase 2, step 1 test shall be passed in addition to this test: EN 13727, EN 13624 and EN 14348 under the following test conditions:

— temperature as recommended by the manufacturer;

— contact time recommended by the manufacturer;

— dirty conditions and

— reduction as recommended for instrument disinfection.

For products used > 60 °C EN 13624 and EN 14348 should be passed with *Aspergillus brasiliensis* and *M. avium*.

a) Processes with temperatures < 60°C

A chemical-thermal textile disinfection process, when tested in accordance with Clause 5, is considered effective when in three test runs (within a given time, with a suggested dosage, at a suggested temperature and liquor ratio) a reduction of bacteria in and on germ carriers of more than 7 log-units, a reduction of *Candida albicans* and *Aspergillus brasiliensis* of 6 lg-units, a reduction of mycobacteria (*M. avium and/or M. terrae*) of 7 lg-units is reached.

NOTE The implementation of spores and viruses was discussed. Further development is necessary to make it technically feasible.

Further, no test organisms are to be detected in 100 ml washing/disinfection liquid.

As a minimum requirement the bactericidal and yeasticidal activity shall be evaluated using the following test organisms: for bactericidal activity *Pseudomonas aeruginosa, Escherichia coli* (K12), *Staphylococcus aureus, Enterococcus hirae* and for yeasticidal activity *Candida albicans* as test organism.

If an additional fungicidal activity is claimed, also *Aspergillus brasiliensis* shall be used as test organism.

In case of an additional proof of tuberculocidal activity, *Mycobacterium terrae* is to be tested.

If a mycobactericidal effect should be confirmed, it is necessary to perform the test also with *Mycobacterium terrae* and *Mycobacterium avium.*

b) Processes with temperatures ≥ 60°C

A chemical-thermal textile disinfection process, when tested in accordance with Clause 5, is considered as effective when in three test runs (within a given time, with a suggested dosage, at a process temperature ≥ 60°C and a defined liquor ratio) a reduction of *Enterococcus faecium* in and on germ carriers of more than 7 lg-units will be achieved.

Further, no test organisms are to be detected in 100 ml washing/disinfection liquid. This includes fungicidal, mycobactericidal / tuberculocidal activity.

5 Test methods

5.1 Principle

Germ carriers made of cotton fabric are contaminated with a test suspension of microorganisms in defibrinated sheep blood. After drying the carriers are transferred into cotton bags and then the disinfection process in the washing machine is performed at test temperatures either t < 60°C or ≥ 60°C. The process refers to the disinfection step without prewash. At the end of the disinfection step of the procedure, the bags with the carriers have to be taken out (see 5.3.2.18). Each carrier is transferred into a separate tube containing neutralizer and glass-beads. The bacteria should be recovered from the carriers by shaking. The number of surviving bacteria in each sample is determined and the reduction rate is calculated.

5.2 Materials and reagents

5.2.1 Test organisms

The bactericidal activity shall be evaluated using the following strains as test organisms[1)]

— *Pseudomonas aeruginosa* ATCC 15442

— *Escherichia coli* (K12) ATCC 10538

— *Staphylococcus aureus* ATCC 6538

— *Enterococcus hirae* ATCC 10541

— *Enterococcus faecium* ATCC 6057

The yeasticidal/fungicidal activity shall be evaluated using the following test organisms:

— *Candida albicans* ATCC 10231

— *Aspergillus brasiliensis* (formerly *Aspergillus Niger* ATCC 16404) ATCC 16404

The mycobactericidal/tuberculocidal activity shall be evaluated using the following test organisms:

— *Mycobacterium avium* ATCC 15769

— *Mycobacterium terrae* ATCC 15755

NOTE See Annex A for strain reference in some other culture collections.

1) The ATCC numbers are the collection numbers of strains supplied by the American Type Culture Collections (ATCC). 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.

The required incubation temperature for these test organisms is (36 ± 1) °C or (37 ± 1) °C (5.3.2.3) [*C. albicans* and *A. brasiliensis*: (30 ± 1) °C]. The same temperature shall be used for all incubations performed during a test and its control and validation.

If additional test organisms are used, they shall be incubated under optimum growth conditions (temperature, time, atmosphere, and media) noted in the test report. If the additional test organisms selected do not correspond to the specified strains/species, their suitability for supplying the required inocula shall be verified. If these additional test organisms are not classified at a reference centre, their identification characteristics shall be stated. In addition, they shall be held by the testing laboratory or national culture collection stored under a reference for five years.

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 growth of 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 limitation for use should be fixed.

5.2.2.2 Water used for preparation of media

a) The water shall be fresh distilled water and not just demineralized water. Sterilize in the autoclave [5.3.2.1a)].

NOTE 1 Sterilization is not necessary if the water is used e.g. for preparation of culture media and subsequently sterilized.

NOTE 2 If distilled water of adequate quality is not available, water for injections (see bibliographic reference [1]) can be used.

NOTE 3 See 5.2.2.3 for the procedure to prepare hard water.

5.2.2.3 Hard water for dilution of products for validation tests

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 (5.3.2.7) or in the autoclave [5.3.2.1a)]. 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 (5.3.2.8) at 2 °C to 8 °C 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 1 000 ml. Sterilize by membrane filtration (5.3.2.7). Store the solution in the refrigerator (5.3.2.8) at 2 °C to 8 °C 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 with the use of a pipette (5.3.2.9) 6,0 ml 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 of the hard water shall be 7,0 ± 0,2, when measured at (20 ± 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 different final water hardness in each test tube. In any case the final hardness is lower than 375 mg/l of calcium carbonate ($CaCO_3$) in the test tube.

5.2.2.4 Tryptone Soy Agar (TSA)

Tryptone, pancreatic digest of casein	15,0 g
Soy 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

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

In case of encountering problems with neutralization (5.5.1.2 and 5.5.1.3) it may be necessary to add neutralizer to the TSA. Annex B gives guidance on the neutralizers that may be used.

5.2.2.5 Tryptone Soy Broth (TSB)

Tryptone, pancreatic digest of casein	15,0 g
Soy 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

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

5.2.2.6 Brain Heart Infusion Agar (BHI)

Example:

Brain heart infusion	12,5 g
Beef heart infusion	5,0 g
Proteose-Peptone	10,0 g
Glucose	2,0 g
Sodium chloride (NaCl)	5,0 g
Dinatriumhydrogenphosphate	2,5 g
Agar	10,0 g
Water (5.2.2.2)	to 1 000,0 ml

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

5.2.2.7 Malt Extract Agar (MEA)

Malt extract	30,0 g
Soy peptone, papaic digest of soybean meal	3,0 g
Agar	15,0 g
Water (5.2.2.2)	to 1 000,0 ml

Sterilize in the autoclave [5.3.2.1a)]. After sterilization the pH of the medium shall be equivalent to 5,6 ± 0,2 when measured at (20 ± 1) °C.

5.2.2.8 Middlebrook and Cohn 7H10 medium incl. 10 % OADC (7H10)

Middlebrook 7H10 agar	19,0 g
Glycerol	5,0 ml
Water (5.2.2.2)	To 900,0 ml

Heat to boiling to dissolve completely. Sterilize for 10 min in the autoclave [5.3.2.1a)] and cool to 50 °C to 55 °C. Add 100 ml Middlebrook OADC enrichment under aseptic conditions. The final pH of the medium shall be equivalent 6,6 ± 0,2 when measured at (25 ± 1) °C.

5.2.2.9 Diluent

Tryptone, pancreatic digest of casein	1,0 g
Sodium chloride (NaCl)	8,5 ml
Water (5.2.2.2)	to 1 000,0 ml

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

5.2.2.10 Neutralizer

The neutralizer shall be validated for the product being tested in accordance with 5.5.1.2, 5.5.1.3 and 5.5.2. It shall be sterile.

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

5.2.2.11 Sterile defibrinated sheep blood

The sterile defibrinated sheep blood can be acquired from a commercial supplier.

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 [5.3.2.1a)];

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

5.3.2 Usual microbiological laboratory equipment[2)]

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^{+3}_{0})°C for a minimum contact 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 contact time of 30 min, at (170 $^{+5}_{0}$)°C for a minimum contact time of 1 h or at (160^{+5}_{0})°C for a minimum contact time of 2 h.

5.3.2.2 Water baths, capable of being controlled at (20 ± 1) °C, at (36 ± 1) °C or (37 ± 1) °C, at (45 ± 1) °C (to maintain melted medium in case of pour plate technique) and at additional test temperatures ± 1 °C, temperatures til 70 °C ± 1°C shall be adjustable.

5.3.2.3 Incubator, capable of being controlled either at (30 ± 1) °C or (36 ± 1) °C. 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 ± 1) °C.

A puncture electrode or a flat membrane electrode should be used for measuring the pH of the agar media (5.2.2.4 to 5.2.2.8).

5.3.2.5 Stopwatch, a digital stopwatch is recommended

5.3.2.6 Shakers

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

b) Orbital shaker

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 0,45 µm pore size for sterilization of hard water (5.2.2.3).

The vacuum source used shall give an even filtration flow rate. In order to obtain a uniform distribution of microorganisms over the membrane and 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)

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2) Disposable sterile 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.

5.3.2.12 Volumetric flasks

5.3.2.13 Centrifuge (3 000 g_N)

5.3.2.14 Coned bottom screw cap tubes (contents of 50 ml, diameter: about 28 mm)

5.3.2.15 Cylindrical screw vial (contents about 15 ml to 50 ml) for recovery of the test organisms from the carriers

5.3.2.16 Cotton carriers, 1 cm^2: The germ carriers are prepared by using standard cotton fabric thoroughly cooked in double-distilled water three times. The fabric is cut into 1 cm^2 cotton carriers and autoclaved [see 5.3.2.1a)]. For practical reasons the 1 cm^2 carriers are put in the pockets of a bigger cotton towel.

NOTE 1 The incorporation of the test suspension can be improved if the carriers are not dried after autoclaving.

Mass per unit area:	(170 ± 10) g/m^2 (real 160 g/m^2)
Fibrous material (wrap and weft):	cotton, double corded
Fibre length (wrap and weft):	at least 27 mm
Yarn linear density (wrap and weft):	(295 ± 10) dtex
Yarn twist (wrap and weft):	Z-twist (700 ± 25) t/m
Weave:	plain weave 1
Threads per unit length:	270 threads/dm each

The cotton control cloth shall be bleached, unfinished and not brightened.

After three washes the maximum tensile strength, wet, in the warp should be (63 ± 5) daN.

NOTE 2 Cotton proofed in accordance with DIN 53919 [6] fulfils the requirements.

5.3.2.17 Machine ballast load and preparation of ballast load

Textile of poly cotton (65 % polyester / 35 % cotton) shall be used as ballast load. For special purposes textile of cotton (100 %) can be used.

NOTE 1 The bounded liquor is higher in pure cotton than in poly cotton.

Amount of ballast load: 70 % ± 10 % of the max. capacity (in kg).

The ballast load should be washed for 30 min at 80 °C to 90 °C without additives following at least 3 rinsing steps and sterilized in the autoclave prior to first use.

The ballast load should be used for not more than 100 washing cycles (incl. preparation and disinfection cycles).

After disinfection testing the ballast load should be washed for 30 min at 80 °C to 90 °C following at least 5 rinsing steps (at least 5 min each) and sterilized in the autoclave prior to use.

NOTE 2 If foam is present in last rinsing step additional rinsing might be necessary.

5.3.2.18 Washing machine and preparation of the machine

The washing machine and additional equipment shall guaranty the following specifications:

a) Heating time to achieve 60 °C shall be < 10 min;

b) Thermostatic temperature control + 0,5°C in the range from 30 °C to 90°C temperature;

c) Switch on temperature ≤ 4 °C below switch-off temperature, accuracy at switch off ± 1 °C;

d) Timer need to be programmable;

e) Equipment for calibration of the water supply, e.g. a transparent tube for the control of the water level in the washing machine;

f) Separate addition of washing agent and disinfectant shall be possible. Dosage possibility for powder surfactants and liquid surfactants is necessary;

g) Program sequence shall be interrupted at any time and possibility for taking samples shall be given;

h) Water supply: Water supply should be controlled with a margin of ± 0,5 l or ± 0,5 kg (this can be achieved by for example a water flow meter or a weighbridge).

An example for description of specification for ordering a washing machine and additional equipment is given in the following:

1) Front loading horizontal rotating machine;

2) Inner drum volume: > 61 l;

3) Diameter of inner drum: > 52 cm;

4) Depth of inner drum > 31 cm);

5) Perforation: 2,5 mm to 5 mm, perforated cage surface min 600 cm^2;

6) Ratio (diameter of inner drum to depth of inner drum): 1,6 + 15 %;

7) Lifting vanes (ribs): 3 having a height 10 % to 12 % of diameter of cage; base width approximately 65 mm, spacing 120°;

8) Electric heating approximately KW 5,4 is recommended, thermostatically controlled;

9) Heating time to achieve 60°C < 10 min;

10) Thermostatic temperature control + 0,5 °C in the range from 30 °C to 90 °C temperature;

11) Switch on temperature ≤ 4 °C below switch-off temperature, accuracy at switch off ± 1 °C;

12) Timer need to be programmable;

13) Liquor ratio need to be adjustable;

14) Reversing rhythm: Normal/Gentle ON Normal/Gentle OFF, programmable (0 to 250) s, Step size 1 s (e.g. 12 s rotation in one direction, stop for 3 s, then reverse);

15) Time rotating at full washing speed to total washing time: 75 %;

16) Cold water supply: at water pressure 240 kPa ± 20 kPa;

17) Level sensing: step size ≤ 3 mm, repeatability ± 5 mm (±1 l);

18) Drain system: drain valve > 30 l/min.

The cleaning and disinfection procedure shall be adapted to the water hardness. Water hardness shall be logged mentioned in the laboratory protocol. The final hardness shall be equal or higher than 4 mmol/L alkaline earth ions (Mg^{2+} and Ca^{2+}). The temperature of the water influx should be between 12 °C and 20 °C. The water should contain less than 100 cfu/ml of bacteria at 36 °C and 22 °C.

It is recommended to keep the machine in an air conditioned room and control environmental conditions in temperature range of 20 °C to 25 °C and between 40 % to 60 % relative humidity.

Before each test run the machine should be run one time for 30 min at 80°C to 90°C without additives. No ballast load is needed for machine preparation.

It is recommended to document adequate machine parameters during the test runs to ensure a proper function of the machine and the process. This includes temperature in the wash liquor, amount of water influx, reversing profile and if possible on/off-status of heating element.

NOTE The temperature measurement with a separate calibrated data logger placed in the ballast load is preferred.

5.3.2.19 Fritted filter, porosity of 40 µm to 100 µm (see ISO 4793)

5.3.2.20 Roux bottles or similar flasks

5.3.2.21 Glass wool for filtration

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 each test organism, two different suspensions shall be prepared: the “test suspension” 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 organisms and their stock cultures shall be prepared and kept in accordance with EN 12353.

5.4.1.3 Working culture and test organisms

5.4.1.3.1 Bacteria

In order to prepare the working culture of the bacteria, subculture from the stock culture by streaking onto at least approximately three plates containing TSA (5.2.2.4) [*E. faecium*: also BHI (5.2.2.6)] can be used and incubate (5.3.2.3).

After 24 h incubation at (36 ± 1) °C or (37 ± 1) °C prepare a second subculture from the first subculture in the same way and incubate.

In the case of *E .faecium*, it is possible to prepare carriers with a shelf life of 12 weeks (storage conditions as below). In order to prepare the working culture of *E. faecium* for 250 test carriers, subculture from the stock culture by streaking onto at least approximately 15 plates or 10 Roux bottles containing TSA (5.2.2.4) (BHI (5.2.2.6) can be used and incubate (5.3.2.3). After 24 h incubation at (36 ± 1) °C or (37 ± 1) °C use these plates or Roux bottles respectively for inoculation of approximately 40 plates or 25 Roux flasks respectively.

Do not take a third subculture.

5.4.1.3.2 Mycobacteria

In order to prepare the working culture of the mycobacteria, subculture from the stock culture by streaking onto at least approximately three plates for each test containing 7H10 (5.2.2.8) and incubate (5.3.2.3).

In the case of M. terrae, it is possible to prepare carrier with a shelf life of 12 weeks (storage conditions as below).

Do not take any subculture.

To prepare 100 carriers with *M. terrae*, use 10 Petri dishes. The first culture will be the working culture. Do not take any subculture.

5.4.1.3.3 Yeasts and moulds

***Candida albicans* (yeast)**

In order to prepare the working culture of *Candida albicans*, subculture from the stock culture by streaking onto MEA (5.2.2.7) and incubate (5.3.2.3). After 42 h to 48 h prepare a second subculture from the first subculture in the same way and incubate for 42 h to 48 h.

From this second subculture, a third subculture may be produced in the same way. The second and third subcultures shall be the working culture(s). Do not take a fourth subculture.

***Aspergillus brasiliensis* (mould)**

In order to prepare the working culture of *Aspergillus brasiliensis*, subculture from the stock culture by streaking onto MEA (5.2.2.7) and incubate (5.3.2.3). For *Aspergillus brasiliensis* use only the first subculture grown on MEA (5.2.2.7) in Roux bottles (5.3.2.20) and incubate for 7 days to 9 days. No further sub culturing is needed.

5.4.1.4 Test suspension *(N)*

5.4.1.4.1 Bacterial test suspension

For harvesting the bacteria, 5 ml or 10 ml of 0,9 % NaCl solution are pipetted into the plates or the Roux bottles respectively and the bacteria are washed off and resuspended. If necessary the suspension is purified by filtration through glass wool (5.3.2.21) (e.g. for *E. faecium* see 5.4.1.3.1). The filtrate is aliquoted in screw cap tubes and centrifuged 15 min at 3 000g_N. The liquid is removed and the pellet is mixed with suitable volume of 0,9 % NaCl. The number of cells in the suspension shall be at least 1,5 x 10^9 cfu/ml[4].

NOTE For preparation of 10 carrier two Petri dishes or one Roux bottle are needed.

For *P. aeruginosa* centrifugation at 4 700 g is recommended.

To get the test suspension at the appropriate cfu/ml, centrifuge it again and resuspend it with defibrinated sheep blood (5.2.2.11) in the same volume.

For preparing 250 carriers, bacteria from 40 Petri dishes or 25 Roux bottles are necessary. Resuspend the bacteria in 1 ml sheep blood for each tube and resuspend carefully. Transfer the contents of all tubes in a graduated tube and fill up to 50 ml.

Spread a loop of this test suspension on a suitable agar plate and incubate for purity control.

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

a) For counting, prepare 10^{-7} and 10^{-8} dilutions of the test suspension using the diluent (5.2.2.9 and the primary used culture medium respectively). Mix [5.3.2.6a)]. 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 (5.2.2.4), 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 (5.2.2.4).

For incubation and counting see 5.5.2.9.

5.4.1.4.2 Fungal test suspension (N)

5.4.1.4.2.1 *Candida albicans*

For harvesting the cells, 5 ml or 10 ml 0,9 % NaCl solution are pipetted into the plates or the Roux bottles respectively and the cells are washed off and resuspended. The suspension is aliquoted in screw cap tubes and centrifuged for 15 min at 3 000g_N. The liquid is removed and the pellet is mixed with suitable volume of 0,9 % NaCl. The number of cells in the suspension shall be at least 1,5 x 10^8 cfu/ml.

To get the test suspension at the appropriate cfu/ml, centrifuge it again and resuspend it with defibrinated sheep blood (5.2.2.11) in the same volume.

a) For counting, prepare 10^{-6} and 10^{-7} dilutions of the test suspension using diluent (5.2.2.9). Mix (5.3.2.6a).

   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 1,0 ml sample into separate Petri dishes and add 15 ml to 20 ml melted MEA (5.2.2.7), cooled to (45 ± 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 MEA (5.2.2.7).

For incubation and counting, see 5.5.2.9.

5.4.1.4.2.2 *Aspergillus brasiliensis*

The procedure for preparing the *Aspergillus brasiliensis* test suspension is as follows:

a) Take the working culture (5.4.1.3.3) and suspend the conidiospores in 10 ml of sterile 0,05 % (*w/v*) polysorbate 80 solution in water (5.2.2.2). Using a glass rod or spatula, detach the conidiospores from the culture surface. Transfer the suspension into a flask and gently shake by hand for one minute together with 5 g of glass beads (5.3.2.11). Filter the suspension through a fritted filter (5.3.2.19);

b) Carry out a microscopic examination under x 400 magnification immediately after the preparation to show:

   1) the presence of a high concentration of characteristic mature conidiospores, i.e. spiny conidiospores (versus smooth spores). See Figures 1 and 2

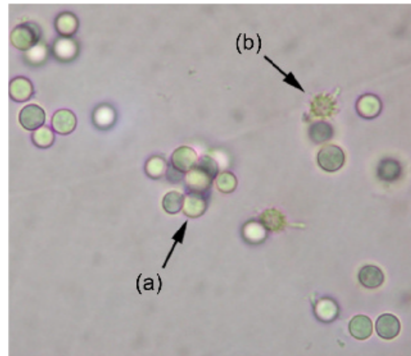

Figure 1 —Observation of conidiospores under light microscope: presence of smooth (a) and spiny (b) spores [inappropriate (not usable) suspension]

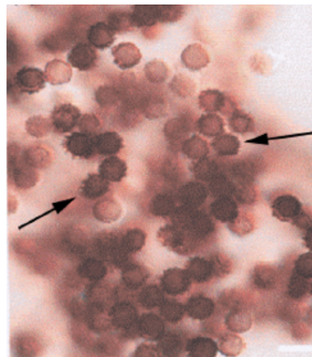

Figure 2 —Observation of conidiospores under light microscope: High concentration of characteristic mature spores with spiny aspect (appropriate suspension)

If there are less than 75 % spiny conidiospores it may be due to the *Aspergillus* strain used or to the media. Therefore use another strain, preferably from another supplier, and/or try out another media;

2) the absence of conidiospore germination (check at least 10 fields of view);

3) if germinated conidiospores are present, discard the suspension;

4) the absence of mycelia fragments (check at least 10 fields of view).

If mycelia are present, proceed to a second fritted filtration. If mycelia are still present, discard the suspension.

c) Adjust the number of conidiospores in the suspension to $1,5 \times 10^7$ cfu/ml to $5,0 \times 10^7$ cfu/ml using the diluent (5.2.2.9), estimating the number of cfu by any suitable means. Use the suspension within 4 h in a water bath controlled at (20 ± 1) °C (5.3.2.2). In any case, adjust the temperature according to 5.5.1.4 only immediately before the start of the test (5.5.2).

The use of a cell counting device for adjusting the number of cells is highly recommended. When using a suitable counting chamber, follow the instructions explicitly.

Each laboratory should therefore produce calibration data to establish the relationship between the counts obtained using the counting device and the counts (5.5.2.9) obtained by the pour plate or the spread plate technique [d)]. Experienced laboratories found a better fit to the required number of conidiospores when the conidiospore suspension count in the device was 10 % to 50 % higher than the number aimed at.

d) To get the test suspension at the appropriate cfu/ml, centrifuge it again and resuspend it with defibrinated sheep blood (5.2.2.11) in the same volume.

e) For counting, prepare 10^{-5} and 10^{-6} dilutions of the test suspension using diluent (5.2.2.9). 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 about half of each 1,0 ml sample into separate Petri dishes (i.e. in duplicate = four plates) and add 15 ml to 20 ml of melted MEA (5.2.2.7), cooled to (45 ± 1) °C;

   2) when using the spread plate technique, spread about one quarter of each 1,0 ml sample on an appropriate number (at least four) of surface dried plates containing MEA (5.2.2.7) (i.e. in duplicate – at least eight plates).

The technique used for counting of the test suspension shall be used for all other countings (5.5.2.2 to 5.5.2.8).

For incubation and counting see 5.5.2.9.

5.4.1.4.3 Mycobacterial test suspension (*N*)

a) Water with 1 % tween 80 should be used for harvesting the mycobacteria. The suspension is aliquoted in screw cap tubes and centrifuged 10 min at 2 000g_N with 4°C. Discard the supernatant. Repeat with step two times. Prepare a suitable homogeneous suspension from the working cultures (5.4.1.3.2) using either

   — the homogenization by glass beads: The pellet is resuspended in a suitable volume into a coned bottom screw cap tube (5.3.2.14) containing 6 g to 7 g of dry glass beads (5.3.2.11). The test organisms are homogenized by mixing (5.3.2.6) for at least 5 min to distribute them homogeneously on the beads and on most of the parts of the screw cap tube's internal surface. Add 5 ml water (5.2.2.2) drop by drop and resuspend them by mixing. After 20 min sedimentation time the supernatant is transferred to a tube.

   — or the homogenization by Potter S 1 apparatus: Resuspend the pellet to 5 ml water containing 0,1 % tween 80. Homogenized this suspension by using the Potter S 1 during ice cooling for 10 min.

NOTE Other methods of homogenization are allowed provided that the number of cfu/ml obtained is appropriate and stable during the time of the test and microscopic examination shows that the suspension is as homogeneous as after the two procedures described above.

   The use of spectrophotometer for counting the number of cells is highly recommended (about 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 found between 2 200 and 2 400. To achieve reproducible results of this measurement it may be necessary to dilute the test suspension, e.g. 1+9. A colourimeter is a suitable alternative.

b) The number of cells in the suspension shall be at least $1{,}5 \times 10^9$ cfu/ml by using water. Maintain this test suspension at 20 °C and use at the day of preparation. Mix with 5 ml sheep blood. These test suspension is sufficient for 100 carriers.

c) For counting prepare 10^{-7} and 10^{-8} dilutions of the test suspension using water (5.2.2.2). Mix (5.3.2.6). Take a sample of 1,0 ml of each dilution in duplicate and transfer divided in nearly equal amounts onto two separate plates (5.3.2.10) containing 7H10 (5.2.2.8), i.e. two plates per 1,0 ml sample, four plates per duplicate 1,0 ml samples.

For incubation and counting see 5.5.2.9.

5.4.1.5 Inoculation of the carriers

The germ carriers are dipped in the test suspension and repeatedly turned by sterilized forceps to wet them completely and remove all air bubbles. The carriers should stay in the suspension for 15 min. Subsequently, the germ carriers are without any delay transferred with a forceps in open Petri dishes for drying on filter paper at room temperature in a safety cabinet until visual dryness. Carrier with *P. areuginosa* shall be dried without blower for 12 h to 14 h.

Each carrier is then transferred by sterilized forceps into a cotton bag (one for each test organism).

Use the prepared carrier immediately after the end of the drying time (except *E. faecium*, *M. terrae*). Note the drying time in the test report.

Carrier contaminated with *E. faecium* or *M. terrae* can be used for 12 weeks, if they are dried at room temperature and by a relative humidity of 20 % (calcium chloride saturated solution) and stored at room temperature and by a relative humidity of 45 % (potassium carbonate saturated solution) in a closed vessel, e.g. a desiccator.

5.4.2 Product test solutions for validation tests

Product test solutions shall be prepared in hard water (5.2.2.3).

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 (5.2.2.3). Subsequent dilutions (=lower concentrations) shall be prepared in volumetric flasks (5.3.2.12) on a volume/volume basis in hard water.

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 validation tests within 2 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 flocculent (for example, through the addition of the interfering substance), it shall be recorded in the test report.

NOTE Counting microorganisms embedded in a precipitate or flocculent is difficult and unreliable.

The concentration of the product stated in the test shall be the desired test concentration. Record the test concentration in terms of weight 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

5.5.1 General

5.5.1.1 Experimental conditions (obligatory and additional)

a) Processes with temperatures < 60 °C

- Test organisms:
  - bactericidal activity: *Pseudomonas aeruginosa*, *Escherichia coli* (K12), *Staphylococcus aureus* and *Enterococcus hirae*
  - yeasticidal activity: *Candida albicans*
  - fungicidal activity (additional): *Aspergillus brasiliensis*

— tuberculocidal activity (additional): *Mycobacterium terrae*

— mycobactericidal activity (additional): *Mycobacterium avium* and *Mycobacterium terrae*

Beside the obligatory test organisms additional test organisms may be selected according to the practical use considered for the product.

— Test temperature:
The tests are carried out at the process temperatures

— Interfering substance:
The obligatory interfering substance to be tested is sterile defibrinated sheep blood (see 5.2.2.11) (12,5 ml sheep blood per kg textile)

b) Processes with temperatures ≥ 60 °C

— Test organism: *Enterococcus faecium*

— Test temperature:
The tests are carried out at the process temperatures

— Interfering substance:
The obligatory interfering substance to be tested is sterile defibrinated sheep blood (see 5.2.2.11) (12,5 ml sheep blood per kg textile)

5.5.1.2 Choice of neutralizer

The neutralizer shall be chosen according to laboratory experience and published data. Some suitable neutralizers are given in Annex B.

In special circumstances it may be necessary to add neutralizer to the growing medium (5.2.2.4, 5.2.2.6, 5.2.2.7, 5.2.2.8).

5.5.1.3 General instructions for validation and control procedures

The neutralization and/or removal of the microbicidal activity of the product shall be controlled and validated for each of the used test organisms and for each experimental condition (interfering substance, temperature, contact time). These procedures (experimental condition control, neutralizer control and method validation) shall be performed at the same time with the test and with the same neutralizer used in the test.

If because of problems with neutralization a neutralizer (5.5.1.2) shall be added to the agar used for the validation and control procedures. The agar used for the test shall contain the same amount of this neutralizer as well.

5.5.1.4 Equilibration of temperature for validation tests

Prior to testing, equilibrate all reagents (product test solutions (5.4.2), validation suspension (5.5.2.6) and diluent (5.2.2.9) to the test temperature (5.5.1.1) using the water bath (5.3.2.2). Check that the temperature of the reagents is stabilized.

The neutralizer (5.2.2.10) and water (5.2.2.2) shall be equilibrated at (20 ± 1) °C.

5.5.1.5 Precautions for manipulation of test organisms

Do not touch the upper part of the test tube sides when adding the test- or the validation suspensions.

5.5.2 Method[5)]

5.5.2.1 General

Ballast load is specified in 5.3.2.16. The type of washing machine used shall be specified in the test report and fulfil the requirements given in 5.3.2.17.

The dosages of the detergent and the disinfectant, as well as the bath parameters, are set according to manufacturer's instructions.

A time dependant temperature profile of the whole cycle shall be recorded and given in the test report.

The disinfection time starts when the required washing temperature is reached. During the disinfection process, attention shall be paid to temperature deviations. Variations shall be specified in the report.

5.5.2.2 Test 'N_a' (Determination of microbicidal concentrations) and reference 'N_0' (Microbial content on carriers)

a) After filling the washing machine with textile (see 5.3.2.16, corresponding to the ballast load recommended) and cotton carriers (10 cotton carriers inside bags per test organism) 12,5 ml defibrinated sheep blood (5.2.2.11) per kg ballast load is added before water inflow.

b) The cleaning and disinfection cycle shall be performed in accordance to the pre-designed procedure [(application concentration of the disinfectant / disinfectant + cleaner (if a combined application is foreseen in the processing procedure], process temperature, contact time, processing procedure, liquor ratio.

   After accomplishing the disinfection step i.e. before rinsing the textile, the washing process shall be stopped and the bags with the carriers shall be removed immediately and directly transferred in vials (one per carrier) containing 5 ml neutralizer in diluent and glass beads (see 5.3.2.11).

   Mix (5.3.2.6b)) for 10 min and transfer 1 ml of the undiluted mixture and additional 1 ml of a 10^{-1} and 10^{-2} dilutions.

   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 (5.2.2.4) *E. faecium*: also BHI can be used; *C. albicans* and *A. brasiliensis*: MEA and for mycobacteria: 7H10, cooled to 45 °C ± 1 °C[6)].

   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 (5.2.2.4). [7)]

   The culture medium shall be the same one which was used for preparing the stock and working cultures. Additionally, the cotton carriers are also placed in separate pouring or on spreading plates.

c) After accomplishing the disinfection step, 100 ml washing liquor is withdrawn and immediately mixed with 100 ml double-concentrated TSB (containing neutralizer).

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5) For a graphical representation of this method see C.1.

6) Only spread plate technique could be used for *P. aeruginosa.*

7) Only spread plate technique could be used for *P. aeruginosa.*

After homogenization, transfer 1 ml of the undiluted mixture and additional 1 ml of a 10^{-1} dilution in TSB see b) 1) and 2).

d) The microbial content of at least 3 non treated carriers shall be determined for each test series.

e) The mean value of the 3 non treated carriers ('N_0') for each test series serves as a basis for calculating the reduction after exposure.

For incubation and counting see 5.5.2.9.

5.5.2.3 Reference control 'N_W' (Test without detergent or disinfectant)

In order to detect the mechanical effect of the washing process, perform the test procedure once with 10 contaminated carriers per test organism ('N_W') using no detergent or disinfectant.

Follow the instructions given in 5.5.2.2 (but use appropriate dilutions for plating e.g. 10^{-2} to 10^{-4}).

5.5.2.4 Reference control '*R*I' (Non contaminated cotton carrier in the test procedure)

6 non contaminated cotton carriers shall be subjected to the procedure in addition to the contaminated carriers in one test procedure series, described in 5.5.2.2.

Follow the instructions given in 5.5.2.2.

No growth of test organisms shall be detected in the corresponding cultures.

5.5.2.5 Reference control '*R*II' (Test with detergent only)

Perform one test series checking the reduction and proof of microorganisms (10 germ carriers per test organism) using detergent, but no disinfectant.

Follow the instructions given in 5.5.2.2 (but use appropriate dilutions for plating e.g. 10^{-2} to 10^{-4}).

5.5.2.6 Validation suspension (N_V, N_{VB})

a) To prepare the validation suspension (N_V), transfer one carrier (5.4.1.5) in a vial containing 5 ml diluent [5.2.2.9 and water (5.2.2.2 for mycobacteria) respectively] and extract the test organisms by shaking and dilute the solution to obtain $3{,}0 \times 10^2$ cfu/ml to $1{,}6 \times 10^3$ cfu/ml.To prepare the validation suspension for the neutralizer control (5.5.2.7) (N_{VB}) dilute the suspension to obtain $3{,}0 \times 10^4$ cfu/ml to $1{,}6 \times 10^5$ cfu/ml.

b) For counting of the validation suspension N_V prepare a 10^{-1} dilution but prepare a 10^{-3} dilution of the validation suspension to count the neutralizer control N_{VB}.

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

For incubation and counting see 5.5.2.9.

5.5.2.7 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 (5.5.2.2) into a tube. Add 1,0 ml of the validation suspension (N_{VB}) (5.5.2.6) containing $3{,}0 \times 10^4$ cfu/ml to $1{,}6 \times 10^5$ cfu/ml. Start the stopwatch at the beginning of the addition, mix. 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}. Place the tubes of the

10^{-2} dilution of N_{VB} in a water bath (5.3.2.2) controlled at (20 ± 1)°C for 5 min ± 10 s. Just before the end of this time, mix.

NOTE The high amount of neutralizer in relation to the test organisms reflects the additional dilutions with neutralizer – in the case of N_a (5.5.2.2) 10^{-1}.

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 using the pour plate or the spread plate technique.

For incubation and counting see 5.5.2.9.

5.5.2.8 Method validation 'C' – dilution-neutralization validation

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

a) Pipette 2,0 ml of the diluent (5.2.2.9) into a tube and then, starting a stopwatch, add 8,0 ml of the product test solution only of the highest concentration used in the test. Mix and place the tube in a water bath (5.3.2.2) controlled at the test temperature θ for t. Just before the end of t, mix 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 5.5.2.2). Restart the stopwatch at the beginning of the addition. Mix and place the tube in a water bath controlled at (20 ± 1) °C for 5 min ± 10 s. Add 1,0 ml of the validation suspension (N_V) (5.5.2.6). Start a stopwatch at the beginning of the addition and mix. 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 again. At the end of this time, take a sample of 1,0 ml of this mixture C in duplicate and inoculate using the pour plate or the spread plate technique.

For incubation and counting see 5.5.2.9.

5.5.2.9 Incubation and counting of the test and the validation suspensions

a) Incubate (5.3.2.3) the plates of test organisms without contact with the disinfectant for 20 h to 24 h (enterococci: 48 h, mycobacteria: 21 days).The culture of test organisms after contact with the disinfectant shall be incubated 7 d longer then the regular incubation time at least. Discard any plates that are not countable for any reason. Count the plates and determine the number of cfu.

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} (neutralizer control 5.5.2.7) using the methods given in 5.6.2.3 and 5.6.2.5. Verify according to 5.7.

d) Calculate the numbers of cfu/ml on the germ carriers in the test mixtures N_a and N_0 and in the reference and validation mixtures N_W, RI, RII, B and C using the methods given in 5.6.2.2 b), 5.6.2.3 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

Table 1 — Number of cells counted per ml

	Number of cells in the microbial suspensions cfu/ml	Number of cells of the controls	Number of cells per untreated carriers cfu/ml	Number of survivors per treated carrier cfu/ml
Test and reference controls	N Test suspension		N_0	N_a N_W, RI, RII
Validation controls	N_V Validation suspension N_{VB} Validation suspension for control B	C B		

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, N_{VB}, N_0, N_{VB0}, N_a, N_W, RI, RII, B and C. The third step is the calculation of the reduction R (5.8).

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, 14 and 165 for *Aspergillus brasiliensis.*

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 limits (330, 165) reflect 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.

b) For counting the test suspension N (5.4.1.4), the validation suspensions N_V and N_{VB} (5.5.2.6) and for all countings of the method, references and validation controls (5.5.2.9) 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 (but substitute by “<14” for further calculations in the case of N_a).

c) Only V_C-values within the counting limits are taken into account for further calculation, except in the case of N_a (5.6.2.4).

5.6.2.3 Calculation of N and N_0 (N_0 is not described)

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

Since two dilutions of the test suspension (5.4.1.4 in connection with 5.6.1.1) are evaluated, calculate the number of cfu/ml as the weighted mean count using the following equation:

$$N = \frac{c}{(n_1 + n_2)d} \quad (1)$$

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, i.e. 10^{-6};

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

d is the dilution factor corresponding to the lower dilution (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 1,00 and 9,90 multiplied by the appropriate power of 10.

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

5.6.2.4 Calculation of N_a

N_a is the number of survivors per ml in the test mixture [5.5.2.2a)] at the end of the contact time and before neutralization.

a) Calculate the mean for each dilution step N_a^0, N_a^{-1} using the following equation:

$$N_a^0,\ N_a^{-1} = 10\ c/n \quad (2)$$

where

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

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

If one or both of the duplicate V_C-values are either below the lower or above the upper limit, express the results as "less than" or "more than".

b) For calculation of N_a use only N_a^0, N_a^{-1} results, where one or both V_C-values are within the counting limits.

c) Use maximum 2 subsequent dilutions for calculating N_a as a weighted mean.

5.6.2.5 Calculation of N_V and N_{VB}

N_V is the number of cells per ml in the validation suspension (5.5.2.6).

N_{VB} is the number of cells per ml in the validation suspension for the neutralization control (5.5.2.7).

Calculate N_V and N_{VB} using the following equations:

$$N_V = 10\ c/n \qquad (3)$$

$$N_{VB} = 1000\ c/n \qquad (4)$$

where

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

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

5.6.2.6 Calculation of N_W, *RI*, *RII*, *B* and *C*

N_W, *RI*, *RII*, *B* and *C* are the numbers of survivors in the reference and validation controls (5.5.2.3 to 5.5.2.8) at the end of the contact time *t*. They correspond to the mean of the V_C-values of the mixtures N_W, *RI*, *RII*, *B* and *C* taken into account.

Calculate N_W, *RI*, *RII*, *B* and *C* using the following equation:

$$N_W, RI, RII, B, C = c/n \qquad (5)$$

where:

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

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

5.7 Verification of methodology

5.7.1 General

A test is valid if:

— all results meet the criteria of 5.7.3; and

— the requirements of 5.9.2 are 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 mean of the two results 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 [5.6.2.2b)] 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

For each test organism check that: N_W, RI, RII, B and C

a) For bacteria and mycobacteria check that:

N is above $1,5 \times 10^9$ ($9,18 \le lg\ N$)

and

N_0 is above $1,5 \times 10^7$ ($7,18 \le lg\ N_0$)

For yeasts and moulds check that:

N is above $1,5 \times 10^8$ ($8,18 \le lg\ N$)

and

N_0 is above $1,5 \times 10^6$ ($6,18 \le lg\ N_0$)

b) For each test organism check that:

N_V is between $3,0 \times 10^2$ and $1,6 \times 10^3$
N_{VB} is between $3,0 \times 10^4$ and $1,6 \times 10^5$

c) For each test organism check that:

N_W	equal or less 10^3
RI	no growth should be detectable
RII	shall be less or equal to $10^3 \ge N_w$
C	are equal to or greater than $0,05 \times N_V$
B (dil. neutr.)	is equal to or greater than $0,0005 \times N_{VB}$ (half of one thousandth)

d) For control of weighted mean counts (5.7.2) check that the quotient is not lower than 5 and not higher than 15.

5.8 Expression of results and precision

5.8.1 Reduction

The reduction ($R = N_0 / N_a$) is expressed in decimal logarithm.

For each test organism record the number of cfu/ml in the carrier N_0 (5.6.2.3) and in the test N_a (5.6.2.4).

For each product concentration and each experimental condition, calculate and record the decimal log reduction (lg) separately using the equation:

$$\lg R = \lg N_0 - \lg N_a$$

For the reference controls, record N_0, the results of N_W and RII (*5.6.2.6*) and their comparison with N_0

For the validation controls, record N_V (5.5.2.8) and N_{VB}, (5.5.2.7) the results of B (and C and their comparison with N_V and N_{VB} respectively.

5.8.2 Repetitions

For precision and repetitions, see EN 14885.

5.9 Interpretation of results – conclusion

5.9.1 General

According to the chosen experimental conditions (obligatory or obligatory and additional) the microbicidal concentrations determined according to this standard may differ (Clause 4).

5.9.2 Microbicidal activity

5.9.2.1 Processes at temperatures < 60°C

a) Bactericidal activity

The product shall be deemed to have passed this standard if it demonstrates in three valid tests for textile disinfection products up to 60 °C under the conditions defined by this standard when the test organisms *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Enterococcus hirae show* at least a 7 lg reduction for each carrier.

b) Yeasticidal activity

The product shall be deemed to have passed this standard if it demonstrates in three valid tests for textile disinfection products up to 60 °C under the conditions defined by this standard when the test organism *Candida albicans shows* at least a 6 lg reduction for each carrier.

c) Fungicidal activity (additional)

The product shall be deemed to have passed this standard if it demonstrates in three valid tests for textile disinfection products up to 60 °C under the conditions defined by this standard when the test organism *Aspergillus brasiliensis shows* at least a 6 lg reduction for each carrier.

d) Tuberculocidal activity (additional)

The product shall be deemed to have passed this standard if it demonstrates in three valid tests for textile disinfection products up to 60 °C under the conditions defined by this standard when the test organism *Mycobacterium terrae shows* at least a 7 lg reduction for each carrier.

e) Mycobactericidal activity (additional)

The product shall be deemed to have passed this standard if it demonstrates in three valid tests for textile disinfection products up to 60 °C under the conditions defined by this standard when the test organism *Mycobacterium avium shows* at least a 7 lg reduction for each carrier.

5.9.2.2 Processes at temperatures ≥ 60°C

The product shall be deemed to have passed this standard if it demonstrates in a valid test for textile disinfection products ≥ 60 °C under the conditions defined by this standard when the test organism *Enterococcus faecium* (obligatory) shows at least a 7 lg reduction, however additional microorganisms can be tested as well.

5.10 Test report

The test report shall refer to this European Standard.

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

Annex A
(informative)

Referenced strains in national collections

- *Pseudomonas aeruginosa*	ATCC	15442
	DSM	939
	CIP	103467
	NCIMB	10421
- *Escherichia coli K12*	DSM	11250
	CIP	54.117
	NCTC	10538
	NCIB	10083
- *Staphylococcus aureus*	ATCC	6538
	DSM	799
	CIP	4.83
	NCTC	10788
	NCIMB	9518
- *Enterococcus faecium*	ATCC	6057
	DSM	2146
	CIP	106742
- *Enterococcus hirae*	ATCC	10541
	DSM	3320
	CIP	58.55
	NCIMB	8192
- *Candida albicans*	ATCC	10231
	DSM	1386
	CIP	48.72
	IFO	1594
	NBRC	1594
	NCPF	3179
	NCYC	1363
- *Aspergillus brasiliensis*	ATCC	16404
	DSM	1988
	CIP	1431.83
	NBRC	9455
	NCPF	2275

- *Mycobacterium avium*	ATCC	15769
	DSM	44157
	CIP	105415
- *Mycobacterium terrae*	ATCC	15755
	DSM	43227
	CIP	104321T
	NCTC	10856

Annex B
(informative)

Suitable neutralizers and rinsing liquids

B.1 General

The weights given in B.2 and B.3 refer to the anhydrous salts (5.2.2.1).

The lists in B.2 and B.3 are not exhaustive and other reagents may be used.

B.2 Neutralizers

Any of the following neutralizers may be used:

— lecithin 3 g/l; polysorbate 80[8] 30 g/l; sodium thiosulphate ($Na_2S_2O_3$) 5 g/l; L-histidine 1 g/l; saponin 30 g/l in diluent (5.2.2.9) or in phosphate buffer 0,0025 mol/l;

— phosphate buffer 0,25 mol/l: potassium dihydrogen phosphate (KH_2PO_4) 34 g; water (5.2.2.2) 500 ml; adjusted to pH 7,2 ± 0,2 with sodium hydroxide (NaOH) 1 mol/l; water (5.2.2.2) up to 1 000 ml;

— fresh egg yolk diluted to 5 % or 0,5 % (v/v);

— 30 g/l polysorbate 80; 4 g/l sodium dodecyl sulphate ($C_{12}H_{25}NaO_4S$); lecithin 3 g/l;

— fresh egg yolk diluted to 5 % (v/v); 40 g/l polysorbate 80;

— 7 % (v/v) ethylene oxide condensate of fatty alcohol; 20 g/l lecithin; 4 % (w/v) polysorbate 80;

— 4 % (v/v) ethylene oxide condensate of fatty alcohol; 4 g/l lecithin;

— 30 g/l polysorbate 80; lecithin 3 g/l; L-histidine 1 g/l;

— glycine as a function of the product concentration;

— 30 g/l polysorbate 80; lecithin 3 g/l;

— phospholipid emulsion (commercial) at 50 mg/ml (diluted 1 to 10);

— sodium thioglycollate at 0,5 g/l or 5 g/l;

— L-cysteine at 0,8 gl or 1,5 g/l;

— thiomalic acid at 0,075 % (v/v) - adjusted to pH 7,0 with sodium hydroxide (NaOH);

— sodium thiosulphate at 5 g/l;

---

[8] Analytical quality, non-hydrolyzed in accordance with the European Pharmacopoeia. TWEEN 80 is 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.

— catalase or peroxidase: one unit (U) of these enzymes catalyses at 25 °C ± 1 °C the decomposition of 1 µmol of hydrogen peroxide per minute at pH 7,0;

— polysorbate 80 30 g/l; saponin 30 g/l; L-histidine 1 g/l; L-cysteine 1 g/l.

— 0,5 % sodium sulfit, 10mM β-cyclodextrine in 0,1M phosphate buffer (stability of 1 h after preparation).

B.3 Neutralizer added to the agar for counting

Any of the following neutralizers may be used:

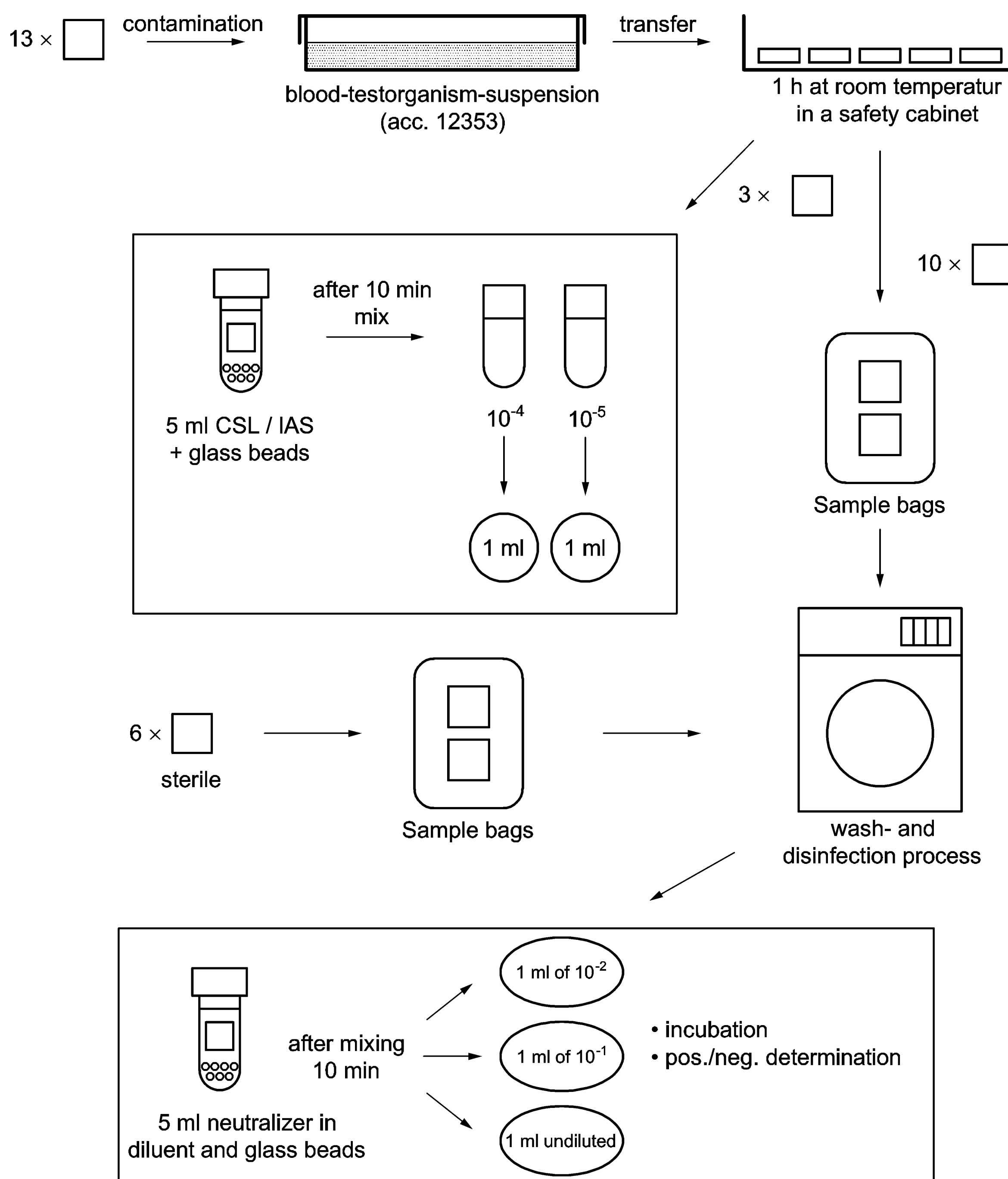
— 10 % (v/v) of a solution containing 0,7 g/l lecithin and 5 % (w/v) polysorbate 80;

— 10 % (v/v) of a solution containing 10 g/l lecithin and 5 % (w/v) polysorbate 80;

— 10 % (v/v) of a solution containing fresh egg yolk 1,5 % (w/v) and 5 % (w/v) polysorbate 80.

Annex C
(informative)

Graphical representations of the test method



Test material: 19 pieces standard cotton, 1 cm × 1 cm

Figure C.1 — Chemical-thermal textile disinfection - Test method

Annex ZA
(informative)

Relationship between this European Standard and the Essential Requirements of EU Directive 93/42/EEC concerning medical devices

This European Standard has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association to provide a means of conforming to the Essential Requirements of Directive 93/42/EEC.

Once this standard is cited in the Official Journal of the European Union under that Directive and has been implemented as a national standard in at least one Member State, compliance with the normative clauses of this standard given in Table ZA.1 confers, within the limits of the scope of this standard, a presumption of conformity with the corresponding Essential Requirements of that Directive and associated EFTA Regulations.

NOTE 1 Where a reference from a clause of this standard to the risk management process is made, the risk management process needs to be in compliance with Directive 93/42/EEC/Directive. This means that risks have to be reduced 'as far as possible', 'to a minimum', 'to the lowest possible level', 'minimized' or 'removed', according to the wording of the corresponding essential requirement.

NOTE 2 The manufacturer's policy for determining **acceptable risk** must be in compliance with essential requirements 1, 2, 5, 6, 7, 8, 9, 11 and 12 of the Directive.

NOTE 3 When an Essential Requirement does not appear in Table ZA.1, it means that it is not addressed by this European Standard.

Table ZA.1 — Correspondence between this European Standard and Directive 93/42/EEC concerning medical devices

Clause(s)/subclause(s) of this European Standard	Essential Requirements (ERs) of Directive 93/42/EEC	Qualifying remarks/notes
Clause 1, Clause 2, Clause 3 and Clause 4	Annex I, 6a (in connection with annex X, 1.1d)	This standard is intended for products whose main efficacy claim is of a microbicidal nature, e.g. chemical disinfectants and antiseptics. Complying with the requirements of this standard demonstrates the bactericidal, mycobactericidal, tuberculocidal, fungicidal or yeasticidal activity of a product for textile disinfection. Generally at least one additional European Standard (phase 2, step 1) has to be complied with to demonstrate a sufficient performance evaluation of such products.

WARNING — Other requirements and other EU Directives may be applicable to the product(s) falling within the scope of this standard.

Bibliography

[1] EUROPEAN PHARMACOPOEIA (EP). Edition 1997 supplement 2000, Water for injections

[2] EN 285, *Sterilization — Steam sterilizers — Large sterilizers*

[3] EN 14561, *Chemical disinfectants and antiseptics — Quantitative carrier test for the evaluation of bactericidal activity for instruments used in the medical area — Test method and requirements (phase 2, step 2)*

[4] EN 14562, *Chemical disinfectants and antiseptics — Quantitative carrier test for the evaluation of fungicidal or yeasticidal activity for instruments used in the medical area — Test method and requirements (phase 2, step 2)*

[5] EN 14563, *Chemical disinfectants and antiseptics — Quantitative carrier test for the evaluation of mycobactericidal or tuberculocidal activity of chemical disinfectants used for instruments in the medical area — Test method and requirements (phase 2, step 2)*

[6] DIN 53919, *Test cotton fabrics for laundering process control — Requirements*

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