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Chemical disinfectants and antiseptics - Methods of airborne room disinfection by automated process - Determination of bactericidal, mycobactericidal, sporicidal, fungicidal, yeasticidal, virucidal and phagocidal activities

Antiseptiques et désinfectants chimiques - Méthodes de désinfection des pièces par voie aérienne par des procédés automatisés - Détermination de l'activité bactéricide, fongicide, levuricide, sporicide, tuberculocide, mycobactéricide, virucide et phagocide

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Chemical disinfectants and antiseptics - Methods of airborne room disinfection by automated process - Determination of bactericidal, mycobactericidal, sporicidal, fungicidal, yeasticidal, virucidal and phagocidal activities

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Chemische Desinfektionsmittel und Antiseptika - Verfahren zur luftübertragenen Raumdesinfektion durch automatisierte Verfahren - Bestimmung der bakteriziden, mykobakteriziden, sporiziden, fungiziden, levuroziden, viruziden, tuberkuloziden, und Phagen-Wirksamkeit

This European Standard was approved by CEN on 13 October 2019.

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European foreword

This document (EN 17272:2020) has been prepared by Technical Committee CEN/ TC 216 "Chemical disinfectants and antiseptics", the secretariat of which is held by AFNOR.

This document shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by October 2020, and conflicting national standards shall be withdrawn at the latest by October 2020.

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

This document describes a Phase 2 step 2 method designed:

— to check, under standardized laboratory conditions close to real-world practice, that the proposed airborne surface disinfection processes meet the objective for which they were devised;

— to cross-compare different processes under reproducible conditions;

— to provide an experimental design within specified limits when real-world-practice conditions depart from the conditions given in the text below.

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

Introduction

The purpose of this document is to describe a test method for assessing the disinfectant activity of airborne surface disinfection processes under a specific experimental condition.

The proposed test method consists of 2 parts:

— Part 1 - Efficacy test: intended to ensure that minimum efficacy requirements are fulfilled for each type of activity claimed and for the targeted application area(s) (CEN/TC 216 WG 1 and/or WG 2 and/or WG 3).

— Part 2 - Distribution test: intended to ensure efficacy of the process throughout the enclosure. It is performed with a reference test organism at 4 sampling positions.

The processes concerned include those involving chemical disinfectants in dispersed gaseous, vapour and/or aerosolised form.

Every automated airborne disinfection cycle/application is unique and the purpose of this document is to provide a defined challenge for the automated airborne disinfection system to successfully meet in order to be considered an efficacious process. This standard method should therefore be regarded as a useful starting point and not as a validation for all intended treatments with a particular automated airborne disinfection system.

The method is used to qualify the process, i.e. the device(s) and product(s) needed for implementation. For such chemical processes, the combination of device and product cannot be separated.

For the defined test conditions the number of carriers and their test positions can be increased according to specific needs of a given application or local requirements.

The manufacturer:

— specifies the limitations and precautions for use of the process;

— ensures that the specified test conditions are representative for the recommended application(s).

The aim of this document is to simulate practical conditions of airborne disinfection in a laboratory situation; obligatory conditions are defined according to the test method defined below. Additional conditions are also proposed.

The test report specifies and summarizes the conditions under which the tests are carried out.

Generally, the processes are implemented after a cleaning procedure and then tested, according to the application areas, under clean or low-level soiling conditions. For specified applications and/or according to the manufacturer recommendations, test methods with other interfering substance can also be envisaged as additional conditions.

The tests described in this document are based on measuring the reduction (expressed as decimal logarithm lg) in terms of numbers of surviving test organisms of different strains of bacteria, mycobacteria, bacterial spores, fungal spores, yeasts, viruses or bacteriophages and under specified conditions. Test organisms may be supplemented by other test organisms. The experimental design described in this document is expected to be followed, but the conditions can be varied according to the needs of the practical application(s).

This method can be used as a basis for biosecurity applications in laboratories.

CEN/TC 216 phase 2, step 1 suspension tests for evaluating the irreversible inactivation by the product cannot be performed as the product is changed by the diffusion through the air (e.g. liquid state vs vapour state).

1 Scope

The test methods described are designed to determine the disinfectant activity of processes used in the 1) medical area, 2) veterinary area, 3) food, industrial, domestic and institutional area using automated processes for distributing chemicals by air diffusion with no operator manually applying the disinfectant. This document covers the disinfection of nonporous surfaces but not that of the air.

The objective of the described processes is to disinfect the surfaces of the overall area including the external surfaces of the equipment contained in such rooms. Air handling and products or processes specifically designed for the disinfection of medical devices are excluded from the scope of this document. The test methods and volumes described provide a defined challenge.

This document is applicable to processes for which activity is claimed against the following groups of microorganisms:

— vegetative bacteria,

— mycobacteria,

— bacterial spores,

— yeasts,

— fungal spores,

— viruses,

— bacteriophages.

This document does not cover processes for which the mode of action is based on immersing and/or circulation, flooding, spraying, wiping or other processes where the product is directly applied to the surfaces and not via air dispersion.

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 10088-2, Stainless steels —Part 2: Technical delivery conditions for sheet/plate and strip of corrosion resisting steels for general purposes

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 of EN 14885 and the following definitions apply:

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

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

3.1
chemical process
process in which the active substance is a chemical agent (product) diffused in gas, liquid and/or solid form

Note 1 to entry: The product and the diffusion system (device) cannot be evaluated separately.

3.2
automated airborne disinfection process
process diffusing a product in the form of a gas, vapour and/or an aerosol (excluding aqueous steam) from a device, without the need for human intervention, targeting surfaces and not the air

3.3
airborne disinfection contact time
ADC time
time from the first release of the product (disinfectant) to the point where carriers are recovered or to the point where aeration starts, if an aeration time is considered necessary

Note 1 to entry: The carriers can be recovered at the end of ADC time, or during the aeration time, with suitable personal protective equipment (PPE) where necessary.

3.4
aeration time
period of time during which an air exchange of the enclosure achieves an appropriate concentration of the product in the enclosure based on the manufacturer's use instructions and risk assessment, to enable the recovery of the carriers

Note 1 to entry: The duration of this aeration time is dependent of the air treatment system characteristics.

3.5
distribution test
placement of test-carriers loaded with test organisms in such a way that the distribution of a product by the combination of a device (machine) and the product achieves its claimed activity throughout the enclosure

Note 1 to entry: This test is performed after or in parallel with the efficacy test.

3.6
supplementary obligatory conditions
test conditions used instead of the obligatory conditions where the practical use of the process and the manufacturers claims are clearly and unambiguously excluding the obligatory test conditions

3.7
sensitive test organism
test organism where the drying causes a lg reduction of more than 1,5 by the end of the aeration time

4 Requirements

4.1 Efficacy tests

The automated airborne disinfection process to be tested under the obligatory experimental conditions defined in 5.5 shall lead to the following reductions in terms of numbers, expressed in decimal log (lg):

Bactericidal activity:

— 5 lg or greater reduction on test-carriers compared to control-carriers not exposed to the process, for each of the four specified bacterial test organisms.

For medical area, refer to 5.2.1.2, 5.2.1.3, 5.2.1.4, 5.2.1.5.

For veterinary area, refer to 5.2.1.1, 5.2.1.2, 5.2.1.3, 5.2.1.6.

For food, industrial, domestic and institutional area, refer to 5.2.1.1, 5.2.1.2, 5.2.1.3, 5.2.1.4.

Mycobactericidal activity:

— 4 lg or greater reduction for medical area and food industry and laboratory area on test-carriers comparative to control-carriers not exposed to the process for the two test organisms implemented, refer to 5.2.1.15, 5.2.1.16.

— 4 lg or greater reduction for veterinary area on test-carriers comparative to control-carriers not exposed to the process for the specified test organism, refer to 5.2.1.15.

Sporicidal activity:

— 4 lg or greater reduction for medical area, 3 lg for veterinary area and food, industrial, domestic and institutional area on test-carriers compared to control-carriers not exposed to the process for the specified bacterial spore test organism, refer to 5.2.1.7.

Fungicidal activity:

— 4 lg or greater for medical area, veterinary area and food, industrial, domestic and institutional area on test-carriers compared to control-carriers not exposed to the process for the two specified fungal test organisms (yeast and fungal spore), refer to 5.2.1.8 and 5.2.1.9.

Yeasticidal activity:

— 4 lg or greater reduction for medical area, veterinary area and food industry and laboratory area on test-carriers comparative to control-carriers not exposed to the process for the specified yeast test organism, refer to 5.2.1.8.

Virucidal activity:

— 4 lg or greater reduction for medical area and food industry and laboratory area on test-carriers comparative to control-carriers not exposed to the process, for the two specified test organisms, refer to 5.2.1.10 and 5.2.1.11.

— 4 lg or greater reduction for veterinary area on test-carriers comparative to control-carriers not exposed to the process, for the specified test organism, refer to 5.2.1.12.

Phagocidal activity:

— 4 lg or greater reduction for food industry and laboratory area on test-carriers comparative to control-carriers not exposed to the process for the specified two test organisms, refer to 5.2.1.13 and 5.2.1.14.

The requirements and test methods are summarized in Annex A.

The reductions are expressed as decimal logarithmic values (lg).

The reductions indicated in the table of Annex A are the minimum reductions to be obtained on test-carriers in comparison to control-carriers not exposed to the process.

These activities may be determined independently, i.e. by separate testing of the different microbiological groups, e.g. mycobacteria, and do not all shall be undertaken simultaneously. Each activity can be claimed independently but passing both bactericidal and yeasticidal activity as described in Annex A is the minimum requirement to claim compliance with this document.

These seven activities shall be determined under the standard-reference experimental conditions defined in 5.5.

Additional specific bactericidal, mycobactericidal, sporicidal, fungicidal, yeasticidal, virucidal, and phagocidal activity can, as appropriate, be determined under other conditions (e.g. other ADC times, type of carriers, test organisms, volume of the enclosure, temperature, humidity), for specific intended uses. These additional conditions shall be described, recorded and reported in the test report.

Even if an automated airborne disinfection system has passed all or part of the test method described here, the system and its delivered cycles shall then be validated in the individual enclosure (e.g. hospital room, or animal house) in practice to be disinfected using appropriate biological or validated chemical indicators.

4.2 Distribution tests

The reduction in the number of viable bacterial cells throughout the enclosure shall be determined with Staphylococcus aureus ATCC 6538 = CIP 4,83 (refer to 5.2.1.2) to achieve 5 lg or greater reduction on each test–carrier. Test-carriers shall be located in accordance with Annex A, part 2. Test conditions shall replicate those used in the efficacy tests.

5 Test method

5.1 Principle

5.1.1 Preliminary test to validate absence of residual effect

The aim of the preliminary test is to identify whether there is any residual activity due to residual product transferred via the carriers into the subculture media (agar plates and/ or membranes) and to find a method for eliminating this effect. This procedure should ensure that the results of the efficacy test are based only on the irreversible inactivation of the test organisms and not on an inhibitory (static) effect.

Non-contaminated carriers are exposed to the process after deposition and drying of the interfering substance used in the assay.

Recovery into 100 ml (20 ml for virucidal activity) of sterile liquid medium, and testing for microbiostatic (e.g. bacteriostatic) effects due to traces of the product residing on the carriers, which could generate an inhibitory effect in agar medium and/ or on the filter membranes or a decrease in the residual viral titre.

5.1.2 Efficacy test

Using test organism suspensions containing interfering substance, deposit 50 µl per carrier prepared as described in 5.2.3.

Spread and air-dry the inoculum as described in 5.5.1.2.2, then expose the prepared test-carriers to the product diffused by the tested automated airborne disinfection process, under defined conditions.

Recover the surviving bacteria, mycobacteria, bacterial spores, yeasts, fungal spores, viruses or bacteriophages by mechanical action, such as scraping (e.g. with a glass pipette or scalpel), or if required sonicate, from the carriers, into the recovery liquid:

For bacteria, mycobacteria, bacterial spores, fungal spores and yeasts, dilute and inoculate the agar with a fraction of the recovery liquid. Incubate and count the colonies. Filtrate the remaining recovery liquid using membrane filtration, then rinse to eliminate as much product as possible. Transfer onto agar medium. Place the carrier into agar medium to capture any remaining surviving test organisms. Incubate and count the number of colonies.

For viruses and bacteriophages, dilute a fraction of the recovery liquid, incubate on a cell line and determine the viral titre.

5.1.3 Distribution test

The distribution test is a replication of the efficacy test except that only Staphylococcus aureus is used as the test organism and the test carriers are located in other defined positions and orientations.

5.2 Materials and reagents

5.2.1 Test organisms

Depending on the type of activity targeted, tests shall use all or some of the following test organisms.

These test organisms can be obtained from culture collections. The test organisms are:

For bactericidal activity tests:

— 5.2.1.1 Pseudomonas aeruginosa ATCC 15442 = CIP 103-467 (DSM 937)

— 5.2.1.2 Staphylococcus aureus ATCC 6538 = CIP 4.83 (DSM 799)

— 5.2.1.3 Enterococcus hirae ATCC 10541 = CIP 5855 (DSM 3320)

— 5.2.1.4 Escherichia coli ATCC 10536 = CIP 54127 (DSM 682)

— 5.2.1.5 Acinetobacter baumanii ATCC 19606 = CIP 70.34 (DSM 30007)

— 5.2.1.6 Proteus hauseri ATCC 13315 = CIP 58.60(DSM 30118)

For sporicidal activity tests:

— 5.2.1.7 Bacillus subtilis spores ATCC 6633 = CIP 52 62

For yeasticidal activity tests:

— 5.2.1.8 Candida albicans ATCC 10231 = IP 4872 (DSM 1386)

For fungicidal activity tests:

— 5.2.1.8 Candida albicans ATCC 10231 = IP 4872 (DSM 1386)

— 5.2.1.9 Aspergillus brasiliensis ATCC 16404 = IP 1431-83 (DSM 1988)

For virucidal activity tests:

— 5.2.1.10 Murine Norovirus souche S99, Friedrich Loefler Institut, Berlin. MNV cultured on RAW 264.7 (ATCC TIB-71) cells line

— 5.2.1.11 Adenovirus type 5, adenoid strain, ATCC VR-5. Adenovirus cultured on HeLa cells or other lines of suitable susceptibility.

— 5.2.1.12 Porcine Parvovirus NADL2 strain cultured on ST cells or other appropriate cells line

For phagocidal activity:

— 5.2.1.13 Bacteriophage for Lactococcus lactis subspecies lactis P001 (DSM 4262)

— 5.2.1.14. Bacteriophage for Lactococcus lactis subspecies lactis P008 (DSM 10567)

The multiplication of these two bacteriophages shall be obtained from the following host strain: Lactococcus lactis subspecies lactis F7/2 (DSM 4366).

For mycobactericidal activity:

— 5.2.1.15. Mycobacterium avium ATCC 15769 = CIP 105415

— 5.2.1.16 Mycobacterium terrae ATCC 15755 (tuberculocidal activity)

Both test organisms shall be tested, except for the veterinary area where only Mycobacterium avium is required.

These strains can be obtained from biological collection resource centres.

The use of other test organisms

If additional test organisms are used, they shall be incubated under optimum growth conditions (temperature, time, atmosphere, media) and noted in the test report. If the additional test organisms selected do not correspond to the specified strains, 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 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 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.

NOTE Commercial information (e.g. product name..) are provided by CEN as a convenience to users of this document and does not represent an endorsement by CEN of this product. Equivalent products can be used if it is demonstrated that they lead to the same results.

5.2.2.2 Culture media for bacteria, mycobacteria, spores, fungal spores and yeasts

5.2.2.2.1 Water

The water shall be freshly glass-distilled water and not demineralized water.

Sterilize in the autoclave (5.3.2.1a).

Sterile distilled water is necessary for the preparation of suspensions for tests. Sterilization is not necessary if the water is used for e.g. preparation of culture media and subsequently sterilized.

NOTE If distilled water of adequate quality is not available, water for injection can be used.

5.2.2.2.2 Agar for bacterial counts (Tryptone Soya Agar: TSA)

For counts of viable bacterial cells (5.2.1.1 to 5.2.1.6)

Tryptone, pancreatic digest of casein	15,0 g
Soya peptone, papaic digest of soybean meal	5,0 g
NaCl	5,0 g
Agar	15,0 g
Water (see 5.2.2.2.1) to	1 000 ml

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

If necessary, add a neutralizer to the medium (and record the addition in the test report).

Agar for the preservation of test organisms 5.2.1.1 to 5.2.1.6 (refer to 5.2.2.2.2) without neutralizer.

5.2.2.2.3 Reconstituted milk for use with sensitive test organisms

Prepare the reconstituted skimmed milk (15 g/l fat) as follows:

Skimmed powdered milk, guaranteed antibiotic-free and additive-free, reconstituted at 100 g powder per 1 l distilled water.

Sterilize 30 min at (105 ± 3) °C or 5 min at (121 ± 3) °C.

5.2.2.2.4 Malt extract agar (MEA)

For counts on the number of viable yeast and fungal spore cells (5.2.1.8 and 5.2.1.9).

Malt extract (technical grade)	30,0 g
Agar	15,0 g
Water (see 5.2.2.2.1) to	1 000 ml

The malt extract shall be of food grade (e.g. Cristomalt powder) or of equivalent grade, not highly purified and not containing only maltose.

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

If necessary, add a neutralizer to the medium (and record the addition in the test report).

5.2.2.2.5 Medium for the preservation of test organisms 5.2.1.8 and 5.2.1.9 without neutralizer (5.2.2.2.4)

5.2.2.2.6 Agar for mycobacteria (refer to 5.2.1.15. and 5.2.1.16)

Middlebrook and Cohn 7H10 medium + 10 % oleic acid dextrose-albumin complex (OADC) (hereinafter referred to as 7H10)

To carry out the counts of viable mycobacteria:

—	Middlebrook 7H10 agar	19 g
—	glycerol	5 ml
—	water (see 5.2.2.2.1) to	895 ml

Heat to boiling point to achieve total dissolution. Sterilize in an autoclave for 10 min at 121 °C and allow to cool to a temperature of 50 °C to 55 °C in a water bath.

Add 100 ml of Middlebrook OADC enrichment medium under aseptic conditions. Final pH = 6,6 at (20 ± 1) °C.

5.2.2.2.7 Medium for spores (refer to 5.2.1.7)

Tryptone Soy Agar (TSA) for counting of viable Bacillus spores:

Tryptone, pancreatic digest of casein	15,0 g
Soya peptone, papaic digest of Soybean meal	5,0 g
Sodium Chloride (NaCl)	5,0 g
Agar	15,0 g
Water (see 5.2.2.2.1) to	1 000 ml

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

Other media may be used for spores: see Annex D.

5.2.2.2.8 Diluent for microbial suspensions

Bacteria, fungi, yeast and spores

Tryptone salt:

—	Tryptone, pancreatic digest of casein	1,0 g
—	Sodium chloride	8,5 g
—	Water (see 5.2.2.2.1) to	1 000 ml

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

Mycobacteria: Tryptone salt (5.2.2.2.9) or distilled water (5.2.2.2.1).

The diluent shall not induce interference between the test organisms and the product under test.

5.2.2.2.9 Liquid for test organism recovery and rinsing liquid for membrane filtration

Composition:

Tryptone, pancreatic digest of casein	1,0 g
Sodium chloride	8,5 g
Water (see 5.2.2.2.1) to	1 000 ml

Preparation:

Dissolve the sodium chloride and tryptone in the water. Add any neutralizing agent specifically designed for the product under test.

Prepare the diluent in large-volume batches, then distribute into smaller adapted flasks. Sterilize in the autoclave.

For mycobacteria, water (refer to 5.2.2.2.1) is used instead of diluents.

5.2.2.3 Culture media and reagents for preparation of virus (test organisms 5.2.1.10 to 5.2.1.12.)

5.2.2.3.1 Growth and preservation media

For viruses, Eagle minimum essential medium (MEM) or equivalent should be used, supplemented with a suitable concentration of mycoplasm-free inactivated foetal calf serum, and antibiotics and other growth factors if required (refer to EN 14476 and EN 14675).

5.2.2.4 Culture media and reagents for preparation of bacteriophages (test organisms 5.2.1.13 to 5.2.1.14.)

5.2.2.4.1 M17 culture broth

For the preservation of the host bacterial strain, bacteriophage multiplication and bacteriophage diluent preparation:

Phytone peptone (derived from soybean meal)	5,0 g
Polypeptone peptone (derived from casein and animal tissue)	5,0 g
Beef extract powder	5,0 g
Yeast extracts	2,5 g
D(+)-lactose	5,0 g
Ascorbic acid	0,5 g
Sodium -glycerophosphate	19,0 g
Magnesium sulfate, 7 H_2O	0,2 g
Water (see 5.2.2.2.1) to	1 000 ml

Sterilize by autoclaving. After sterilization, pH of the medium shall be equivalent to (7,0 ± 0,2) when measured at (20 ± 1) °C.

When M17 broth is the diluent used to prepare the neutralizer (refer to EN 13610), double-concentrated prepared M17 broth should be used (i.e. the concentration of all the ingredients added to the 1 000 ml of water should be doubled).

5.2.2.4.2 M17 agar (underlay agar)

Underlay agar for quantitative counting of the lysis zones (plaques) each obtained from a single infectious bacteriophage particle, formed in the host bacterial lawn.

Add 15 g of agar to 1 000 ml of M17 culture broth. Dissolve the agar by bringing it to the boil while stirring continuously.

Sterilize by autoclaving. After sterilization, pH of the medium shall be equivalent to 7,0 ± 0,2 when measured at (20 ± 1) °C. Once the agar has cooled to (45 ± 1) °C, add 10 ml of sterile 1 mol/l $CaCl_2$ stock solution (refer to 5.2.2.4.6). Mix gently and pour 15 ml to 18 ml of agar into Petri dishes.

5.2.2.4.3 Overlay agar (surface agar, soft agar)

For counts of bacteriophages: dissolve 6,5 g of agar in 1 000 ml of M17 culture broth (bring to the boil while stirring continuously). Distribute the molten agar into test tubes (2,5 ml to 3 ml per tube).

Sterilize by autoclaving.

5.2.2.4.4 Bacteriophage diluent (based on Ringer's solution diluted to 1/4)

To prepare the series of dilutions intended to determine the titre of the bacteriophage suspensions (counts of bacteriophage-induced lysis zones):

Ringer's solution diluted to 1/4:

—	sodium chloride	2 250 g
—	potassium chloride	0,105 g
—	anhydrous calcium chloride	0,060 g
—	sodium hydrogen carbonate	0,050 g
—	water (see 5.2.2.2.1) to	1 000 ml

Add 10 ml of M17 culture broth to 90 ml of Ringer's solution diluted to 1/4.

Sterilize by autoclaving. Before use, add 1 ml of 1 mol/l $CaCl_2$ stock solution to 100 ml of dilution broth.

NOTE Ringer's solution can be prepared using ready-to-use tablets according to the supplier's recommendations.

5.2.2.4.5 SM buffer

For resuspension and intact preservation of bacteriophage particles:

Tris/HCl	2,4 g
sodium chloride	5,8 g
Magnesium sulfate, 7 H_2O	2,5 g
Water (see 5.2.2.2.1) to	1 000 ml.

Adjust the pH of the buffer to (7,4 ± 0,1) when measured at (20 ± 1) °C. Sterilize by autoclaving.

5.2.2.4.6 $CaCl_2$ stock solutions (1 mol/l and 0,05 mol/l)

In water (refer to 5.2.2.2.1), dissolve 110,99 g or 5,55 g of anhydrous $CaCl_2$ and make up to 1 000 ml to obtain the 1,0 mol/l or 0,05 mol/l stock solution, respectively. Sterilize by autoclaving.

5.2.2.4.7 Lactic acid solution (10 % volume fraction)

For the acidification of low-fat milk with a view to preparing the acid whey.

Dilute a 90 % volume fraction lactic acid stock solution in water (refer to 5.2.2.2.1) in order to obtain a 10 % volume fraction working solution. For this purpose, add 8 parts water to 1 part stock solution. Sterilize by autoclaving.

5.2.2.5 Interfering substance (See Annex C)

The interfering substance should be selected according to the application area of the process (see Annex C).

When an excessive drying loss rate is found for sensitive test organisms (for instance Pseudomonas aeruginosa, Escherichia coli, Candida albicans and/or other additional strains as Proteus hauseri), the addition to the recommended interfering substance(s) of skimmed milk at the dilution 1/20 is accepted to limit the drying loss.

The interfering substance shall be sterile and prepared concentrated 10-fold relative to the final concentration in the test.

The preparation and sterilization methods and the composition shall be recorded in the test report.

5.2.3 Carriers

5.2.3.1 General

New carriers shall be used for each test.

5.2.3.2 Obligatory carriers

301 stainless steel disks (EN 10088-1) 3 cm to 4 cm in diameter, rated grade 2B under EN 10088-2, surface-finished on both sides. The surfaces should be made as flat as possible using a stainless steel 1,2 mm or 1,5 mm gauge. They shall be used only once, then discarded.

Prior to use the surfaces should be placed in a beaker (minimum size: 50 ml) containing not less than 20 ml of 5 % (V/V) Decon ®[1] for 60 min. Immediately rinse the discs with water (refer to 5.2.2.2.1) for 10 s.

The surface shall not be allowed to dry to any extent. The discs shall only be handled with forceps. Rinse the discs with water (refer to 5.2.2.2.1) for a further 10 s to ensure complete removal of the surfactant. To supply a satisfactory flow of water, a sterilized fluid dispensing pressure vessel with suitable hose and connectors or other suitable method can be used and regulated to supply approximately 2000 ml per min. Treat the clean disc in a bath containing 70 % (V/V) iso-propanol for 15 min. Remove the disc and dry by evaporation under laminar air flow.

5.2.3.3 Additional carriers

Any carrier may be used provided it is nonporous and described in detail in the test report.

The carriers shall be suitable for spreading a 50 µl inoculum on a surface in the region of 3 cm^2 (2 cm in diameter). Verify the spreading feasibility on this carrier. They shall then be suitable for introduction into a 150 ml to 500 ml recipient and into a Petri dish for plating.

Cleaning, disinfection and/or sterilization requirements shall be determined according to carrier format and type of materials, and detailed in the test report.

5.3 Apparatus and glassware

5.3.1 General

Sterilize all glassware and parts of 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:

— by moist heat, in the autoclave (5.3.2.1 a)

— by dry heat, in a hot-air oven (5.3.2.1 b).

5.3.2 Usual microbiological laboratory equipment, and in particular, the following:

5.3.2.1 Apparatus for sterilization

a) For moist heat sterilization, an autoclave capable of being held at (121 ± 3) °C for a minimum holding time of at least 15 min;

[1] Decon® 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.

b) for dry heat sterilization, a hot air oven capable of being held at (180 ± 5) °C for a minimum holding time of 30 min, at (170 ± 5) °C for a minimum holding time of 1 h, or at (160 ± 5) °C for a minimum holding time of 2 h.

5.3.2.2 Water bath, capable of being controlled at (45 ± 1) °C.

5.3.2.3 Incubators

— Incubator, for drying the contaminated carriers in less than 2 h with or without air exchange, controlled at (37 ± 1) °C.

— Incubator (for bactericidal, mycobactericidal, sporicidal, virucidal, phagocidal activity), capable of being held at (36 ± 1) °C or at (37 ± 1) °C. Incubator (for fungicidal and yeasticidal activity), capable of being held at (30 ± 1) °C.

— CO_2 incubator (95 % air, 5 % CO_2), capable of being held at (36 ± 1) °C or at (37 ± 1) °C for cell culture and virus incubation.

5.3.2.4 pH-meter accurate to ± 0,1 pH units at (20 ± 1) °C, for measuring the pH of the agar media. A puncture electrode or a flat membrane electrode should be used.

5.3.2.5 Stopwatch

5.3.2.6 Mechanical or electromechanical shaker

5.3.2.7 Membrane filtration apparatus, built in a material compatible with the filtrate substances.

The apparatus shall be equipped with an analytical filter funnel with a capacity of at least 50 ml. It shall be suitable for use with 47 mm to 50 mm-diameter, 0,45 µm pore-size filters.

The vacuum source used shall give an even filtration flow rate. In order to obtain a uniform distribution of the microorganisms over the membrane and prevent overlong filtration, the device shall be set to filter 100 ml of rinsing liquid in 20 s to 40 s.

5.3.2.8 Refrigerator, temperature-controllable to between 2 °C and 8 °C

5.3.2.9 Graduated, pipettes of nominal capacities of 10 ml, 2 ml, 1 ml and 0,1 ml. Calibrated automated pipettes may be used.

5.3.2.10 Petri dishes of size 90 mm to 100 mm in diameter and 55 mm for the membranes

5.3.2.11 Glass beads (diameter: 3 mm to 4 mm)

5.3.2.12 Fritted glass filter, pore size in the range 40 µm to 100 µm (ISO 4793) = pore size 2

5.3.2.13 Laboratory centrifuge, capable of 2 000 g_N acceleration (4 000 g_N for bacteriophages)

5.3.2.14 Roux flask, straight-necked

5.3.2.15 Analytical balance of adapted operating range

5.3.2.16 Spectrophotometer fitted with a monochromator

5.3.2.17 Vacuum pump

5.3.2.18 Hydrophobic-grid 0,45 µm pore-size sterile filter membranes (diameter 47 mm to 50 mm) in cellulose ester or any other material adapted to the test product.

5.3.2.19 System for holding the test-carriers at a set distance from the source

5.3.2.20 Microscope, preferably a phase-contrast microscope (for the sporicidal activity tests) and inverse microscope for virucidal activity

5.3.2.21 Forceps

5.3.2.22 Test tubes with screw top (diameter 18 mm or 20 mm)

5.3.2.23 Recipients: flasks or vials of adequate capacity

5.3.2.24 Conical flasks, larger-rimmed, 250 ml or 500 ml capacity

5.3.2.25 Screw-capped flasks

5.3.2.26 Micropipettes with disposable tips, dispensing 0,05 ml = 50 µl for depositing the inoculum

5.3.2.27 Ultrasonic vibrator, between 30 kHz and 43 kHz

5.3.2.28 Magnetic stirrer to keep the cells suspended prior to inoculation

5.3.2.29 Petri dishes, sterile test tubes, culture vials of suitable capacity

5.3.2.30 Volumetric flask calibrated at 20°C

5.3.2.31 96-well sterile micro-titre plates, six- to eight-well plates for cell culture and vials of cell culture.

5.3.2.32 Ice machine or commercially available ice to cool the cell preservation medium and the reaction mixtures during the test

5.3.2.33 Tray for ice bath with ice and water

5.3.2.34 Biological safety cabinet, class II (also may be used during the inoculum drying step)

5.3.2.35 Freezer with temperature less than or equal to – 70°C

5.3.2.36 Desiccator with vacuum pressure gauge or use of MSS with air exchanged as alternative for inoculum drying operation on carriers.

5.3.2.37 High-speed homogenizer e.g. POTTER homogenizer

5.3.2.38 Vortex

5.3.2.39 PPE and/or air treatment system for aeration

5.4 Preparation and counting of test suspensions

5.4.1 Preparation of test suspensions (bacteria, mycobacteria, spores, fungal spores, yeasts)

5.4.1.1 Preservation of test organisms and stock cultures

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

5.4.1.2 Working culture of test organisms

5.4.1.2.1 General

For additional test organisms, any departure from the culture method or test suspension preparation method described below shall be recorded, with the rationale, in the test report.

5.4.1.2.2 Bacterial suspensions

To prepare the working culture of the test bacteria, subculture from the stock culture on TSA and incubate at (37 ± 1) °C. After 18 h to 24 h, prepare a second subculture from the first subculture in the same way and incubate for 18 h to 24 h. From this second subculture, a third subculture may be produced in the same way.

NOTE The second and/ or third subculture are the working cultures.

If it is not possible to prepare the second subculture on a particular day, a 48 h subculture may be used for subsequent subculturing, provided that the subculture has been kept in the incubator over the 48 h period. In this case, prepare another 24 h subculture before running the test.

Incubate at (37 ± 1) °C.

5.4.1.2.3 Mycobacterial suspensions

For the preparation of the working culture of Mycobacterium avium and Mycobacterium terrae prepare a first subculture from the stock culture (5.4.1.1).

Inoculate at least two plates of 7H10 medium (see 5.2.2.2.6) and incubate them in an incubator at (36 ± 1) °C or (37 ± 1) °C, to prevent the plates from drying out, use a CO_2 incubator or place the plates in wrappers made of polyethylene or sealed with insulating tape to prevent the medium from drying and incubate in an oven at (36 ± 1) °C.

After 21 days prepare a second subculture from the first subculture in the same way and incubate for 21 days. The first and/ or second subculture is/ are the working culture(s).

Never produce and use a third subculture.

5.4.1.2.4 Spore suspensions

The bacterial spores are prepared according to Annex D. They are not subcultured but kept in stock suspension format.

5.4.1.2.5 Fungal spores and yeasts

To prepare the working culture of Candida albicans, subculture from the stock culture on MEA and incubate at (30 ± 1) °C. 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.

NOTE The second and/ or third subculture are the working cultures.

If it is not possible to prepare the second subculture on a particular day, a 72 h subculture may be used for subsequent subculturing, provided that the subculture has been kept in the incubator over the 72 h period. In this case, prepare another 48 h subculture before running the test. Never produce and use a fourth subculture.

For Aspergillus brasiliensis, use the first subculture grown on MEA in Roux flasks and incubate at (30 ± 1) °C for seven to nine days.

5.4.2 Preparation of suspensions in interfering substance

5.4.2.1 Bacteria (see 5.2.1.1 to 5.2.1.6)

Take 10 ml of diluent (5.2.2.2.8) and place in a 100 ml flask with 5 g of glass beads.

Take a loopful of working culture and transfer it into the diluent (refer to 5.2.2.2.8).

The cells should be suspended in the diluent by rubbing the loop against the wall of the flask to dislodge the cells before immersing in the diluent. Shake the flask for 3 min using a mechanical shaker.

Aspirate the suspension from the glass beads and transfer to another tube.

Adjust the number of cells in the suspension using the diluent (refer to 5.2.2.2.8), estimating the number of colony forming units (cfu) either spectrophotometrically or by any other suitable means between 5×10^7 and 2×10^9 cfu/ml.

Then add the interfering substance in order to obtain a 1/10 dilution.

Keep this test suspension in the water bath at (20 ± 1) °C and use within 2 h after preparation.

For protecting test organisms sensitive to drying (refer to 3.6), such as Pseudomonas aeruginosa, Escherichia coli or additional test organisms such as Proteus hauseri, methodological adaptations may be allowed as follows when standard conditions cannot be achieved (control titre loss greater than 1,5 lg and final control titre less than 10^6 after drying and the end of the ADC time and aeration time):

These adaptations are additional to the interfering substance that generally have a certain protective effect.

1st adaptation step[2]:

Adding a 1/20 dilution rate of skimmed milk to the interfering substance (refer to 5.2.2.2.5).

2nd adaptation step[2]:

Allowed if standard conditions laid down cannot be achieved when applying the 1st adaptation step.

Increase the initial inoculum up to 5×10^9 in order to obtain a final control titre of 10^6 after drying and the end of the ADC time and the aeration time. Then add the interfering substance to obtain a final concentration of 10 % V/V (refer to Annex C).

3rd adaptation step:

Allowed if standard conditions cannot be achieved when applying the 2nd adaptation step.

Increase the initial inoculum up to 5×10^8 and add to the interfering substance a 1/20 dilution rate of skimmed milk.

[2] 1st or 2nd adaptation step could be waived and 2nd or 3rd adaptation steps directly applied for strains whose sensitivity to drying has been previously demonstrated.

5.4.2.2 Bacterial spores (refer to 5.2.1.7)

— Stock test suspension of the bacterial spores: the stock suspension of Bacillus subtilis spores is prepared at the test lab, in conformance with Annex D.

— Test spore suspension: to prepare the test spore suspension, dilute the spore stock suspension with the diluent 5.2.2.2.8.

Add the interfering substance to this suspension to obtain a final concentration of 10 % V/V (refer to Annex C).The final spore titre shall be between 2×10^6 and 5×10^6 cfu/ml regarding medical area and 2×10^5 and 5×10^5 cfu/ml for other areas. The laboratory conducting the tests is required to ensure the accuracy of the titres obtained for preliminary tests.

Maintain this test suspension in the water bath at (20 ± 1) °C and use within 2 h.

Microscopic analysis under 400x magnification shall be carried out immediately after the preparation step and just before the test, to show the absence of vegetative cells and germinative spores.

In the event of evidence of germinative spores ≥ 1 %, the suspension shall be discarded.

5.4.2.3 Candida albicans (refer to 5.2.1.8)

Take 10 ml of diluent (5.2.2.2.8), and place in a 100 ml flask with 10 g of glass beads.

Take a loopful of working culture and transfer it into the diluent. The cells should be suspended in the diluent by rubbing the loop against the wall of the flask to dislodge the cells before immersing in the diluent. Shake the flask for 3 min using a mechanical shaker. Aspirate the suspension from the glass beads and transfer to another tube. Adjust the number of cells in the suspension using the diluent, estimating the number of units either spectrophotometrically or by any other suitable means.

Add to this suspension according to the case defined in Annex C, the interfering substance to obtain a final concentration of 10 % V/V (refer to Annex C). The final titre of Candida albicans shall be between 2×10^7 and 1×10^8 cfu/ml.

Keep this test suspension in the water bath at (20 ± 1) °C and use within 2 h after preparation.

For drying sensitive test organisms (3.6), e.g. Candida albicans and/or additional test organisms, methodological adaptations may be allowed as follows when standard conditions cannot be achieved (control titre loss greater than 1,5 lg and final control titre less than 10^5 after drying and the end of the ADC time and aeration time):

These adaptations are additional to the interfering substance that generally have a certain protective effect.

1st adaptation step[2]:

Adding a 1/20 dilution rate of skimmed milk to the interfering substance (5.2.2.2.5).

2nd adaptation step[2]:

Allowed if standard conditions laid down cannot be achieved when applying the 1st adaptation step.

Increase the initial inoculum up to 5×10^8 in order to obtain a final control titre of 10^5 after drying and the end of the ADC time and the aeration time. Then add the interfering substance in order to obtain a final concentration of 10 % V/V (refer to Annex C).

3rd adaptation step:

Allowed if standard conditions cannot be achieved when applying the 2nd adaptation step.

Increase the initial inoculum up to 5×10^8 and add to the interfering substance (refer to Annex C) a 1/20 dilution rate of skimmed milk.

5.4.2.4 Aspergillus brasiliensis (refer to 5.2.1.9)

Place the working culture cells in suspension in 10 ml of sterile 0,05 % (m/V) polysorbate 80 % solution in water (refer to 5.2.2.2.1). Using a sterile glass spatula, dislodge the conidiospores from the culture surface. Transfer the suspension into a conical flask together with glass beads, and shake gently for 1 min. Filter the suspension through a fritted glass filter.

Carry out microscopic analysis under 400 × magnification immediately after the preparation to ensure:

1) the presence of a high concentration (at least 75 % spiny spores) of characteristic mature spores, i.e. spiny spores (as opposed to smooth spores) [see Figure 1 and Figure 2];

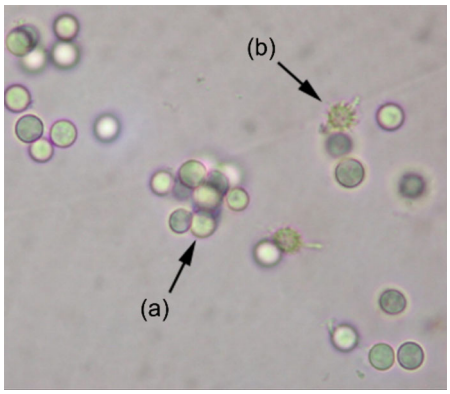
2) the absence of spore germination (check at least 10 fields-of-view);

3) if there is evidence of spore germination, discard the suspension;

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

If there is evidence of mycelia, perform a second fritted filtration.

If there is still evidence of mycelia, discard the suspension.



Key

(a) smooth spores

(b) spiny spores

Figure 1 — View of conidiospores using an optical microscope — presence of smooth and spiny spores (unacceptable suspension)

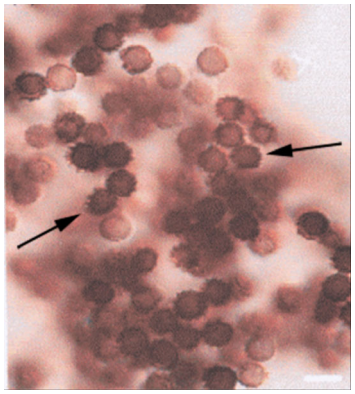


Figure 2 —View of conidiospores using an optical microscope — high concentration of characteristic mature spiny spores (acceptable suspension)

The inability to obtain 75 % spiny spores may be due to the Aspergillus brasiliensis culture or the medium used to produce these spores. In such cases, a culture from another culture collection and/or malt extract agar from another supplier should be used.

Then, by adding sterile water (refer to 5.2.2.2.1), adjust the number of spores in the suspension, estimating the number of colony-forming units by any suitable means.

This test suspension shall be preserved for no longer than 4 h, and at a temperature in the range 2 °C to 8 °C.

Re-mix the test suspension immediately before use to resuspend the spores.

Add to this suspension, the interfering substance to obtain a final concentration of 10 % V/V (refer to Annex C). The final titre of Aspergillus brasiliensis spores shall be between 5×10^6 and 1×10^7 cfu/ml. The laboratory conducting the tests is required to ensure the accuracy of the titres obtained for preliminary tests.

5.4.2.5 Mycobacterium avium and Mycobacterium terrae (refer to 5.2.1.15 and 5.2.1.16)

a) Prepare a suitable homogeneous suspension from the working culture (refer to 5.4.1.2.3) using either:

— homogenizing using glass beads. Using a plastic loop, transfer the test organisms from at least two plates of the working culture (refer to 5.4.1.2.3) into a screw-capped tube with a conical base containing 6 g to 7 g of glass beads (refer to 5.3.2.11). The test organisms along with 2 ml of water (refer to 5.2.2.2.1) are homogenized by mixing (refer to 5.3.2.6) for at least 5 min to ensure homogeneous distribution on the beads and on most of the parts of the inner surface of the screw-capped tube (refer to 5.3.2.22). Add 10 ml of water (refer to 5.2.2.2.1) drop by drop and resuspend by mixing (refer to 5.3.2.6). After a sedimentation phase of 20 min, the supernatant is transferred into a tube.

— or homogenization with Potter type apparatus (refer to 5.3.2.37). Using a pipette, transfer 5,0 ml of water (refer to 5.2.2.2.1) onto each of the plates (at least two) of working culture (refer to 5.4.1.2.3) and recover the test organisms using a glass spatula. Using a pipette, introduce all the liquid from the plates into a 25 ml centrifugation tube. Make up to 18 ml of water (refer to 5.2.2.2.1). Rinse three times in succession with water (refer to 5.2.2.2.1), (centrifuge each time at approximately 2 000 g_N for 15 min). After each centrifugation, remove the supernatant, resuspend by mixing and fill with water to the original volume.

After the final centrifugation, remove the supernatant and transfer the sediments into a 15 ml recipient of the Potter apparatus (refer to 5.3.2.37). Fill with water (refer to 5.2.2.2.1) to 15 ml. Mix, cool with ice and homogenize for 15 min.

After a sedimentation phase of 20 min during which it is necessary to have sufficient ice for cooling, the supernatant is transferred into a tube.

NOTE Other homogenization methods are allowed provided that the number of colony-forming units per millilitre obtained is suitable and stable for the duration of the test and microscopic analysis demonstrates that the suspension is as homogeneous as after both methods described above.

b) Add the interfering substance that is relevant for one of the three areas (e.g. medical) to the supernatant (refer to Annex C).

Nine volumes of supernatant are mixed with one volume of interfering substance.

Adjust the number of cells in the test suspension using diluent (refer to 5.2.2.2.8). The number of cells may be estimated using a spectrophotometer or a nephelometer.

The final titre of the test suspension shall be between 1×10^7 cfu/ml and 1×10^8 cfu/ml.

Store this test suspension in the water bath at (20 ± 1) °C and use the preparation on the same day.

5.4.3 Counting of suspensions prepared in interfering substance

5.4.3.1 Bacteria

Counting of bacterial test suspensions:

Dilute the adjusted bacterial suspensions (refer to 5.4.2.1) to 10^{-6}, 10^{-7} and 10^{-8} with diluent (refer to 5.2.2.2.8).

Counts are carried out in parallel by pour plate technique N1 and by filtration N_2.

Pour plate technique: take a duplicate sample of 1,0 ml of each dilution and inoculate using the pour plate technique N1. Pipette each 1,0 ml sample into separate Petri dishes and add 15 ml to 20 ml of molten agar TSA cooled to (45 ± 1) °C for bacteria testing.

Filtration: take a duplicate sample N_2 of 1,0 ml of each dilution and transfer each of these 1,0 ml samples to a separate membrane filtration set (refer to 5.3.2.7). Filter immediately. During filtration, flush through rinsing liquid (refer to 5.2.2.2.9) in the same way as for the test (refer to 5.5.1.4.3). Then lay each membrane onto the surface of the agar medium (refer to 5.2.2.2.2).

Incubate the plates at (36 ± 1) °C or at (37 ± 1) °C for 24 h. Discard any plates which are not countable (for any reason). Incubate the plates for a further 24 h. Do not recount plates which no longer show well-separated colonies. Recount the remaining plates.

Count N1 the number of colonies obtained by agar-plating and N_2 the number of colonies obtained by filtration, which shall be run in parallel.

5.4.3.2 Mycobacteria

Counting and incubation of test suspension in the presence of interfering substance:

a) For counting the test suspension carried out in parallel on the surface (N1) and by filtration (N_2)

Prepare 10^{-5} and 10^{-6} dilutions of the test suspension using diluent (refer to 5.2.2.2.8). Mix (refer to 5.3.2.6).

Surface:

Take a duplicate sample of 1,0 ml of each dilution. Divide each sample into two portions of approximately equal size and distribute them onto dried plates containing 18 ml to 20 ml of 7H10 medium on the surface (refer to 5.2.2.2.6), i.e. a total of four plates per 1,0 ml in duplicate.

Filtration:

Take a duplicate sample of 1,0 ml of each dilution and transfer two portions of approximately equal size of each sample into a membrane filtration apparatus (refer to 5.3.2.7).

Filter and rinse with 50 ml of rinsing water (refer to 5.2.2.2.1).

Place each membrane (four in total) on the surface of plates containing 18 ml to 20 ml of 7H10 agar (refer to 5.2.2.2.6).

b) Incubation

Incubate the plates for 21 days at (36 ± 1) °C or (37 ± 1) °C (refer to 5.4.1.2.3.). Discard any plates which are not countable. Count the plates to determine the number of colony-forming units (cfu) on each plate.

For each plate, record the exact number of colonies:

On agar: record 330 whenever the number is over 330.

If a value is under 14, record the number (but replace by "< 14" for subsequent calculations).

On membranes: Record 165 whenever the number is over 165.

If a value is under 14, record the number (but replace by "< 14" for subsequent calculations).

Calculate the number of cfu/ml in the test suspension N1 and N_2 using the method stated in subclause 5.6.

5.4.3.3 Bacterial spores

For counting the bacterial spore test suspension, prepare, 10^{-5} and 10^{-6} dilutions regarding medical area and 10^{-4} and 10^{-5} dilutions for the other areas, of the test suspension using the diluent (refer to 5.2.2.2.8).

Counts are carried out in parallel by pour plate technique N1 and by filtration N_2.

Pour plate technique: take a duplicate sample of 1,0 ml of each dilution and transfer each of these 1,0 ml samples into separate Petri dishes, then add between 12 ml and 15 ml melted TSA (refer to 5.2.2.2.2), cooled to (45 ± 1) °C.

Filtration: take a duplicate sample N_2 of 1,0 ml of each dilution and transfer each of these 1,0 ml samples to a separate membrane filtration set (5.3.2.7). Filter immediately. During filtration, flush through rinsing liquid (refer to 5.2.2.2.9) in the same way as for the test (refer to 5.5.4.3). Then lay each membrane onto the surface of the agar medium (refer to 5.2.2.2.7).

Incubate the Petri dishes at (30 ± 1) °C for three days. Determine the highest colony count for each plate. Calculate the number of cfu/ml in the test suspension.

Count N1 the number of colonies obtained by agar-plating and N_2 the number of colonies obtained by filtration, which shall be run in parallel.

5.4.3.4 Fungal spores and yeasts

Counting of fungal test suspensions:

Dilute the adjusted fungal suspensions (see 5.4.2.2 and 5.4.2.3) to 10^{-5}, 10^{-6} and 10^{-7} with diluent (refer to 5.2.2.2.9).

Counts are carried out in parallel by pour plate technique N1 and by filtration N_2.

Pour plate technique: take a duplicate sample of 1,0 ml of each dilution and inoculate using the pour plate technique N1. Pipette each 1,0 ml sample into separate Petri dishes and add 15 ml to 20 ml of molten agar MEA cooled to (45 ± 1)°C for fungal testing.

Filtration: take a duplicate sample N_2 of 1,0 ml of each dilution and transfer each of these 1,0 ml samples to a separate membrane filtration set (refer to 5.3.2.7). Filter immediately. During filtration, flush through rinsing liquid (refer to 5.2.2.2.9) in the same way as for the test (refer to 5.5.4.3). Then lay each membrane onto the surface of the agar medium (refer to 5.2.2.2.4).

For Aspergillus brasiliensis, divide the sample into two, three or four roughly equal-sized aliquots and transfer each aliquot portion into a separate membrane filtration recipient (refer to 5.3.2.7), which means inoculating four, six or eight membranes for each duplicate sample.

NOTE The reason the sample is divided up is the upper limit of the count (see 5.6.2).

For the fungal test organisms, incubate the plates at (30 ± 1) °C for 24 h for Candida albicans and 42 h to 48 h for Aspergillus brasiliensis. Discard any plates which are not countable (for any reason). Run counts on the plates and determine the number of cfu. Incubate the plates for a further 24 h. Do not recount plates which no longer show well-separated colonies. Recount the remaining plates.

For Aspergillus brasiliensis, leave incubation to continue for a further 20 h to 24 h, and if the number of colonies (small colonies) has increased, for another 20 h to 24 h period.

Count N1 the number of colonies obtained by agar-plating and N_2 the number of colonies obtained by filtration, which shall be run in parallel.

5.4.4 Preparation and counting of viral suspensions

5.4.4.1 Viral stock suspension

The virus shall be multiplied on a large scale to obtain a viral suspension having the same characteristics as the standard viral suspension. For safety reasons, only 10 passages from the original virus are authorized.

For viral suspension propagation and preparation, refer to 5.5.2.

The viral suspension is stored in aliquots at – 70 °C or preferentially at – 196 °C in liquid nitrogen.

5.4.4.2 Viral test suspension

The viral stock suspension is multiplied in a suitable host cell line and capable of producing a high concentration of viral particles. The cell debris is removed by centrifugation (400 g_N for 15 min). This preparation is referred to as the “viral test suspension”.

The viral test suspension is used undiluted in the test.

The viral suspension titre shall be between 1×10^7 and 1×10^9 PFU/ ml, or shall be sufficiently high so as to be suitable for determining a 4 lg reduction.

In some exceptional cases, the viral suspension may be concentrated using suitable methods (i.e. ultracentrifugation).

To prevent aggregation, sonication may be required.

To verify the quality of the viral suspension, a protein assay (i.e. Lowry method) may be carried out for superior reproducibility.

The viral suspension titre shall account for the loss caused by the contaminated carrier drying step (5.5.1.2 and 5.5.2).

5.4.4.3 Infectivity test

5.4.4.3.1 Virus titre on suspended cells in microtitration plates

Dilute the viral suspension by 1/10 dilutions in a 0,1 ml volume of viral suspension + a suitable volume of culture medium (e.g.: MEM + 2 % FCS). Pipette tips shall be changed after each dilution step.

Transfer 0,1 ml of each dilution into six or eight wells of a microtitration plate, starting with the highest dilution. Add 0,1 ml of cell culture suspension at a density enabling the formation of a monolayer (90 %) in at least 2 days. Six or eight wells are used as cell controls and receive no viral suspension.

The viral cytopathic effect is analysed using an inverted microscope once the suitable incubation period has elapsed (according to the type of virus).

5.4.4.3.2 Virus titre on pre-established mono-cellular lawns in microtitration plates

Transfer 0,1 ml of each dilution into six or eight wells of a microtitration plate containing confluent cells (90 %) in a monolayer containing no medium. The final row of six or eight wells receives 0,1 ml of culture medium (MEM + 2 % FCS) and is used for cell control purposes.

After 1 h of incubation at (37 ± 1) °C, 0,1 ml of cell culture medium is added to each well and the plates are placed under incubation at (37 ± 1) °C for a suitable period. The viral cytopathic effect is analysed using an inverted microscope.

5.4.4.3.3 Plaque assay method

Wells of plastic plates (surface diameter 30 mm to 35 mm) with confluent monolayer cells are washed once with phosphate buffer solution (PBS) and inoculated with 0,2 ml of serial dilutions of the virus in a culture medium (MEM 2 % FCS). Three wells are generally used per dilution.

After an adsorption period of 1 h at (37 ± 1) °C, during which the moisture of the monolayer cells is maintained by inclining the plates every 8 min to 10 min, the inoculum is removed and the monolayer cells are washed once with phosphate buffer solution (PBS). Subsequently, the wells are covered with 3 ml of a mixture containing 2 % molten agarose or any other suitable semi-solid medium and MEM concentrated twice with 4 % FCS.

The cultures are incubated for 2 days to 3 days at (37 ± 1) °C in a CO_2 incubator (see 5.3.2.3).

The plaques can be counted after incorporating a second layer (2 ml) having the same composition as the first and also containing 5 % of a neutral red solution (1/1 000) and further incubation (protected from light) at (37 ± 1) °C for 24 h to 48 h in a CO_2 incubator (see 5.3.2.3).

Counting can also be carried out after adding methyl violet. The monolayer cells are fixed by adding 2 ml of 10 % trichloroacetic acid (TCA) onto the agar layer for 10 min to 15 min at ambient temperature. The agar layer is then removed and 2 ml of 0,1 % methyl red in 20 % ethanol added. After 10 min to 15 min at ambient temperature, the wells are washed completely with water and the plaques (white spots) are counted.

NOTE The CPE determination can be used as an alternative to the plaque assay method.

5.4.5 Preparation and counting of bacteriophage test suspensions

5.4.5.1 Host bacteria stock culture

Inoculate the reconstituted skimmed milk with a 1 % volume fraction with culture in liquid medium (refer to 5.2.2.4.1) or with a loopful of bacteria from inclined or plated M17 agar (refer to 5.2.2.4.2). Incubate for 2 h at (30 ± 1) °C and store this host strain stock culture in reconstituted skimmed milk in a

refrigerator set to a temperature between 2 °C and 8 °C. In a two-week interval, leave these stock cultures to grow overnight at (30 ± 1) °C and repeat the procedure to obtain a fresh stock culture.

If extended storage is required, freeze the cultures in skimmed milk at temperatures between – 18 °C and – 20 °C, or lower.

Freeze-dried cultures may also be used.

5.4.5.2 Host bacteria working culture

To prepare the working culture of the host bacteria, subculture from the stock culture in M17 culture broth (refer to 5.2.2.4.1) and incubate at (30 ± 1) °C. Use a volume fraction of 1 % inoculum from liquid culture or a loop of bacteria from a M17 slope or agar plate.

After 16 h to 24 h, prepare a second subculture from the first in M17 culture broth (1 % volume fraction of inoculum) and incubate for 16 h to 24 h at (30 ± 1) °C.

From this second subculture, a third subculture may be produced in the same way. The second and/or third subculture are the working cultures.

If it is not possible to prepare the second subculture on a particular day, a 48 h subculture may be used for preparing this second subculture, provided that the subculture has been stored, after incubation for 2 h at (30 ± 1) °C, in a refrigerator set to a temperature between 2 °C and 8 °C. In this case, before continuing, a third subculture to be incubated for 16 h to 24 h should be prepared. Never produce and use a fourth subculture.

5.4.5.3 Bacteriophage stock suspension

In a test tube, add 0,1 ml of standard bacteriophage lysate to 0,1 ml of a working culture of host bacteria (16 h to 24 h culture in M17 culture broth). Supplement the sample with 10 mmol/L of $CaCl_2$ by adding 0,05 ml of sterile 50 mmol/l $CaCl_2$ stock solution (see 5.2.2.4.6). Shake briefly and incubate for 10 min at ambient temperature (i.e. between 20 °C and 21 °C) to enable adsorption of the bacteriophages on the bacterial cells.

In the test tube, add 10 ml of M17 culture broth (preheated to (30 ± 1) °C), mix briefly and incubate at (30 ± 1) °C (see 5.3.2.3) until cell lysis occurs. The growth of the host bacteria (i.e. turbidity) and cell lysis shall be monitored with the naked eye, or, preferably, by measuring the optical density of the culture with a suitable spectrophotometer set to a defined wavelength (e.g. 620 nm).

Prepare a control test tube in the same way, but replace the bacteriophage lysate with 0,1 ml of M17 culture broth (see 5.2.2.4.1). Compare the turbidity in the test tube with that in the control tube.

After lysis of the host bacteria in the test tube containing the bacteriophages, place the supernatant containing the bacteriophages on a filter membrane (pore size of 0,45 µm).

Store the bacteriophage stock suspensions between 2 °C and 8 °C until use.

5.4.5.4 High-titre bacteriophage suspension

From the bacteriophage stock suspensions (refer to 5.4.4.5), prepare decimal dilutions in bacteriophage diluent (refer to 5.2.2.4.4). For this purpose, add 1,0 ml of bacteriophage stock suspension to 9,0 ml of bacteriophage diluent. Mix and prepare the following dilutions to the 10^{-5} dilution in the same way.

Take a duplicate sample of 0,1 ml of each dilution and transfer each of these 0,1 ml samples to a separate test tube. Add 0,3 ml of a 16 h to 24 h working bacterial culture, grown in M17 culture broth. Add 0,1 ml of 50 mmol/l $CaCl_2$ solution (refer to 5.2.2.4.6), mix briefly and incubate for 10 min at ambient temperature to enable adsorption of the bacteriophages on the host bacterial cells.

Add 2,5 ml to 3,0 ml of M17 overlay agar (refer to 5.2.2.4.3), previously molten in a boiling water bath or in a microwave oven and cooled in a water bath at (45 ± 1) °C. Mix briefly, then spread the samples

evenly in plates containing M17 underlay agar (refer to 5.2.2.4.2). To obtain homogeneous sample distribution, the plates should be inclined slowly by hand before the overlay agar sets.

Take any necessary precautions to prevent uncontrolled overheating of the oven from causing excessive uncontrolled boiling of the liquefied agar. The recipient caps should be unscrewed to prevent any risk of explosion.

Incubate the plates at (30 ± 1) °C for at least 6 h, or until the following day for 16 h to 24 h.

Examine the plates to check the appearance of clear and uniform bacteriophage-induced lysis zones (plaques) in the bacterial lawn.

NOTE 1 Bacteriophage P008 plaques have sharp edges and a diameter of 1 mm to 2 mm. Bacteriophage P001 plaques are larger (2 mm to 3 mm in diameter) and surrounded with turbid halos (EN 13610).

Select the plates exhibiting confluent lysis from those inoculated using the cascade dilutions. On these plates, the plaques shall touch and a fine sheet of non-lyzed residual cells shall be barely visible.

Sample[3] the bacteriophages on agar plates showing confluent lysis by scraping with a sterile angled glass rod, and transfer into a centrifugation tube or flask.

Rinse each plate thoroughly with 5 ml of SM buffer (see 5.2.2.4.5) and pour these buffers into the test tubes (for centrifugation) corresponding to the plates.

Shake the tubes gently from time to time for at least 15 min at ambient temperature.

Sediment the soft agar and cell debris by centrifugation (i.e. for 15 min at 4 000 g_N).

The supernatant shall be filtered through a filter membrane (see 5.3.2.7).

Plaques from the same strain but collected on different plates shall be pooled to determine the bacteriophage suspension titre.

NOTE 2 A bacteriophage lysate prepared using this method is practically free from protein contamination. As a general rule, the bacteriophage suspension titres obtained range between 1×10^{10} and 5×10^{11} PFU/ml.

Store the high-titre bacteriophage suspension between 2 °C and 8 °C for not more than three months.

For counting on high-titre bacteriophage suspensions, prepare serial dilutions in bacteriophage diluent (refer to 5.2.2.4.4). For this purpose, add 1,0 ml of high-titre bacteriophage suspension to 9,0 ml of bacteriophage diluent. Mix and prepare the following dilutions to the 10^{-9} dilution in the same way. Take a duplicate sample of 0,1 ml in the 10^{-8} and 10^{-9} dilutions and pour each of these 0,1 ml samples into a separate test tube. Add 0,3 ml of a 16 h to 24 h working bacterial culture in M17 culture broth (refer to 5.2.2.4.1). Add 0,1 ml of a 0,05 mol/l $CaCl_2$ solution (refer to 5.2.2.4.6), mix briefly and incubate for 10 min at ambient temperature to enable adsorption of the bacteriophages on the host bacterial cells.

Add 2,5 ml to 3 ml of M17 overlay agar (refer to 5.2.2.4.3), previously molten in a boiling water bath or in a microwave oven and cooled in a water bath at (45 ± 1) °C. Mix briefly, then spread the samples evenly in plates containing M17 underlay agar (refer to 5.2.2.4.2). To obtain homogeneous sample distribution, the plates should be inclined slowly by hand before the overlay agar sets.

5.4.5.5 Preparation of bacteriophage suspension

Before applying the test procedure to determine the virucidal activity of the products, the bacteriophage titre of the high-titre bacteriophage suspension (refer to 5.4.4.4) is adjusted between

[3] Do not select plates on which the bacterial lawn has been completely removed or on which individual plaques do not touch (i.e. on which the plaques are completely isolated), as it is not possible to obtain high-titre lysates from these plates.

8×10^8 and 3×10^9 PFU/ml with SM buffer (refer to 5.2.2.4.5) in order to obtain a final test bacteriophage suspension suitable for implementing the procedure.

Before carrying out the efficacy test, verify the actual titre of the bacteriophage suspension by preparing a series of dilutions of the test bacteriophage suspension using bacteriophage diluent (refer to 5.2.2.4.4). For this purpose, add 1,0 ml of test bacteriophage suspension to 9,0 ml of bacteriophage diluent. Mix and prepare the following dilutions to the 10^{-7} dilution in the same way. Take a duplicate sample of 0,1 ml from the 10^{-6} and 10^{-7} dilutions and transfer each of these 0,1 ml samples into a separate test tube. Add 0,3 ml of a 16 h to 24 h working bacterial culture in M17 culture broth (refer to 5.2.2.4.1). Add 0,1 ml of 50 mmol/l $CaCl_2$ solution (refer to 5.2.2.4.6), mix briefly and incubate for 10 min at ambient temperature to enable adsorption of the bacteriophages on the host bacterial cells.

Add 2,5 ml to 3 ml of M17 overlay agar (refer to 5.2.2.4.3), previously molten in a boiling water bath or a microwave oven and cooled in a water bath at (45 ± 1) °C. Mix briefly, then spread the samples evenly in plates containing M17 underlay agar (refer to 5.2.2.4.2). To obtain homogeneous sample distribution, the plates should be inclined slowly by hand before the overlay agar sets.

Take any necessary precautions to prevent uncontrolled overheating of the oven from causing excessive uncontrolled boiling of the liquefied agar. The recipient caps should be unscrewed to prevent any risk of explosion.

5.4.5.6 Preparation of bacteriophages in interfering substance

Using the test bacteriophage suspension, add the acid whey or skimmed milk to obtain a 1/10 dilution. The final bacteriophage titre shall be between 8×10^8 and 3×10^9 PFU/ml.

Keep this test suspension in the water bath at (20 ± 1)°C and use within 2 h after preparation.

For counting in acid whey or skimmed milk, follow the same procedure as described in 5.4.4.1.

5.5 Procedure for evaluating the automated airborne disinfection process activity

5.5.1 Procedure for evaluating the process activity according to conditions of use furnished by the manufacturer (bacteria, mycobacteria, spores, fungal spores, yeasts, viruses, bacteriophages)

5.5.1.1 Experimental conditions (obligatory, supplementary obligatory and additional)

The test enclosure shall be sealable to allow the product to be safely diffused inside, thermally-insulated, offer homogeneous temperature and relative humidity, and shall be of a volume in the range 30 m^3 to 150 m^3.

“Supplementary” obligatory conditions may be set up for processes with an intended use clearly excluding the above obligatory conditions.

Draught-proof any gas or dispersed product escape routes. If gaps around doors, windows or other apertures require sealing, make sure that gas impermeable tape is used. Duct tape, clear parcel tape and/or masking tape will not prevent leakage of vaporized product.

Besides the obligatory conditions, supplementary obligatory and additional experimental conditions may be selected according to the intended practical use and manufacturer’s claims for the process, i.e.:

a) ADC time:

- according to the manufacturer's recommendations;
- apply product for an ADC ≤ 15 h;

— additional condition: Any other ADC times ≤ 48 h according to strains desiccation sensitivity and manufacturer's claims for the process.

b) Temperature:

— obligatory test temperature: (20 ± 2) °C when starting the test;

— supplementary obligatory test temperature if customary practice excludes the temperature conditions above. It shall only be applied when none of the manufacturer's use claims clearly and unambiguously require the obligatory test conditions. This temperature will then replace the obligatory temperature;

— any other temperature according to the requirements of the different application areas. The justification for the choice of this temperature should be given in the test report.

c) Humidity:

— obligatory test relative humidity: 50 % to 75 % (ambient hygrometry conditions) at the start of the test;

— supplementary obligatory relative humidity if customary practice excludes the relative humidity conditions above. It shall only be applied when none of the manufacturer's use claims clearly and unambiguously require the obligatory test conditions. This relative humidity will then replace the obligatory humidity.

d) Volume of the enclosure:

Testing shall be carried out based on the volume claims made by the manufacturer.

— obligatory enclosure volume: 30 m^3 to 150 m^3 and 0,25 m^3 to 4 m^3;

— If the manufacturer does not claim use in volumes of 4m^3 or smaller, only the obligatory large enclosure test needs to be carried out. If the manufacturer only claims use in volumes of 4m^3 or smaller, only the obligatory small enclosure test needs to be carried out.

Table 1 describes the required tests in accordance with the manufacturers intended use volume claims.

Table 1 — Manufacturers' intended use volume claims

Manufacturer recommendations	Volumes	
	Small enclosures	Large enclosures
Intended use volume	Between 0,25 m^3 and 4,0 m^3	> 4 m^3
Required tests	Tests under obligatory conditions in an enclosure between 0,25 m^3 and 4,0 m^3	Tests under obligatory conditions in an enclosure 30 m^3 to 150 m^3
Distribution test	A distribution test as per A.2 shall be carried out	A distribution test as per A.2 shall be carried out

If a process cannot be tested in accordance with the obligatory conditions due to it being physically unable to operate in an enclosure volume 30 m^3 to 150 m^3 it shall be tested in a larger volume

according to the conditions and test organisms defined in this document as a supplementary obligatory condition.

e) type of carriers, orientation and positioning:

— obligatory carriers: stainless steel disks;

— additional carriers: any non-porous carrier, provided that it is described in the test report.

Automated airborne disinfection process:

For efficacy test:

— obligatory orientation and positioning for large enclosures: distance as per Annex B at a height between 1 m and 1,5 m, vertical position, facing away from the release source;

— obligatory orientation and positioning for small enclosures: at a height equal to 50 % of the average vertical height of the enclosure and at a distance from the release source > 40 % of the enclosure diagonal. Test-carriers shall be in the vertical position, facing away from the release source;

— additional orientation and positioning: any configuration according to the intended practical use of the process.

NOTE For distribution test see Annex A

f) test organisms

— obligatory test organisms: see 5.2.1;

— additional test organisms: any test organism according to the intended practical use of the process.

g) interfering substance:

— obligatory interfering substances: see Annex C;

— for some specific applications, and according to the manufacturer's recommendations, tests with another interfering substance may also be envisaged under additional conditions.

5.5.1.2 Survival controls of test organisms on carriers (efficacy and distribution test)

5.5.1.2.1 General

To be run in parallel with the efficacy test.

5.5.1.2.2 Contamination and drying of the control-carriers

Contaminate carriers (refer to 5.2.4.1) placed in sterile Petri dishes by depositing 0,05 ml = 50 µl of suspension (refer to 5.4.1) in a single drop in the centre of the carrier.

Then spread the inoculum evenly on the carrier, covering an area of (3,0 ± 0,5) cm^2.

For this purpose, use a sterile inoculation loop and working from the centre spread the inoculum using several circular movements in order to cover a consistent circular area of approximately (2,0 ± 0,2) cm in diameter. The loop shall be changed between each spreading operation.

For each test organism, contaminate two identical carriers for survival control.

Dry the carriers.

The carriers shall be dried according to 5.3.2.3. The carriers are placed in a Petri dish, with the lid open, and then dried for not more than 120 min at a temperature not exceeding 37 °C (samples which are not dry at the end of this period cannot be used for the tests).

Monitor regularly (about every 10 min) the status of the deposits. Once visibly dry, the carriers are removed and the Petri dish lid is closed again.

Before drying the carriers, it is important to take the following points into account: the sensitivity to drying of the different test organisms may vary significantly. In addition, the adjustment of the suspension titre shall account for potential loss during the drying step. The target values for the control titres after drying and the end of the ADC time and the aeration time, if applicable, are specified in the summary table in Annex A.

NOTE It has been observed during tests that a low temperature (23 ± 1) °C and gentle drying are less traumatic for sensitive test organisms.

Once drying has been carried out, the automated airborne disinfection process starts, i.e. the ADC time, according to the manufacturer's claim/ recommendation. Control-carriers shall not be exposed to the product and shall be kept in the laboratory during the ADC time. At the end of the ADC time and aeration time if applicable, counting shall be performed on the two carriers (5.5.1.2.3).

Given that the initial titre and the quantity of non-viable test organisms on the carriers may impact the results (not high inactivation in case of higher numbers), it may be necessary for some processes to accurately adjust the initial titres just above the limits set by the standard. Similarly, the drying method should be selected carefully, particularly for test organisms known for their sensitivity to drying.

Regardless of the drying method selected, it should be documented in the test report and be suitable for obtaining a sufficient number of test organisms in line with the criteria of this document.

5.5.1.2.3 Counting of the control-carriers

At the end of the drying time, the two carriers are left in their lidded plates for the ADC time under temperature and relative humidity conditions simulating as close as possible those recorded in the enclosure at the start of the test.

At the end of the ADC time, transfer the two carriers into two screw top tubes containing 100 ml of sterile recovery liquid (refer to 5.2.2.2.9).

Shake by hand for a few seconds. To dislodge the test organisms from the surface, use either a glass stirrer, a pipette tip or glass beads (5 mm diameter) or other equivalent apparatus. Apply mechanical action to the carrier surfaces for at least 1 min. This mechanical action step may be completed by running the sample through an ultrasonic vibrator for 1 min (specify the power and frequency of the vibrator used, and run a check for any antimicrobial effect before use) and/ or by scraping. If the ultrasound vibrator and/ or scraping is used for the tests, it shall also be used for the control runs under the same conditions.

After another quick shake, dilute 1 ml of recovery liquid in 9 ml of diluent (refer to 5.2.2.2.9) and continue running serial dilutions until achieving the 10^{-3} dilution (dilution to be adapted according to the titres of the suspensions prepared). Transfer two sets of 1 ml of the 10^{-2} and 10^{-3} recovery liquid dilutions thus obtained into Petri dishes to carry out the counts by agar-plating in agar medium (refer to 5.2.2.2.2) for bacteria (see 5.2.1.1 to 5.2.1.6), agar-plating in agar medium (refer to 5.2.2.2.4) for yeast and fungi (see 5.2.1.8 and 5.2.1.9), agar-plating in agar medium (refer to 5.2.2.2.7) for spores (refer to 5.2.1.7) and agar-plating in agar medium (refer to 5.2.2.2.6) for mycobacteria (see 5.2.1.15 and 5.2.1.16).

For mycobacteria (see 5.2.1.15 and 5.2.1.16), once the ADC time and the aeration time if applied have elapsed, transfer each carrier into 100 ml of recovery liquid (refer to 5.2.2.2.9).

Shake by hand for a few seconds and using a sterile glass stirrer, scrape every surface of the carrier to dislodge any remaining dried suspension residues.

After a final quick shake, dilute 1 ml of the solution in 9 ml of diluent (refer to 5.2.2.2.9) and continue running serial dilutions until achieving a 10^{-3} dilution.

Take a duplicate sample of 1ml of the 10^{-2} and 10^{-3} dilutions according to surface counting technique (refer to 5.4.3)

For each test organism, count the colony numbers in the agar medium, determine the means for each of the pre-listed dilutions, and express the result in proportional terms as the number of bacteria, bacterial spores, yeasts or fungal spores recovered on the control-carriers (= T) (refer to 5.6).

The loss of viability (after drying and the end of the ADC time and aeration time, if applied) shall be calculated and recorded in the test report (N – T) (refer to 5.6).

5.5.1.2.4 Bacteriophage counts on the control-carriers

At the end of the drying time, the two carriers are left in the laboratory in their lidded plates for the scheduled ADC time and aeration time if applied under temperature and relative humidity conditions mirroring as closely as possible those recorded in the place where the actual automated airborne disinfection process is carried out.

Then transfer the two carriers stored in the laboratory into two conical recipients containing 100 ml of sterile recovery liquid (5.2.2.2.9).

Shake by hand for a few seconds. Using a glass stirrer or a pipette tip, scrape every surface of the carrier for at least 1 min to dislodge the bacteriophages.

NOTE Using an ultrasonic vibrator helps dislodge the bacteriophages from the carrier.

After another quick shake, dilute 1 ml of recovery liquid in 9 ml of bacteriophage diluent (see 5.2.2.4.9) and continue running serial dilutions until achieving the 10^{-3} dilution (dilution to be adapted according to the titres of the suspensions prepared).

Take a duplicate sample of 0,1 ml from the 10^{-2} and 10^{-3} dilutions and transfer each of these samples into a separate test tube. Add 0,3 ml of a 16 h to 24 h working bacterial culture in M17 culture broth (5.4.5.2).

Add 0,1 ml of 50 mmol/l $CaCl_2$ solution (see 5.2.2.4.6), mix briefly and incubate for 10 min at ambient temperature to enable adsorption of the bacteriophages on the host bacterial cells.

Add 2,5 ml to 3 ml of M17 overlay agar (see 5.2.2.4.3), previously molten in a boiling water bath and cooled in a water bath at (45 ± 1) °C. Mix briefly, then spread the samples evenly on plates containing M17 underlay agar (see 5.2.2.4.2). To obtain homogeneous sample distribution, the plates should be inclined slowly by hand before the overlay agar sets.

For each test organism, count the number of bacteriophages in the agar medium, determine the means for each of the pre-listed dilutions, and express the result in proportional terms as the number of bacteriophages recovered on the control carriers (= T) (see 5.6).

5.5.1.3 Preliminary test to validate absence of residual effect (efficacy test and distribution test)

5.5.1.3.1 Recovering product residues on the carrier and testing for any potential antimicrobial effect

Test at least three carriers at distance D as defined in the table given in Annex B, with the inoculum in vertical position facing away from the release source.

a) Preparation of the carriers

Using a calibrated precision pipette, deposit 0,05 ml = 50 µl of interfering substance only prepared at 10 % (V/V) in diluent 5.2.2.2.9

For sensitive test organisms as defined in 3.6, deposit 50 µl of reconstituted milk diluted to 1/5 for tests selecting this option.

Leave the carriers to air-dry as described in 5.5.1.2.2.

b) Exposure of the carriers to the product under test

Expose the carriers to the automated airborne disinfection process in the test enclosure.

c) Recovering the deposited product

Once the ADC time stated by the manufacturer and the aeration time (if applicable) have elapsed, remove the carriers and transfer into 100 ml of recovery liquid.

After contact of the carrier with the recovery liquid, the recovery liquid is called S.

For mycobacteria (see 5.2.1.15 and 5.2.1.16):

d) Contamination of the carriers

Inoculate two sterilized carriers placed in Petri dishes by depositing 0,05 ml = 50 µl of diluent containing the interfering substance at the final concentration in the test.

Nine volumes of diluent are mixed with one volume of interfering substance.

Spread the microdroplet and dry.

e) Exposure of the carriers to the product under test

Expose the carriers to the product for the same ADC time as for the test to achieve the same adherence of the product on the carrier.

f) Recovering the product deposited on the carrier

Once the ADC time has elapsed, transfer the carriers into 100 ml of recovery liquid (5.2.2.2.9) to resuspend the product adhering to the carrier as above.

After contact of the carrier with the recovery liquid, the recovery liquid is called S.

5.5.1.3.2 Screening for an inhibitory effect in the agar medium

Add 1 ml of the test organism suspension appropriate dilution (refer to 5.4.2) to an empty Petri dish, along with 1 ml of solution S obtained in 5.5.1.3.1 without allowing the two liquids to contact. For bacteriophages, use the same counting method as described in 5.5.1.2.3 (for the dilutions in question).

Pour the agar medium appropriate for the test organism, mix well, cool down, and incubate.

Compare the number n1 of colonies obtained to the number counted in agar medium non-exposed to contact with the residual product recovery solution N1 to the same dilution as the one chosen for n1 (refer to 5.6).

For mycobacteria (see 5.2.1.15 and 5.2.1.16), take 1 ml of the recovery solution S, divide into two portions of approximately equal size and inoculate the 7H10 agar plates (refer to 5.2.2.2.6). Leave to air-dry, then deposit on the surface of the same agar media 1 ml of the 10^{-5} or 10^{-6} dilution divided into portions of equal size using the surface counting technique (refer to 5.4.3).

For incubation and counting, refer to 5.4.3.

Compare the number n1 of colonies obtained against the count on the test suspension N1.

5.5.1.3.3 Screening for an inhibitory effect in filter membranes

Membrane-filter 98 ml of the solution S obtained in step 5.5.1.3.1 and rinse three times with 50 ml of recovery liquid (refer to 5.2.2.2.9) to remove the product.

Re-immerse the membrane a fourth time in 50 ml of recovery liquid (refer to 5.2.2.2.9) and add 1 ml of the suspension diluted to 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and/or 10^{-8}. Filter and deliver the membrane to the agar medium for counting.

Run this test in duplicate, and repeat for each test organism.

After incubation, count the number of colonies developed and record the mean result n2 (see 5.6) of the two tests performed.

Compare N_2 (refer to 5.6) against the number of colonies obtained at the chosen dilution when filtering 1 ml, of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and/or 10^{-8} diluted suspensions on membranes non-exposed to contact with the product.

The filtration technique is not applicable for bacteriophages and thus shall not be carried out.

For mycobacteria (see 5.2.1.15 and 5.2.1.16.), membrane–filter 98 ml of solution S and rinse three times with 50 ml of liquid.

Re-immerse the membrane with 50 ml of rinsing liquid and add 1 ml of the 10^{-5} dilution or 10^{-6} dilution, filter and transfer the membrane onto 7H10 agar plate (refer to 5.2.2.2.6).

For incubation and counting refer to 5.4.3 and record the mean of both tests as n2.

Compare N_2 against at the chosen dilution the number of colonies obtained.

5.5.1.3.4 Screening for a carrier-related inhibitory effect in the agar medium

In a Petri dish, deposit 1 ml of the 10^{-4} 10^{-5} 10^{-6} 10^{-7} and/or 10^{-8} microbial suspension dilutions and the exposed carrier obtained in 5.5.1.2.2.

Pour in the agar medium for the test organism, mix well, cool down, and incubate.

Compare the number n3 (refer to 5.6) of colonies obtained to the number N1 (refer to 5.6) at dilution retained by counting in agar plates without carriers being added.

For bacteriophages:

Deposit the carriers on an M17 agar underlay.

In a tube, mix 0,1 ml of each of the bacteriophage dilution with 0,3 ml of a 16 h to 24 h working bacterial culture in M17 culture broth (refer to 5.2.2.4.1) and 0,1 ml of 50 mmol/l $CaCl_2$ solution (refer to 5.2.2.4.6), mix briefly and incubate for 10 min at ambient temperature. Add 2,5 ml to 3 ml of M17 overlay agar (refer to 5.2.2.4.3), previously molten in a boiling water bath or a microwave oven and cooled in a water bath at (45 ± 1) °C. Mix briefly, then spread the samples evenly on M17 underlay agar (refer to 5.2.2.4.2) plates containing the carriers.

For mycobacteria (see 5.2.1.15 and 5.2.1.16), in a Petri dish containing 18 ml to 20 ml of solidified 7H10 agar (refer to 5.2.2.2.6), deposit the carrier exposed to the product.

Deposit 1 ml of 10^{-5} or 10^{-6} dilution onto the surface of the agar (refer to 5.4.3).

Compare N1 against the number of colonies obtained.

5.5.1.4 Efficacy and distribution test

5.5.1.4.1 Contamination of the test-carriers

Complete the steps in 5.5.1.2.1.

Prepare three carriers per test organism for efficacy test.

Prepare four carriers per test organism for distribution test.

5.5.1.4.2 Exposure of the test-carriers to the automated airborne disinfection process under test

The environmental conditioning system shall be stopped for the course of the test to avoid any undue influence or interference of the automated airborne disinfection system.

In between each test, use any suitable method (e.g. with ventilation / extraction) to remove the product that could have effects on the following test.

Distribute the three carriers strain-by-strain at a height (H) in the range 1 to 1,5 m and at a distance (D) as defined in the table in Annex B according to the volume of the enclosure.

Position of the carriers: vertical.

Orientation: microbial inoculum facing away from the release source.

Start the process system and deliver the product for the time stipulated and according to manufacturer's instructions; this is usually based on volume of the enclosure. Determine the precise quantities implemented or diffused by weight differentials or volumetric measurements.

Once the ADC time claimed by the manufacturer (combined with aeration time, if applicable, and PPE for workers safety) elapsed recover the carriers and transfer them immediately into recovery liquid.

The enclosure shall be well ventilated after each test.

Provide a scale drawing of the test set-up, giving the precise layout of the enclosure, the position of the device, and the position of the carriers for each step.

5.5.1.4.3 Counting of bacteria, mycobacteria, bacterial spores, yeasts, fungal spores, and bacteriophages recovered

Immediately after removal from the enclosure, each test-carrier shall be transferred into a 100 ml container of sterile recovery liquid (refer to 5.2.2.2.9).

Shake by hand for a few seconds. Using a glass stirrer or a pipette tip or any other appropriate scraper, scrape every surface of the steel disk for at least 1 min to dislodge the test organisms. An ultrasonic vibrator (refer to 5.3.2.27) may also be used.

After another quick shake, dilute 1 ml of recovery liquid in 9 ml of diluent 5.2.2.2.9 and continue running serial dilutions until a 10^{-3} dilution is achieved.

Transfer two sets of 1 ml of the pure recovery liquid and/or 10^{-1} 10^{-2} and/or 10^{-3} dilutions (according to expected process efficacy) into Petri dishes to carry out the counts by pour plating in agar medium (refer to 5.2.2.2.2, 5.2.2.2.4, 5.2.2.2.6, 5.2.2.2.7). For bacteriophages, use the same counting method as described in 5.5.1.2.3. Pour 10 ml of the recovery liquid into a sterile filtration apparatus without knocking the carrier over, then pour the remaining liquid (around 87 ml) into another separate filtration apparatus. (The filtration technique is not applicable for bacteriophages and thus shall not be carried out.)

Filter, rinse the membrane three times using 50 ml of recovery liquid (refer to 5.2.2.2.9) previously poured into a conical flask and shaken with the carrier.

Then transfer the membrane onto the agar medium for counting (refer to 5.2.2.2.2, 5.2.2.2.4, 5.2.2.2.6, 5.2.2.2.7), solidified into a 4 mm thick layer.

Remove each carrier under aseptic conditions and place them in Petri dishes with inoculum facing upward. Pour in a sufficient quantity of agar medium to evenly cover the carrier.

For bacteriophages: Deposit the carriers on an M17 agar underlay (refer to 5.2.2.4.2). In a tube, mix 0,3 ml of a 16 h to 24 h working bacterial culture in M17 culture broth (refer to 5.2.2.4.1) and 0,1 ml of 50 mmol/l $CaCl_2$ solution (refer to 5.2.2.4.6), mix briefly and incubate for 10 min at ambient temperature. Add 2,5 ml to 3 ml of M17 overlay agar, previously molten in a boiling water bath or a microwave oven and cooled in a water bath at (47 ± 1) °C. Mix briefly, then spread the samples evenly on M17 underlay agar plates containing the carriers.

Repeat this procedure for each exposure, each test organism, and each carrier.

5.5.1.4.4 Incubation and counting of the test mixture

Incubate:

— 48 h at 37 °C or (36 ± 1) °C for bacteria (See 5.2.1.1 to 5.2.1.6);

— 21 days at 36 °C or (37 ± 1) °C (if possible in a CO_2 incubator or under moisture conditions) for mycobacteria (refer to 5.2.1.15 and 5.2.1.16).

— 72 h at (30 ± 1) °C with a reading taken at 48 h for bacterial spores (refer to 5.2.1.7);

— 48 h at (30 ± 1) °C for C. albicans (refer to 5.2.1.8);

— 72 h at (30 ± 1) °C with a reading taken at 48 h for A. brasiliensis (refer to 5.2.1.9);

— 6 h or 16 h – 24 h at (30 ± 1) °C for bacteriophages (refer to 5.2.1.13 and 5.2.1.14);

For each test organism and each carrier type, count the number of colonies on the Petri dishes and/or the membranes.

Calculate result n'1 (mean of the triplicate test-carriers, corresponding either to the mean number of bacteria, the mean number of yeasts/fungal spores, or the mean number of bacterial spores found in 100 ml of recovery liquid).

Similarly, count the number of colonies on carriers on agar, i.e. n'2, being the mean of the triplicate test runs.

5.5.2 Virucidal activity tests

5.5.2.1 General

The experimental conditions (obligatory and additional) are described in 5.5.1.1.

Before running a test, stabilize all the reagents — with the exception of the viral test suspension —, i.e. the interfering substance, water, cell culture medium, diluent and test solution of the product at (20 ± 2) °C using a thermostatically-controlled bath.

The tubes are filled with chilled medium (MEM 2 % FCS) for the various titres to be conducted after the different exposure times and placed in a molten ice bath. The microtitration plates are labelled for identification.

5.5.2.2 Preliminary test to validate absence of residual effect (efficacy test and distribution test)

5.5.2.2.1 Recovering product residues on the carrier and testing for any potential virucidal effect

Test at least three carriers.

Situated at distance D as defined in the table given in Annex B, with the inoculum in vertical position facing away from the release source.

a) Preparation of the carriers

Using a precision pipette, deposit 50 µl of diluent containing the reconstituted interfering substance at the suitable final concentration on the carriers, then spread and dry (refer to 5.5.1.2.2).

b) Exposure of carriers to process under test

Expose the carriers to the automated airborne disinfection process in the enclosure during the test phase.

c) Recovery of carriers and neutralization of activity of product applied

Once the ADC time and the ensuing aeration time, if applicable, have elapsed, recover the carriers and transfer them to 20 ml of recovery liquid to neutralize the action of the product.

This gives the recovery solution S.

Neutralization by means of dilution effect or by adding a specific agent should be explained in the report. Incomplete neutralization of the residue shall invalidate the tests.

5.5.2.2.2 Susceptibility of cells to viruses

The reduction in susceptibility of the cells to viruses is evaluated by means of comparative titres of the virus on cells treated or not treated with product:

Cell treatment:

a) cell monolayer: 0,1 ml of solution S or PBS is dispensed into each well of the 96-well microplate. After 1 h at (37 ± 1) °C, the supernatant is removed;

b) suspended cells: 0,1 ml of solution S or PBS is added to one volume of double concentrated cell suspension. After 1 h at (37 ± 1) °C, the cells are centrifuged and resuspended in the culture medium.

Comparative virus titre:

The virus is diluted from 10^{-2} to 10^{-10} and titrated on the treated and untreated cells in parallel. There is no loss of susceptibility when solution S:

c) indicates a low cell kill rate (25 % of the monolayer cells); or

d) induces a reduction of the virus titre < 1 lg

5.5.2.2.3 Evaluating inhibition of product activity

A volume of solution S (or PBS as control) is to be added to 1 volume of double concentrated cell suspension mixed with the interfering substance. After 30 min at (20 ± 1) °C, a series of dilutions is prepared and the viral titres are compared.

The difference between the titres shall be less than 0,5 lg.

5.5.2.3 Efficacy test

5.5.2.3.1 Contamination of the carriers

Prepare three carriers per test organism:

Nine volumes of viral suspension are mixed with 1 volume of interfering substance. For each test, prepare three test carriers and three control-carriers by depositing 50 µl of the suspension described above. Then spread and dry the carriers as defined above (refer to 5.5.1.2.2).

Use the dried carriers within one hour to avoid inactivation of the virus over time.

5.5.2.3.2 Exposure of the test-carriers to automated airborne disinfection process

Refer to 5.5.1.4.2

Expose the carriers to the automated airborne disinfection process in the test enclosure during the test phase.

5.5.2.3.3 Determination of viral titre after exposure

Immediately after recovery, once the ADC time and the ensuing aeration time have elapsed, each test-carrier shall be transferred into a flask containing 20 ml of recovery liquid. The quantity of recovery liquid may be reduced provided that its neutralizing potential remains adequate.

NOTE Test laboratories are advised to conduct preliminary tests to ensure complete residue neutralization.

Mix by hand for a few seconds. Using a sterile glass stirrer or other means described in 5.5.1.2.3, scrape each side of the disk to dislodge any trace of dried residue. Ultrasonic vibrations (refer to 5.3.2.27) may also be used.

After a final quick shake, dilute 1 ml of recovery solution in 9 ml and continue the serial dilutions. The viral titre is determined as per 5.4.4.

5.5.2.4 Calculation and expression of results

The cell culture results are recorded as “0” in the absence of cytopathic effects (CPE), and “1” (approximately 25 % of cells with CPE) to “4” (all cells with CPE) (CPE titre) or as the number of lysis plaques (plaque-forming units, PFU).

The estimated virus concentration should be calculated using suitable methods:

— the viral concentration may be calculated using the Spearman-Kärber method (see EN 14476 and EN 14675);

— PFU calculation: the number of plaque-forming units (PFU/ml) shall be calculated. All cell culture units with non-confluent lysis plaques shall be used for the calculation.

The reduction in viral titre is calculated using the difference between the dried viral titres before and after treatment with the automated airborne disinfection process.

Verification of methodology

A test is only valid if the following criteria are met:

a) the virus test suspension has a $TCID_{50}$ in the range 1×10^7/ml and 1×10^9/ml, or has a titre suitable for determining a 4 lg reduction in the viral titre;

b) the detectable reduction is at least 4 lg;

c) cell susceptibility test: viral titre reduction lg < 1;

d) neutralization test: difference between tests and controls < 0,5 lg.

Calculation of virucidal activity of products

The inactivation of the virus induced by the virucidal activity of each test solution of the product is determined for each exposure time compared to viral controls not exposed to the product and kept for the exposure time period. The reduction, expressed in lg, is then calculated using the difference between the logarithmic titre of the control samples and the logarithmic titres of each test.

All the results are reported as raw data and are further presented as negative $TCID_{50}$ or PFU values.

5.5.3 Procedure for evaluating the mycobactericidal activity

5.5.3.1 General

The experimental conditions of the tests are described in 5.5.1.4.2.

5.5.3.2 Contamination of the carriers

Follow the procedure in 5.5.1.2.2 and prepare three carriers.

5.5.3.3 Exposure of the test-carriers to the product under test

Refer to 5.5.1.4.2. Expose the carriers to the automated airborne disinfection process in the enclosure during the test.

5.5.3.4 Counts on the carriers

Once the ADC time and the aeration time, if applicable, have elapsed, transfer each carrier into 100 ml of recovery liquid (refer to 5.2.2.2.9).

Shake by hand for a few seconds and using a sterile glass stirrer, scrape each surface of the carrier to dislodge any remaining dried suspension residues.

After a final quick shake, dilute 1 ml of the solution in 9 ml of diluent (refer to 5.2.2.2.8) and continue running serial dilutions until a 10^{-3} dilution is achieved.

Transfer two sets of 1 ml of pure recovery liquid and/ or 10^{-1}, 10^{-2} and 10^{-3} dilutions according to the surface counting or inclusion technique (refer to 5.4.3.4 a).

Incubation of 7H10 agar plates (refer to 5.2.2.1.10), refer to 5.4.3.4 b.

Filter the 97 ml of recovery liquid. Pour 10 ml of the recovery liquid into a filtration apparatus. Rinse the membrane three times using 50 ml of recovery liquid (refer to 5.2.2.2.9). Pour the remaining 87 ml of liquid into another separate filtration apparatus. Rinse the membrane three times using 50 ml of recovery liquid (refer to 5.2.2.2.9) previously poured into a conical flask and shaken with the carrier.

For filtration counting and incubation see 5.4.3.

Remove the carrier under aseptic conditions and place it in the Petri dish containing 18 ml to 20 ml of solidified 7H10 agar (refer to 5.2.2.1.6) or proceed by inclusion.

The value n'1 is the mean number of viable colonies detected in 100 ml of recovery liquid and n'2 the mean number of viable colonies detected on test-carriers.

5.6 Experimental data and calculations (bacteria, yeasts, fungal spores, bacterial spores, bacteriophages and mycobacteria)

5.6.1 General

— The first step is to determine the mean number of test organisms or lysis plaques per ml based on the raw values.

— The second step is to determine N values (number of test organisms/ ml of working culture suspension), T values (number of test organisms on controls), n1 – n2 – n3 (screening for an inhibitory activity), and n'1 and n'2 (number of surviving test organisms).

— The third step is to calculate the lg reduction d.

5.6.2 Colony counting and limit thresholds

— On agar: do not count plates containing over 330 for bacteria, the yeast and mycobacteria, or plates containing over 165 for A. brasiliensis;

— on membranes: do not count plates containing over 165 for bacteria, mycobacteria and the yeast, or plates containing over 55 colonies for A. brasiliensis.

Counts on carriers (by agar-plating) and membranes obtained from tests representing all surviving test organisms.

In case of a low count (< 14) of surviving test organisms after the test, each colony obtained shall be included in the efficacy calculations.

The bacteriophage titres shall be calculated on the basis of the lysis plaques in the host bacterial cell lawns as follows:

— only plaque counts under 300 PFU (Plaque-Forming Unit per ml) per plate for bacteriophage P008 or 200 PFU per plate for bacteriophage P001 shall be used to calculate the plaque counts.

— for a result to be taken into account, a plate shall contain 14 or more plaques.

— at least two plates are required to calculate the bacteriophage titres: one of the two plates or both contain over 14 plaques and both contain under 300.

NOTE Counting individual plaques on a plate is limited by the start of confluent lysis in the host bacterial cell lawn. As bacteriophage P001 plaques are substantially larger than those of bacteriophage P008, the upper limit for counting plaques derived from bacteriophage P001 is actually less than 300 plaques per plate (i.e. 200 plaques per plate).

5.6.3 Determining number of test organisms N in bacterial, mycobacterial, bacterial spores, yeasts, fungal spores and bacteriophage suspensions (refer to 5.4)

Calculate the mean test organisms count obtained for one dilution or for two successive dilutions if it is possible to calculate a weighted mean, and multiply by the dilution factor to get rate N in test organisms/ ml of suspension.

To calculate the weighted mean count, use the following formula:

$$N = \frac{c}{\left(n1 + 0,1\,n2\right)} \times d \times V \text{ in CFU or PFU / ml}$$

where:

c is the sum of CFU or PFU counted on all the plates included;

n1 is the number of plates included taken into account in the lower dilution, i.e. 10^{-6};

n2 is the number of plates included taken into account in the higher dilution, i.e. 10^{-7};

d is the dilution factor corresponding to the lower dilution;

V is the volume of the sample (1 ml or 0,1 ml).

EXAMPLE For 1 ml of test organism suspension, we obtain: 233 and 215 colonies at 10^{-6} and 27 and 21 colonies at 10^{-7}.

The rate of test organism/ ml in suspension N is obtained as follows:

$$N = \frac{233 + 215 + 0{,}1\left(27 + 21\right)}{2{,}2} \times 10^{6} = 205{,}8 = 2{,}1 \times 10^{8} \text{ .Microorganisms / ml}$$

5.6.4 Determining number of microorganisms T on control-carriers (refer to 5.5.1.2)

Calculate the mean number of colonies obtained for one dilution or for two successive dilutions if it is possible to calculate a weighted mean, and multiply by the dilution factor of the carrier recovery diluent then by 100.

Plates with a colony number under 14 shall not be counted.

EXAMPLE: two carriers contaminated with C. albicans give the following means: 225 and 232 colonies at 10^{-2} of recovery medium and 21 and 23 colonies at 10^{-3}.

The number of test organisms per carrier, T, is obtained as follows:

$$T = \frac{225 + 232 + 21 + 23}{2{,}2} \times 10^{2} \times 100 = 228 \times 10^{4} = 2{,}3 \times 10^{6} \text{ Test organisms / carrier}$$

To calculate the number of bacteriophages on the control-carriers (T), use a specific calculation formula for bacteriophages:

The number of bacteriophages per carrier, T, is obtained as follows:

c/ [(n1 + 0,1 n2) x d x V] × 100 where V= 0,1 ml for bacteriophages counted on carriers

5.6.5 Determining values obtained in the preliminary test (refer to 5.5.1.3)

— Screening for an inhibitory effect in the agar medium: calculate the mean of the n1 values obtained.

— Screening for an inhibitory effect in membranes: calculate the mean of the n2 values obtained (Except for unfiltered bacteriophages).

— Screening for a carrier-vectored inhibitory effect in the agar medium: calculate the mean of the n3 values obtained.

5.6.6 Determining the number of test organisms and plaques on test-carriers post- exposure and the reduction R (refer to 5.5.1.4.3)

For the test-carriers, n'1 represents the number of test organisms (or plaques) surviving in 100 ml of recovery liquid. It can be obtained:

— either from the filtration of 87 ml of recovery liquid (by applying the rule of three);

If the colony count on a membrane is over 165 for bacteria and yeasts or over 55 for fungal spores, record the number as > 165 (or > 55).

— or from the filtration of 10 ml of recovery liquid;

— or from the number of colonies or plaques obtained on 1 ml of recovery liquid, or on a dilution of recovery liquid;

— or from two successive dilutions if it is possible to calculate a weighted mean.

The number n'2 is the number of colonies (or plaques) obtained directly by pour-plating the test-carrier.

If the colony count on a plate is over 330 for bacteria and yeasts or over 165 for fungal spores and bacteriophages, record the number as > 330 (or > 165). For bacteriophages, note > 165 PFU.

The sum n'1 + n'2 corresponds to the total number of surviving test organisms- per test carrier.

The lg reduction, d, is obtained by integrating the number of test organisms (or bacteriophages) on control-carriers, T, and the number of n'1 + n'2 surviving microorganisms (or bacteriophages).

The equation for obtaining the lg reduction is written:

$$d = \lg T - \lg (n'1 + n'2) = \lg [T/ (n'1 + n'2)]$$

Once the lg reduction has been obtained for each test-carrier, the mean for the three carriers shall be calculated.

To pass the test the mean lg reduction shall be greater than or equal to the required values given in Table 1.

If the counts used to calculate n'1 and n'2 all equal 0, then the readout value for R = lg T.

EXAMPLE 1 Numbers of colonies in the recordable interval.

i.e. a test with C. albicans and a control $T = 4{,}5 \times 10^6$ test organisms/ carrier.

The counts for surviving test organisms are:

— a mean of 22 colonies for 1 ml of pure recovery liquid;

— a mean of 15 colonies on pour-plated carriers.

$n'1 = 22 \times 100 = 2{,}2 \times 10^3$ test organisms/ 100 ml;

n'2 = 15.

Hence

$$R = \lg [T / (n'1+n'2)] = \lg [4{,}5 \times 10^6 / (2{,}2 \times 10^3 +15)] \text{ i.e. } R = 3{,}3 \text{ lg.}$$

NOTE In cases where the n'1 value and/ or the n'2 value cannot be determined (>one of the limit thresholds specified in 5.6.2), it remains possible to complete the calculation by working to the hypothesis that n'1 or n'2 = at the stated limit threshold and gives an activity < the lg base-10 value calculated for this threshold

EXAMPLE 2: Colony numbers > 330 and low kill rate R.

i.e. a test with C. albicans and a control $T = 4{,}5 \times 10^6$ microorganisms/ carrier.

The counts for surviving test organisms (for the three carriers) are:

— > 330 colonies for 1 ml of the 10^{-1} dilution of recovery liquid;

— > 330 colonies on pour-plated carriers.

Then:

$n'1 > 330 \times 10 \times 100$ i.e. $> 3{,}3.10^5$ test organisms/ 100 ml;

n'2 > 330.

This gives R = lg [T / (n'1 + n'2)] = lg [4,5 × 10^6 / (3,3 × 10^5 + 330)] i.e. R < 1,1 lg.

EXAMPLE 3 Low colony numbers and high kill rate R.

i.e. a test with C. albicans and a control T = 4,5 × 10^6 test organisms / carrier.

The counts for surviving test organisms (for the three carriers) are:

— 3 colonies in 87 ml of filtered recovery liquid;

— 0 colonies in 10 ml of filtered recovery liquid;

— 0 colonies in 1 ml of recovery liquid and 10^{-1} diluted filtered recovery liquid;

— 0 colonies on pour-plated carriers.

Then:

n'1 = (3/ 87) × 100 = 3,4≈3;

n'2 = 0.

This gives R = lg [T / (n'1 + n'2)] = lg [4,5 × 10^6 / (3 + 0)] i.e. R = 6,18 lg.

5.7 Interpretation of results

Key values:

— number of the working culture suspensions: N shall be in the range 5 × 10^7 to 2 × 10^9 cfu/ ml for bacteria (see 5.2.1.1 to 5.2.1.6) expected for P. aeruginosa (5 × 10^7 to 5 × 10^9 cfu/ ml), between 2 × 10^7 and 1 × 10^8 cfu/ ml for Candida albicans (refer to 5.2.1.8), between 5 × 10^6 and 1 × 10^7 cfu/ ml for Aspergillus brasiliensis (5.2.1.9), 2 × 10^5 and 5 × 10^5 cfu/ ml (2 × 10^6 and 5 × 10^6 cfu/ ml for medical area) for the Bacillus subtilis spores (refer to 5.2.1.7), 8 × 10^8 and 3 × 10^9 PFU/ ml for bacteriophages (refer to 5.2.1.13 and 5.2.1.14), 1 × 10^7 = and 1 × 10^9 PFU/ ml for viruses (refer to 5.2.1.10, 5.2.1.11 and 5.2.1.12)and 1 × 10^7 and 1 × 10^8 CFU/ ml for mycobacteria (refer to 5.2.1.15 and 5.2.1.16).

— control-carriers not exposed to the product: for all test organisms, T shall be at least 1 lg above the target reduction as defined in Annex A;

— preliminary tests (refer to 5.5.1.3).

The experimental conditions are applicable for the test in the defined conditions if:

n1 > 0,5 N1

and

n3 > 0,5 N1

and

$n2 > 0{,}5\ N_2$

If the agar medium or the filter membranes still contain a sufficient quantity of product to create an inhibitory effect:

$n1 \leq 0{,}5\ N1$

or

$n3 \leq 0{,}5\ N1$

or

$n2 \leq 0{,}5\ N_2$

Repeat the test either adding neutralizer to the agar medium, and/or adding a neutralizer into the carrier recovery liquid, and/or increasing the number of times the membranes are rinsed and/or increasing the volume of liquid used for each rinsing.

($N1 - N_2 - n1 - n2 - n3$ are defined in 5.5.2).

— the efficacy test (see summary table for each application area in Annex A)

* Minimum lg reduction required to pass standard: refer to Annex A

* Lg reduction on additional test organisms: according to the intended practical use of the process, the product activity can be set at $dx \geq 3$, 4 or 5 lg depending on the type of microorganism tested: bacterial spores (≥ 4 lg regarding medical area and ≥ 3 lg for other areas) – yeasts/fungal spores (≥ 4 lg) – bacteria (≥ 5 lg) – viruses (≥ 4 lg) – mycobacteria (≥ 4 lg) on various test organisms suitable for the application areas.

5.8 Test report

TEMPLATE TEST REPORT:

— identification of the testing laboratory;

— identification of the sample: name of the process (product and device), type of device (vaporisation, nebulisation, misting, liquid spray, etc.), batch number and — if available — serial number, manufacturer, date of delivery, storage conditions, active ingredients (optional);

— neutralizers added to media or diluents, type of filter membranes, ultrasonic vibrator;

— test start date and test end date;

— limits to use, especially in terms of ability to diffuse throughout the enclosure.

Attach a scale drawing of the test set-up for each procedural step.

Observations

— type of carriers;

— device-to-test-carrier distance;

— dispersed product release time (if measurable);

— activity period (if measurable);

— aeration time (describe any technical conditions such as air exchange rate, time before carriers are recovered, disinfectant concentration monitoring apparatus if used and disinfectant concentration at point if carrier recovery if measured);

— amount of product diffused;

— exact size of the enclosure (all three dimensions).

Results

See Table 2.

Conclusions

— Mark “conforming” or “non-conforming” for each group of test organisms along with the lg reductions obtained for each test organism.

— For the distribution test, do not average the carrier results. Results shall be expressed in lg reductions in comparison to the control carrier. Each carrier shall demonstrate a ≥ 5 lg reduction.

Table 2 — RESULTS: example for an automated process

Add raw data N1, N_2, n1, n2, n3, n'1, n'2, T

Test organisms	N Initial suspension titre	Test validation			T Controls	Tests: lg reductions
		n1/N1	n2/ N_2	n3/N1		
		n1 > 0,5 N1	n2 > 0, 5 N_2	n3 > 0, 5 N1		see interpretation (5.7)
BACTERIA						
P. aeruginosa	5×10^7 to 5×10^9 cfu/ml				1×10^6 per carrier	R1
S. aureus	5×10^7 to 2×10^9 cfu/ml				1×10^6 per carrier	R2
E. hirae	5×10^7 to 2×10^9 cfu/ml				1×10^6 per carrier	R3
E. coli	5×10^7 to 2×10^9 cfu/ml				1×10^6 per carrier	R4
Acinetobacter baumannii	5×10^7 to 2×10^9 cfu/ml				1×10^6 per carrier	R5
P. hauseri	5×10^7 to 2×10^9 cfu/ml				1×10^6 per carrier	R6
SPORES						
B. subtilis (spores) (medical area)	2×10^6 to 5×10^6 cfu/ml				1×10^4 per carrier	R7
B. subtilis (spores) (other areas)	2×10^5 to 5×10^5 cfu/ml				1×10^4 per carrier	R7
FUNGAL SPORES AND YEASTS FUNGAL SPORE						
C. albicans	2×10^7 to 1×10^8 cfu/ml				1×10^5 per carrier	R8
A. brasiliensis	5×10^6 to 1×10^7 cfu/ml				1×10^5 per carrier	R9
VIRUSES						
Murine norovirus	1×10^7 to 1×10^9 PFU/ml				1×10^5 per carrier	R10
Adenovirus	1×10^7 to 1×10^9 PFU/ml				1×10^5 per carrier	R11
Porcine parvovirus	1×10^7 to 1×10^9 PFU/ml				1×10^5 per carrier	R12

Add raw data N1, N_2, n1, n2, n3, n'1, n'2, T

Test organisms	N Initial suspension titre	Test validation			T Controls	Tests: lg reductions
		n1/N1	n2/ N_2	n3/N1		
BACTERIOPHAGES						
P001	8 x10^8 to 3 x10^9 PFU/ml				2 x10^5 per carrier	R13
P008	8 x10^8 to 3 x10^9 PFU/ml				2 x10^5 per carrier	R14
MYCOBACTERIA						
Mycobacterium avium	1 x10^7 to 1 x10^8 cfu/ml				1 x10^5 per carrier	R15
Mycobacterium terrae	1 x10^7 to 1 x10^8 cfu/ml				1 x10^5 per carrier	R16

Annex A
(normative)

Summary of test requirements

A.1 Efficacy tests

Table A.1 — Efficacy tests

Part 1: Efficacy tests							
Test organisms and efficacy objectives	Obligatory conditions	- The reduction is expressed as lg values in comparison to control-carriers not exposed. - Initial load on carrier is ≥ 1 lg above the required lg reduction. - Each activity can be claimed independently but bactericidal and yeasticidal activities are the minimum to be passed. An activity for a group of microorganisms (e.g. virucidal) can only be claimed if all specified microorganisms of this group have been successfully tested. - In case of sensitive test organisms: refer to 5.4.2.1 and 5.4.2.3					
	Additional conditions	Additional test organisms can also be used if relevant for a given application.					
		Medical area		Veterinary area		Food, industrial, domestic and institutional area	
	Categories	Test organisms	R	Test organisms	R	Test organisms	R
				Pseudomonas aeruginosa	5	Pseudomonas aeruginosa	5
		Staphylococcus aureus	5	Staphylococcus aureus	5	Staphylococcus aureus	5
		Enterococcus hirae	5	Enterococcus hirae	5	Enterococcus hirae	5
		Escherichia coli	5			Escherichia coli	5
		Acinetobacter baumanii	5				
				Proteus hauseri	5		
	Bacterial spores	Bacillus subtilis	4	Bacillus subtilis	3	Bacillus subtilis	3
	Fungi and yeast	Candida albicans	4	Candida albicans	4	Candida albicans	4
		Aspergillus brasiliensis	4	Aspergillus brasiliensis	4	Aspergillus brasiliensis	4
	Virus	Murine norovirus	4			Murine norovirus	4
		Adenovirus type 5	4			Adenovirus type 5	4
				Porcine parvovirus	4		
	Bacteriophages					Lactobacillus lactis P001	4
						Lactobacillus lactis P008	4
	Mycobacteria	Mycobacterium avium	4	Mycobacterium avium	4	Mycobacterium avium	4
		Mycobacterium terrae	4			Mycobacterium terrae	4

<table>
<tr><th colspan="5">Part 1: Efficacy tests</th></tr>
<tr><td rowspan="4">Interfering and protective substances</td><td colspan="4">Obligatory conditions</td></tr>
<tr><td>Clean conditions:
BSA 0,3 g/l
BSA 0,3 g/l + milk 1/20 for sensitive test organisms acc. to 5.2.2.2.3</td><td colspan="2">Dirty Conditions (low level)
1. BSA 3 g/l
2. BSA 3 g/l + milk 1/20 for sensitive test organisms acc. to 5.2.2.2.3</td><td>Clean conditions:
1. BSA 0,3 g/l
2. BSA 0,3 g/l + milk 1/20 for sensitive test organisms acc. to 5.2.2.2.3</td></tr>
<tr><td colspan="4">Additional conditions</td></tr>
<tr><td>Dirty conditions:
BSA 3 g/l + 3 mg/l erythrocytes</td><td colspan="2">Dirty conditions (high level):
1. BSA 10 g/l + yeast extracts 10g/ L
2. BSA 10 g/l + yeast extracts 10 g/l + milk 1/20 for sensitive test organisms acc. to 5.2.2.2.3</td><td>Dirty conditions:
1. BSA 3 g/l
2. BSA 3 g/l + milk 1/20 for sensitive test organisms acc. to 5.2.2.2.3</td></tr>
<tr><td rowspan="2">Carrier</td><td>Obligatory conditions</td><td colspan="3">Stainless steel disc. Diameter 3 cm to 4 cm</td></tr>
<tr><td>Additional conditions</td><td colspan="3">Any other non-porous material. Disc of 3 cm to 4 cm diameter.</td></tr>
<tr><td>Deposit</td><td colspan="4">50 µl - spread the inoculum on 3 cm^2 (diameter of 2 cm – visual control)</td></tr>
<tr><td>Drying</td><td colspan="4">Preference should be given to the method leading to the lowest loss, as accumulation of non-viable microorganisms can be a significant additional protective challenge. Maximum temperature: (37 ± 1) °C</td></tr>
<tr><td>Diffusion device</td><td colspan="4">- Only one per enclosure</td></tr>
<tr><td rowspan="2">Test enclosure</td><td colspan="2">Large enclosure (30 m^3 to 150 m^3)</td><td colspan="2">Small enclosure (0,25 m^3 to 4 m^3)</td></tr>
<tr><td colspan="2">-T °C and relative humidity at the beginning of the test:
-T °C: (20 ± 2) °C or supplementary obligatory conditions max. or min. T °C with a range of ± 2 °C
-RH: 50 % to 75 % or supplementary obligatory conditions</td><td colspan="2">-T °C and relative humidity at the beginning of the test:
-T °C: (20 ± 2) °C or supplementary obligatory conditions max. or min. T°C with a range of ± 2 °C
-RH: 50 % to 75 % or supplementary obligatory conditions</td></tr>
<tr><td>Carrier position and number of samples</td><td colspan="2">-Vertical, inoculum facing away from the release source
Height - 1 m to 1,5 m above the floor
-Distance from source: > or equal to 40 % of enclosure diagonal
-Test in triplicate i.e. three carriers for each test organism</td><td colspan="2">Vertical, inoculum facing away from the release source
Height - 50 % of the average height of the enclosure
Distance from source: > or equal to 40 % of enclosure diagonal
Test in triplicate i.e. three carriers for each test organism</td></tr>
<tr><td colspan="5">Additional conditions are possible with other test organisms and higher lg reduction requirements. For some additional test organisms, the remaining inoculum post-drying or after the ADC time has elapsed may not be sufficient to demonstrate the lg reduction as required above. The test result is not valid and cannot be used for claiming compliance with the document, however the results may be used for evidence of efficacy if total inactivation is achieved.
Preliminary tests to validate absence of residual effect – see 5.1.1. See 5.2.1 and Annex B for detail on test organisms.</td></tr>
</table>

A.2 Distribution tests

Table A.2 — Distribution tests

Part 2: Distribution tests	The test can be done in parallel to the efficacy test, at the same time in the same test enclosure. If not done in parallel, it shall be done in the same test enclosure.	
Obligatory	Staphylococcus. aureus Reduction 5 lg	
Test organism and efficacy requirement		
Diffusion device	Only one device in the test enclosure. Its position shall be the same as in the efficacy test. The position of the diffusion device shall be described in the test report.	
Enclosure	Large (30 m^3 to 150 m^3)	Small (0,25 m^3 to 4 m^3)
Carriers position and orientation	- Two carriers per corner in opposite corners, at 0,50 m from the floor and walls (two vertically positioned facing away from the source, and two horizontally positioned facing the floor) - Two carriers per corner in the other opposite corners, at 0,5 m from the ceiling and walls (two vertically positioned facing away from the source and two horizontally positioned facing the ceiling)	- Two carriers per corner in opposite corners, at 0,15 m from the floor and walls (two vertically positioned facing away from the source and two horizontally positioned facing the floor) - Two carriers per corner in the other opposite corners, at 0,15 m from the ceiling and walls (two vertically positioned facing away from the source and two horizontally positioned facing the ceiling)

Annex B
(normative)

Distance between diffusion device and test-carriers

Table B.1 — Values applied with a tolerance of ± 10 % for volumes between 30 m^3 and 150 m^3

Volume of the enclosure (in 10 m^3 increments)	distance between diffusion device and test-carriers (m)
[30 m^3 to 40 m^3]	2,6
[40 m^3 to 50 m^3]	3,0
[50 m^3 to 60 m^3]	3,3
[60 m^3 to 70 m^3]	3,6
[70 m^3 to 80 m^3]	3,9
[80 m^3 to 90 m^3]	4,1
[90 m^3 to 100 m^3]	4,4
[100 m^3 to 110 m^3]	4,6
[110 m^3 to 120 m^3]	4,8
[120 m^3 to 130 m^3]	5,0
[130 m^3 to 140 m^3]	5,2
[140 m^3 to 150 m^3]	5,4

Volume from 0,25 m^3 to 4 m^3
Distance Emission point – Test-carrier: D: > or equal to 40 % of enclosure diagonal facing away from the release source ********** Height of the Test-carriers: H = 50 % of the enclosure height

Annex C
(normative)

Interfering substance

Table C.1 — Conditions for products

Conditions for products used in medical area	
Clean conditions	
For bactericidal, fungicidal, yeasticidal, sporicidal, virucidal and mycobactericidal, activities,	Commercially available bovine albumin (Cohn fraction V) should be used and prepared as follows: — dissolve 0,3 g of bovine albumin in 100 ml of water; — make up to the mark with distilled water; — sterilize by means of 0,22 µm membrane-filtration; — store in a refrigerator for not more than one month; — the final bovine albumin (BSA) concentration in the test is 0,3 g/l.
For sensitive test organisms as defined in 3.6.	Dissolve 0,6 g of Bovine albumin fraction V in 100 ml of water in a 100 ml volumetric flask. Make up to the mark with distilled water. Sterilize by means of 0,22 µm membrane-filtration and store in a refrigerator for a period of no more than one month. The final bovine albumin concentration during the test is 0,6 g/l. Mix volume for volume with reconstituted skimmed milk (refer to 5.2.2.2.3). The final bovine albumin concentration during the test is 0,3 g/l and the reconstituted skimmed milk is diluted to 1/20.
Dirty conditions (bovine albumin and blood)	
Bovine albumin and blood: Commercially available bovine albumin (Cohn fraction V, suitable for microbiological requirements) should be used and prepared as follows: — dissolve 3 g of bovine albumin in 100 ml of water; — make up to the mark with distilled water; — sterilize by means of 0,22 µm membrane-filtration; — the final bovine serum albumin (BSA) concentration in the test is 3 g/l; — store in a refrigerator for a period of one month. Sheep erythrocytes: Commercially available sterile defibrinated sheep blood should be used. — centrifuge the erythrocytes using at least 8 ml of fresh defibrinated sheep blood at 800 g for 10 min; — after removing the supernatant, resuspend the erythrocytes in the diluent; — repeat this procedure at least three times such that the supernatant is colourless. Bovine albumin and sheep erythrocyte solution: — suspend 3 ml of concentrated erythrocytes in 97 ml of 3 % (m/v) bovine albumin solution; — the final bovine albumin and sheep erythrocyte concentration in the test procedure is 3 g/l and 3 ml/l, respectively; — to prevent contamination, this mixture should be divided into the number of aliquots required for one day and stored in separate containers for not more than seven days between 2 °C and 8 °C. NOTE The preparation of the soiling condition in the presence of skimmed milk may require a specific protocol and an inoculum/interfering substance ratio other than 1/10 so as to prevent red blood cell aggregation. In this case, the mixture ratio should be taken into account in the calculations.	

Conditions for products used in the veterinary area	
Low-level soiling conditions (bovine albumin solution)	
For bactericidal, fungicidal, yeasticidal, sporicidal, virucidal and mycobactericidal, activities,	Commercially available bovine albumin (Cohn fraction V) should be used and prepared as follows: — dissolve 0,3 g of bovine albumin in 100 ml of water; — make up to the mark with distilled water; — sterilize by means of 0,22 µm membrane-filtration; — store in a refrigerator for not more than one month; — the final bovine albumin (BSA) concentration in the test is 0,3 g/l.
For sensitive test organisms as defined in 3.6.	Dissolve 6 g of Bovine albumin (Cohn fraction V) in 100 ml of water in 90 ml of water in a 100 ml volumetric flask. Make up to the mark with distilled water. Sterilize by means of 0,22 µm membrane-filtration and store in a refrigerator for a period of no more than one month. The final bovine albumin concentration during the test is 6 g/l. Mix volume for volume with reconstituted skimmed milk (refer to 5.2.2.2.4). The final bovine albumin concentration during the test is 3 g/l and the reconstituted skimmed milk is diluted to 1/20
Bacteriophages	See above
High-level soiling conditions (mixture of bovine albumin solution and yeast extract)	
For bactericidal, fungicidal, yeasticidal, sporicidal, virucidal and mycobactericidal, activities,	Dissolve 50 g of yeast extract in 150 ml of water in a 250 ml volumetric flask and allow the foam to subside. Make up to the mark with water. Transfer into a clean flask and sterilize in an autoclave. Allow to cool to ambient temperature. Take up 25 ml of this solution in a 50 ml volumetric flask and add 10 ml of water. Dissolve 5 g of bovine albumin (Cohn fraction V) in the solution while stirring and allow the foam to subside. Make up to the mark with water and sterilize by means of 0,22 membrane-filtration. Store in a refrigerator (2°C to 8°C) and use within one month. The final concentration in the test is 10 g/l of bovine albumin and 10 g/l of yeast extract.
For sensitive test organisms as defined in 3.6.	The preparation of the high level soiling condition in the presence of skimmed milk may require a specific protocol and an inoculum/interfering substance ratio other than 1/10 due to ingredient solubility and the high concentration of the stock solution. In this case, the mixture ratio should be taken into account in the calculations.
Conditions for products used in the food, industrial, domestic and institutional area	
Clean conditions	
For bactericidal, fungicidal, yeasticidal, sporicidal, virucidal and mycobactericidal, activities,	Dissolve 0,3 g of Bovine albumin (Cohn fraction V) in 100 ml of water in a 100 ml volumetric flask. Make up to the mark with distilled water. Sterilize by means of 0,22 µm membrane-filtration and store in a refrigerator for a period of no more than one month. The final bovine albumin concentration during the test is 0,3 g/l.
For sensitive test organisms as defined in 3.6.	Dissolve 0,6 g of Bovine albumin (Cohn fraction V) in 100 ml of water in a 100 ml volumetric flask. Make up to the mark with distilled water. Sterilize by means of 0,22 µm membrane-filtration and store in a refrigerator for a period of no more than one month. The final bovine albumin concentration during the test is 0,6 g/l. Mix volume for volume with reconstituted skimmed milk (refer to 5.2.2.2.4). The final bovine albumin concentration during the test is 0,3 g/l and the reconstituted skimmed milk is diluted to 1/20.
Bacteriophages	See above

Conditions for products used in the food, industrial, domestic and institutional area	
Dirty conditions	
For bactericidal, fungicidal, yeasticidal, sporicidal, virucidal and mycobactericidal, activities,	Dissolve 3 g of Bovine albumin (Cohn fraction V) in 100 ml of water in a 100 ml volumetric flask. Make up to the mark with distilled water. Sterilize by means of 0,22 µm membrane-filtration and store in a refrigerator for a period of no more than one month. The final bovine albumin concentration during the test is 3 g/l.
For sensitive test organisms as defined in 3.6.	Dissolve 6 g of Bovine albumin (Cohn fraction V) in 90 ml of water in a 100 ml volumetric flask. Make up to the mark with distilled water. Sterilize by means of 0,22 µm membrane-filtration and store in a refrigerator for a period of no more than one month. The final bovine albumin concentration during the test is 6 g/l. Mix volume for volume with reconstituted skimmed milk (refer to 5.2.2.2.4). The final bovine albumin concentration during the test is 3 g/l and the reconstituted skimmed milk is diluted to 1/20.

Annex D
(normative)

Preparation of spore stock suspensions of Bacillus subtilis

D.1 Material and reagents

D.1.1 Tryptone Glucose Broth (TGB):

For preparation of the inoculum of Bacillus subtilis:

Yeast extract	2,5 g
Tryptone	5,0 g
Glucose	1,0 g
Water (see 5.2.2.2.1)	1 000 ml

Distribute of 10 ml per tube. Sterilize in the autoclave. After sterilization the pH of the medium shall be equivalent to (7,2 ± 0,2) when measured at (20 ± 1) °C.

D.1.2 Yeast extract Agar (MYA):

For preparation of Bacillus spores:

Meat extract	10,0 g
Yeast extract	2,0 g
$MnSO_4, H_2O$	0,04 g
Agar	15,0 g
Water (see 5.2.2.2.1)	1 000 ml

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

D.1.3 Centrifuge, capable of 10 000 g_N acceleration

D.2 Preparation of Bacillus spore stock suspensions

Seed the TGB medium (refer to D.1.1) with approximately 10^6 spores using bacteria preserved as indicated in EN 12353. Incubate at (30 ± 1) °C in order to obtain a culture in the exponential growth stage (about 24 h) containing approximately 10^7 CFU/ml. Transfer 2 ml or 3 ml of this culture into a Roux flask containing MYA (refer to D.1.2) and tilt it several times so that the inoculum comes into contact with all of the surface of the agar. Remove excess inoculum.

Incubate the Roux flasks at (30 ± 1) °C. After the third day of incubation, note the state of the culture under a microscope, preferably a phase-contrast type. If sporulation has not started, it is better to begin again. Otherwise, continue the incubation until the plant cells have dissolved (8 to 10 days) and until 80 % of the vegetative cells have sporulated (at least 10 days). Recover the spores and place them in suspension in water (see 5.2.2.2.1). Carry out four successive flushing operations by centrifuging during 20 min (see D.1.3) in water (refer to 5.2.2.2.1). Transfer the last suspension to a flask with screw top and heat it for 10 min at (75 ± 1) °C.

Titrate the spores on TSA (refer to 5.2.2.2.2) after suitable dilution in water (refer to 5.2.2.2.1). Incubate the plates for 72 h at (30 ± 1) °C. Store the spore stock suspension in water (refer to 5.2.2.2.1) in sterile closable vessel (for example falcon tube or glass flasks) with sterile glass beads (diameter: 3 mm to 4 mm) for at least 4 weeks in a refrigerator (2 °C to 8 °C) before use to achieve mature spores or store as a frozen stock suspension.

Annex E
(normative)

Graphical representation of the test procedure

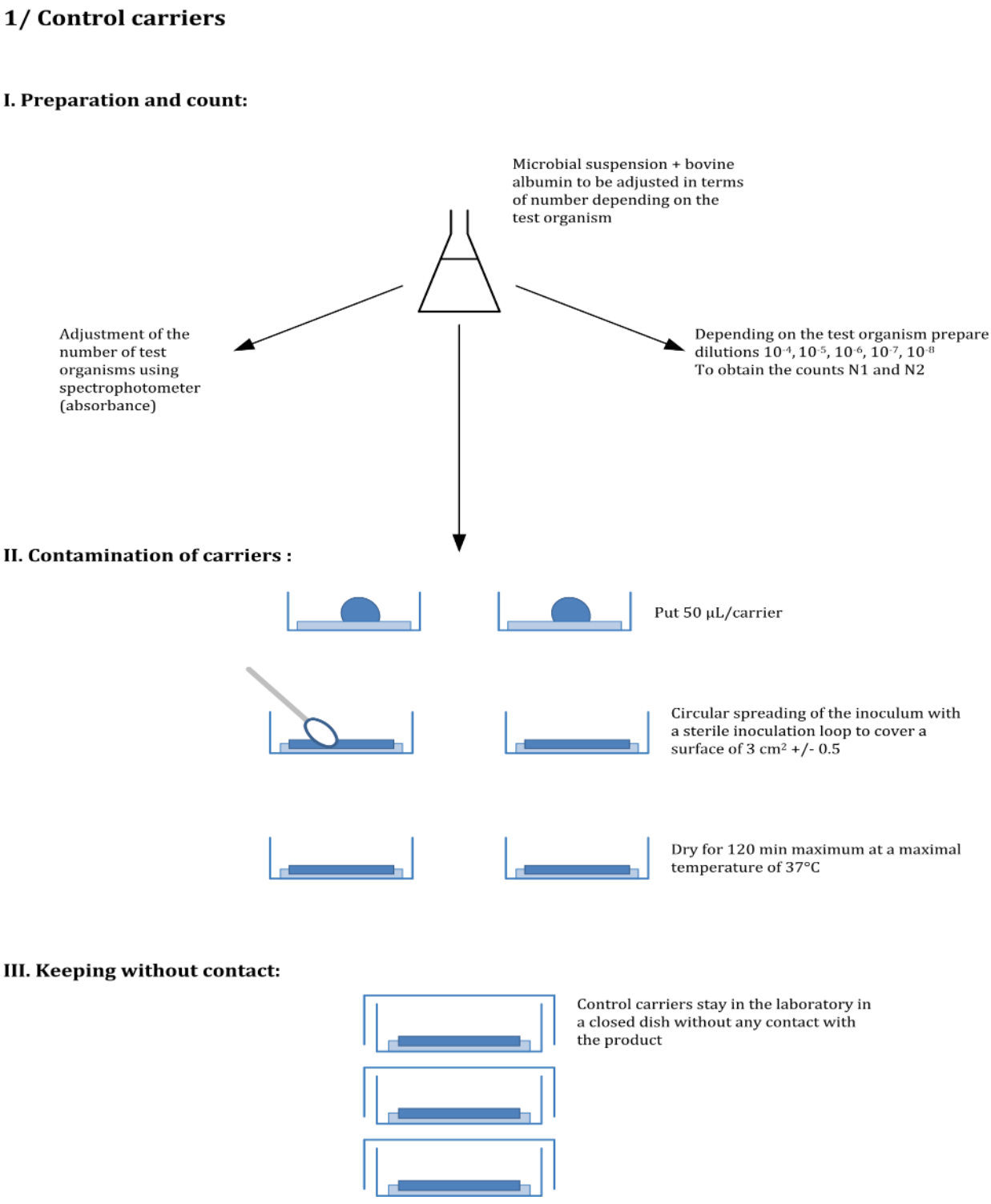
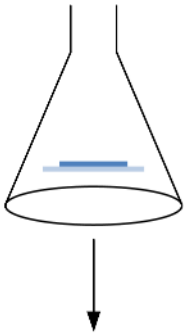


Figure E.1 — Control-carriers

IV. Recovery of viable microorganisms :



100 mL of Recovery liquid, mix + scrap + falcutative use of ultrasounds

Dilutions + plating + count
T = microorganisms count per control carrier

2/ Preliminary Test

I. Carrier preparation:

50 µL of interfering substance alone

Dry for 120 minutes maximum at 37°C maximum

II. Carrier exposed to product:

Expose carriers to the product under conditions similar to the efficacy test to fixate the product on the carrier(s)

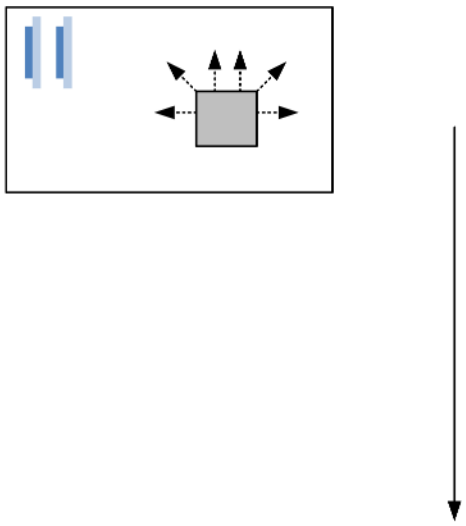


Figure E.2 — Preliminary test

Compare n1 and n3 to N1 dilutions and n2 to N_2 dilutions

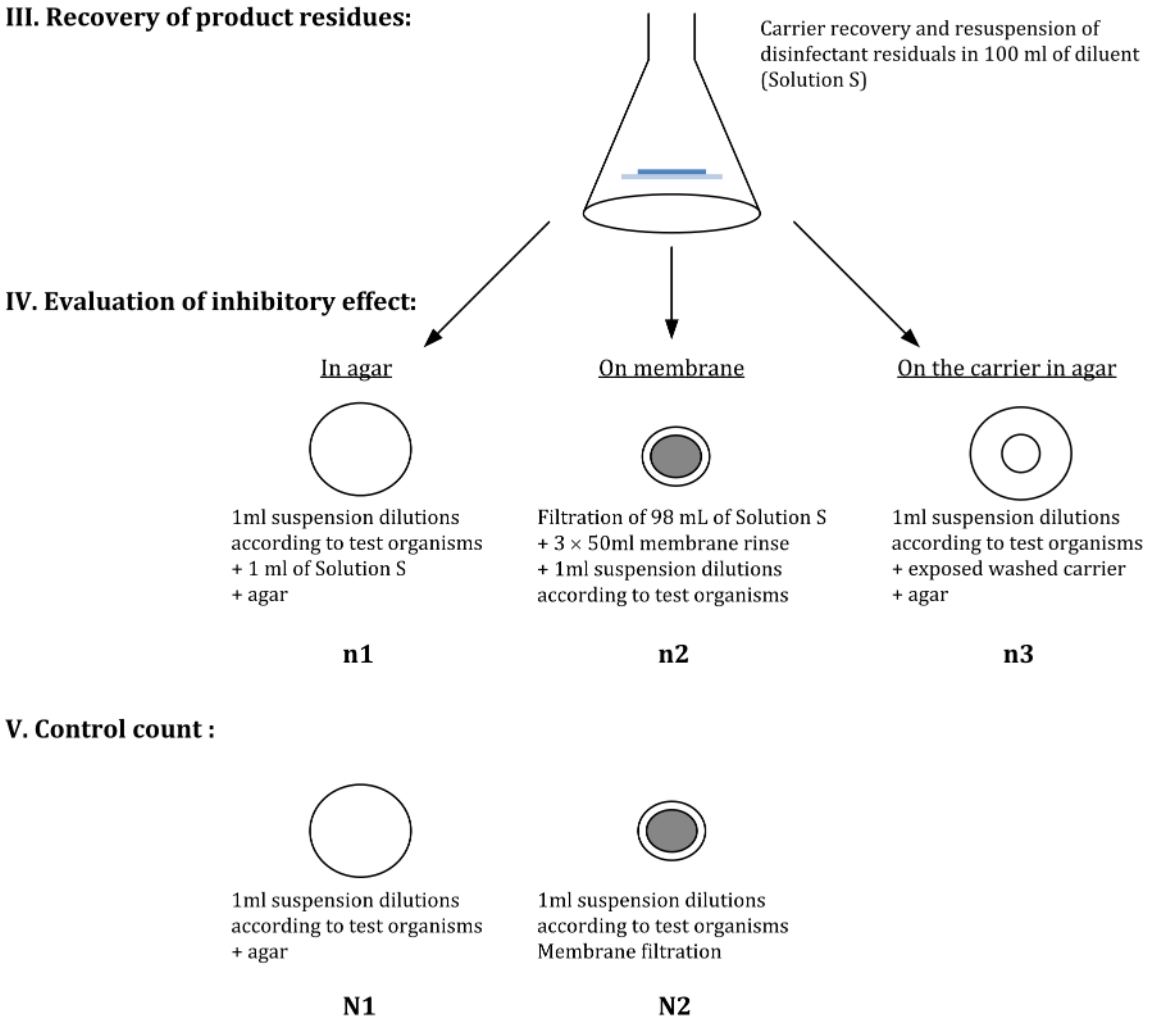
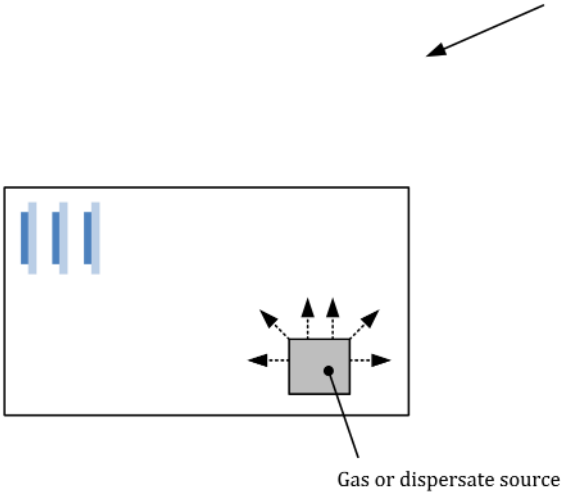


Figure E.3 — Preliminary test – Recovery of product residues

VI. Carrier exposure to disinfectant:



During exposure, the carriers shall be facing away from the disinfectant source at a distance defined in Annex B. The contact time shall be <15 hours. Test conditions shall be noted as describe in this standard.

VII. Viable microorganisms recovery :

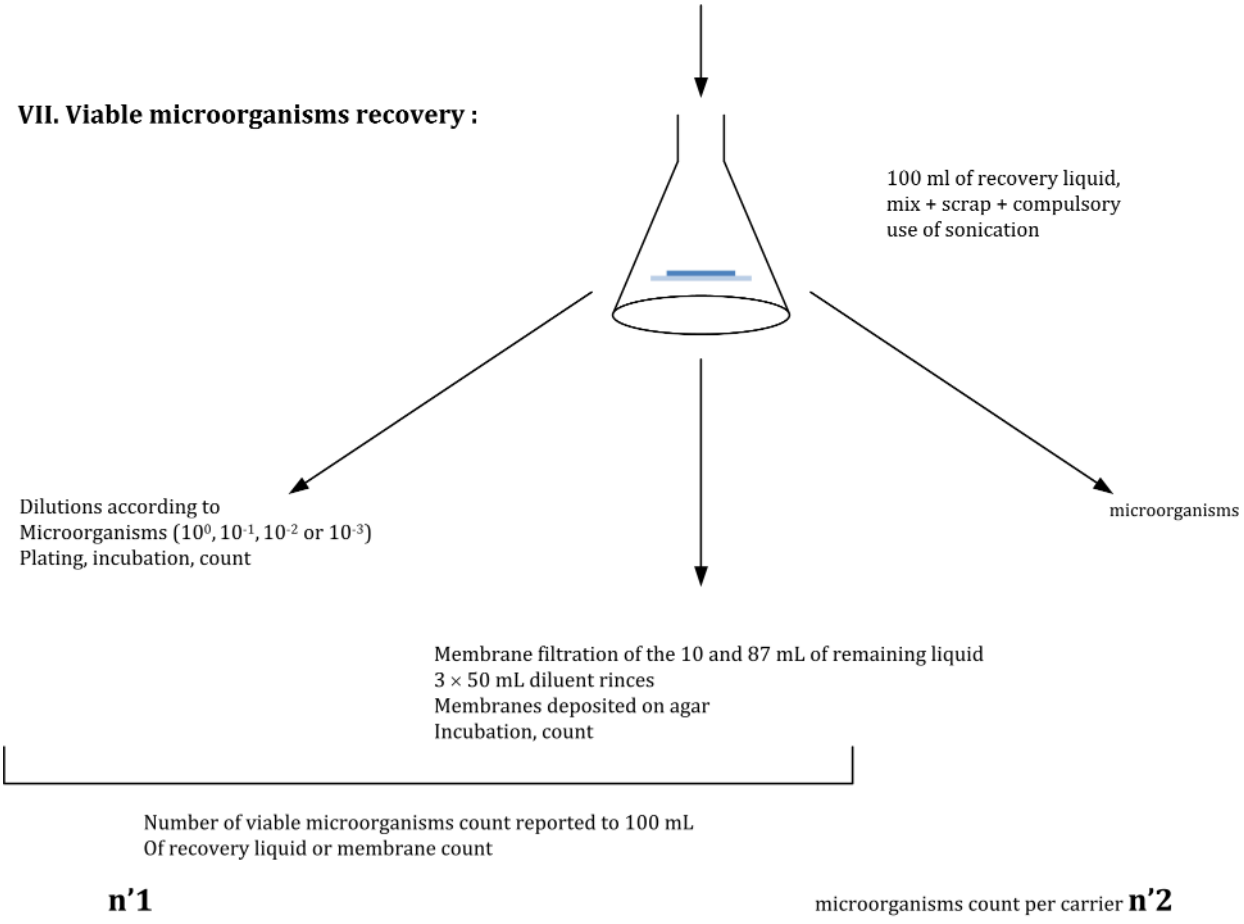


Figure E.4 — Preliminary test – Exposure and recovery

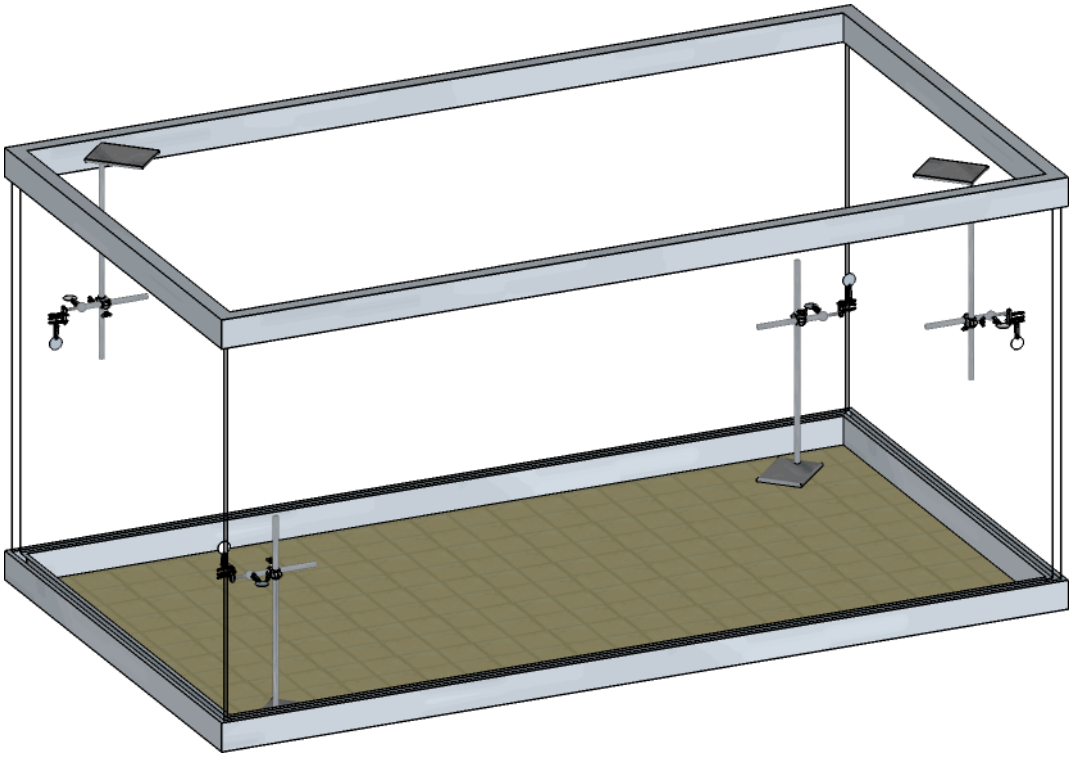


Figure E.5 — Scheme of a test room

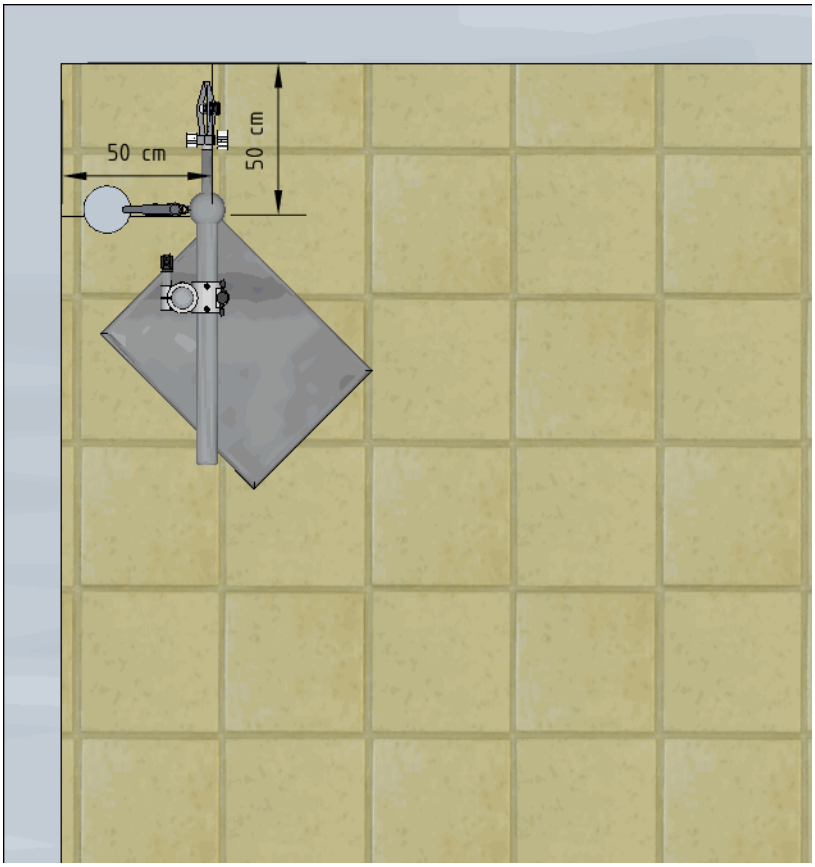


Figure E.6 — Scheme 1 of an example carrier position

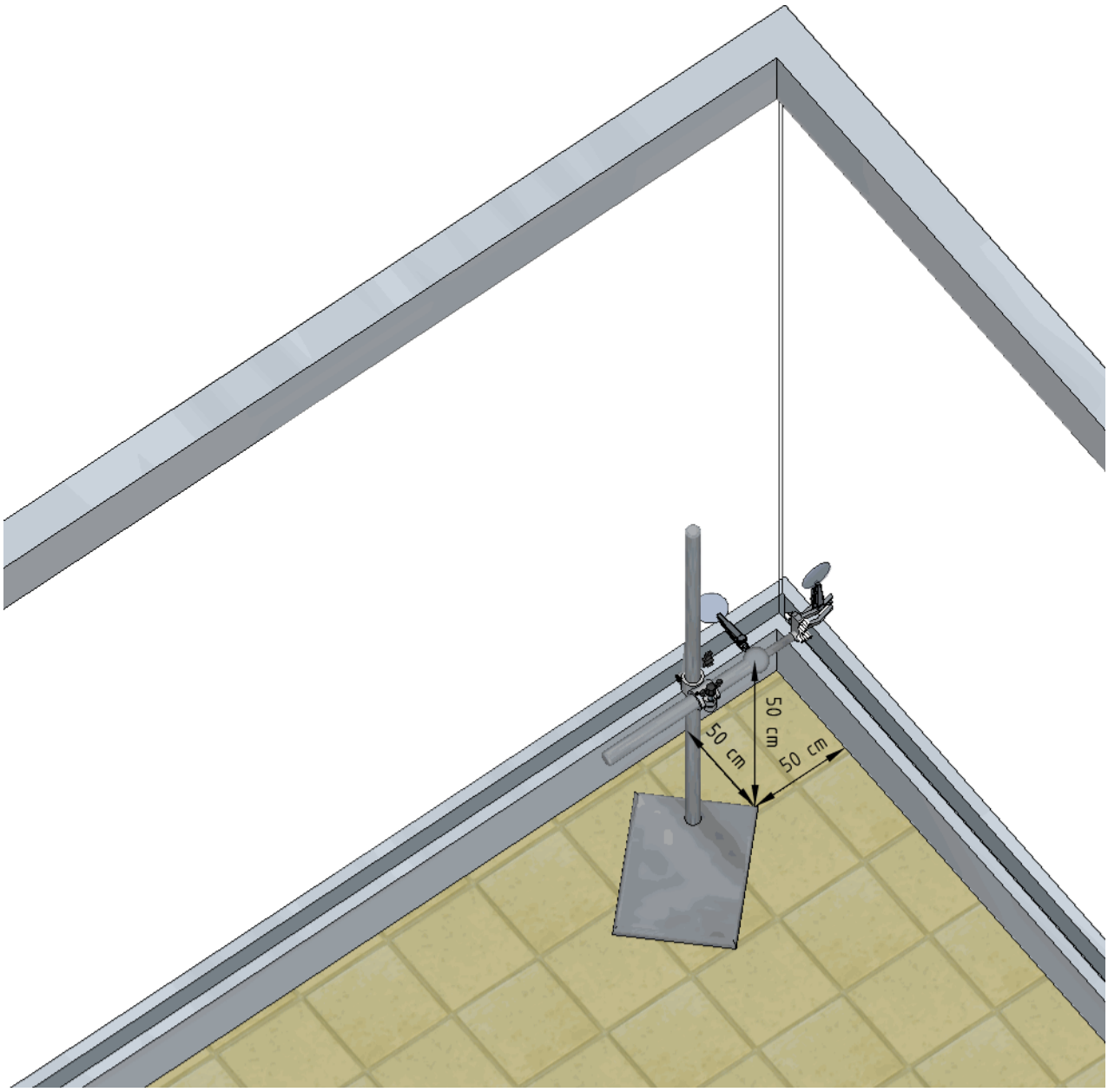


Figure E.7 — Scheme 2 of an example carrier position (not to scale)

Bibliography

[1] EN 10088-1, Stainless steels — Part 1: List of stainless steels

[2] EN 13610, Chemical disinfectants — Quantitative suspension test for the evaluation of virucidal activity against bacteriophages of chemical disinfectants used in food and industrial areas — Test method and requirements (phase 2, step 1)

[3] EN 14204, Chemical disinfectants and antiseptics —Quantitative suspension test for the evaluation of mycobactericidal activity of chemical disinfectants and antiseptics used in the veterinary area — Test method and requirements (phase 2, step 1)

[4] EN 14476, Chemical disinfectants and antiseptics —Quantitative suspension test for the evaluation of virucidal activity in the medical area — Test method and requirements (Phase 2/ Step 1)

[5] EN 14675, Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of virucidal activity of chemical disinfectants and antiseptics used in the veterinary area — Test method and requirements (Phase 2, step 1)

[6] ISO 4793, Laboratory sintered (fritted) filters — Porosity grading, classification and designation