



BSI Standards Publication

**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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## National foreword

This British Standard is the UK implementation of EN 17272:2020+A1:2025. It supersedes BS EN 17272:2020, which is withdrawn.

The start and finish of text introduced or altered by amendment is indicated in the text by tags. Tags indicating changes to CEN text carry the number of the CEN amendment. For example, text altered by CEN amendment A1 is indicated by A1 A1.

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

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

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English Version

**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, virucide et  
phagocid

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

This European Standard was approved by CEN on 13 October 2019 and includes Amendment 1 approved by CEN on 7 April 2025.

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COMITÉ EUROPÉEN DE NORMALISATION  
EUROPÄISCHES KOMITEE FÜR NORMUNG

**CEN-CENELEC Management Centre: Rue de la Science 23, B-1040 Brussels**

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## European foreword

This document (EN 17272:2020+A1:2025) has been prepared by Technical Committee CEN/TC 216 “Chemical disinfectants and antiseptics”, the secretariat of which is held by AFNOR.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by May 2026, and conflicting national standards shall be withdrawn at the latest by May 2026.

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 includes Amendment 1 approved by CEN on 07 April 2025.

This document supersedes A1 EN 17272:2020 A1.

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

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.

A1 Results obtained using the 2020 version of the standard prior to the amendment remain valid. A1

Any feedback and questions on this document should be directed to the users’ national standards body. A complete listing of these bodies can be found on the CEN website.

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, Türkiye 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 5.2.1.7, 5.2.1.8.
- 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 5.2.1.7.

#### **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 5.2.1.9.

#### **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 5.2.1.10 and 5.2.1.11.

#### **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 5.2.1.10.

#### **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 5.2.1.12 and 5.2.1.13.
- 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 5.2.1.14.

#### **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 5.2.1.15 and 5.2.1.16.

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  $\overline{A_1}$  are not required to  $\overline{A_1}$  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. Filter 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 939)
- 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 baumannii* ATCC 19606 = CIP 70.34 (DSM 30007)
- 5.2.1.6 *Proteus hauseri* ATCC 13315 = CIP 58.60 (DSM 30118)

#### **A1** For mycobactericidal activity:

- 5.2.1.7 *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. **A1**

For sporicidal activity tests:

- 5.2.1.9 *Bacillus subtilis* spores ATCC 6633 = CIP 52 62

For yeasticidal activity tests:

- 5.2.1.10 *Candida albicans* ATCC 10231 = IP 4872 (DSM 1386)

For fungicidal activity tests:

- 5.2.1.10 *Candida albicans* ATCC 10231 = IP 4872 (DSM 1386)
- 5.2.1.11 *Aspergillus brasiliensis* ATCC 16404 = IP 1431-83 (DSM 1988)

For virucidal activity tests:

- **A1** 5.2.1.12 Murine Norovirus strain S99, Friedrich Loeffler Institut, Isle de Riems. MNV cultured on RAW 264.7 (ATCC TIB-71) cells line **A1**
- 5.2.1.13 Adenovirus type 5, adenoid strain, ATCC VR-5. *Adenovirus* cultured on HeLa cells or other lines of suitable susceptibility.
- 5.2.1.14 Porcine Parvovirus NADL2 strain cultured on ST cells or other appropriate cells line

**A1** Murine Norovirus strain S99 and Adenovirus type 5 shall be tested for medical area and domestic and institutional area. Porcine Parvovirus NADL2 shall be tested for veterinary area. **A1**

For phagocidal activity:

- 5.2.1.15 Bacteriophage for *Lactococcus lactis* subspecies *lactis* P001 (DSM 4262)
- 5.2.1.16. 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).

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  $(7,2 \pm 0,2)$  when measured at  $(20 \pm 1) ^\circ\text{C}$ .

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 ( $\leq 15$  g/l fat) as follows:

Skimmed powdered milk, guaranteed for the absence of antimicrobial activity .

Sterilize 30 min at  $(105 \pm 3) ^\circ\text{C}$  or 5 min at  $(121 \pm 3) ^\circ\text{C}$ .

#### 5.2.2.2.4 Agar for mycobacteria (refer to 5.2.1.7. and 5.2.1.8)

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  $121 ^\circ\text{C}$  and allow to cool to a temperature of  $50 ^\circ\text{C}$  to  $55 ^\circ\text{C}$  in a water bath.

Add 100 ml of Middlebrook OADC enrichment medium under aseptic conditions. Final pH =  $6,6$  at  $(20 \pm 1) ^\circ\text{C}$ .

#### 5.2.2.2.5 Medium for spores (refer to 5.2.1.9)

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 a)). After sterilization the pH of the medium shall be equivalent to  $(7,2 \pm 0,2)$  measured at  $(20 \pm 1) ^\circ\text{C}$ .  
 Other media may be used for spores: see Annex D.

#### 5.2.2.2.6 Malt extract agar (MEA)

For counts on the number of viable yeast and fungal spore cells (5.2.1.10 and 5.2.1.11).

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  $5,6 \pm 0,2$  when measured at  $(20 \pm 1) ^\circ\text{C}$ .  
 If necessary, add a neutralizer to the medium (and record the addition in the test report).

A1 Deleted text A1

#### 5.2.2.2.7 Diluent for microbial suspensions

A1 Bacteria, yeast, fungi and spores A1

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  $(7,2 \pm 0,2)$  when measured at  $(20 \pm 1) ^\circ\text{C}$ .

Mycobacteria: Tryptone salt A1 (5.2.2.2.7) A1 or distilled water (5.2.2.2.1).

The diluent shall not induce interference between the test organisms and the product under test. A1 In this case, water (5.2.2.2.1) can be used as diluent A1.

#### 5.2.2.2.8 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 to                                                                        | 1 000 ml                                                               |
| <span style="border: 1px solid black; padding: 0 2px;">A1</span> Polysorbate 80 | 5,0 g <span style="border: 1px solid black; padding: 0 2px;">A1</span> |

##### Preparation:

Dissolve the sodium chloride, tryptone A1 and polysorbate A1 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.

**A1** For viruses, preservation media (refer to 5.2.2.3) is used instead of diluents. **A1**

### 5.2.2.3 Culture media and reagents for preparation of virus (test organisms 5.2.1.12 to 5.2.1.14.)

**A1** Deleted text **A1**

For viruses, Eagle minimum essential medium (MEM) or equivalent should be used, supplemented with a suitable concentration of mycoplasma-free inactivated foetal calf serum, and antibiotics and other growth factors if required **A1** deleted text **A1**.

### 5.2.2.4 Culture media and reagents for preparation of bacteriophages (test organisms 5.2.1.15 to 5.2.1.16.)

#### 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 <sub>2</sub> O                       | 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  $(7,0 \pm 0,2)$  when measured at  $(20 \pm 1) ^\circ\text{C}$ .

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  $7,0 \pm 0,2$  when measured at  $(20 \pm 1) ^\circ\text{C}$ . Once the agar has cooled to  $(45 \pm 1) ^\circ\text{C}$ , add 10 ml of sterile 1 mol/l CaCl<sub>2</sub> 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  $\text{CaCl}_2$  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 $\text{H}_2\text{O}$ | 2,5 g     |
| Water (see 5.2.2.2.1) to                  | 1 000 ml. |

Adjust the pH of the buffer to  $(7,4 \pm 0,1)$  when measured at  $(20 \pm 1)^\circ\text{C}$ . Sterilize by autoclaving.

**5.2.2.4.6  $\text{CaCl}_2$  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  $\text{CaCl}_2$  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.

**A1**

**5.2.2.4.8 Bacteriophage and virus recovery and rinsing liquid**

For bacteriophage and virus, growth and preservation media (5.2.2.3.1) are used. **A1**

**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.

▢<sup>A1</sup> The composition and sterilization method shall be recorded in the test report ▢<sup>A1</sup>.

### 5.2.3 Carriers

#### 5.2.3.1 General

New carriers shall be used for each test.

#### 5.2.3.2 Obligatory carriers

▢<sup>A1</sup> 304 ▢<sup>A1</sup> 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 ▢<sup>A1</sup> thick ▢<sup>A1</sup>. 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 ®<sup>1</sup> 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<sup>2</sup> (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

<sup>1</sup> 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.

- a) For moist heat sterilization, an autoclave capable of being held at  $(121 \pm 3)^\circ\text{C}$  for a minimum holding time of at least 15 min;
- b) for dry heat sterilization, a hot air oven capable of being held at  $(180 \pm 5)^\circ\text{C}$  for a minimum holding time of 30 min, at  $(170 \pm 5)^\circ\text{C}$  for a minimum holding time of 1 h, or at  $(160 \pm 5)^\circ\text{C}$  for a minimum holding time of 2 h.

**5.3.2.2 Water bath**, capable of being controlled at  $(45 \pm 1)^\circ\text{C}$ .

**5.3.2.3 Incubators**

- Incubator, for drying the contaminated carriers in less than 2 h with or without air exchange, controlled at  $(37 \pm 1)^\circ\text{C}$ .
- Incubator (for bactericidal, mycobactericidal, sporicidal, virucidal, phagocidal activity), capable of being held at  $(36 \pm 1)^\circ\text{C}$  or at  $(37 \pm 1)^\circ\text{C}$ . Incubator (for fungicidal and yeasticidal activity), capable of being held at  $(30 \pm 1)^\circ\text{C}$ .
- $\text{CO}_2$  incubator (95 % air, 5 %  $\text{CO}_2$ ), capable of being held at  $(36 \pm 1)^\circ\text{C}$  or at  $(37 \pm 1)^\circ\text{C}$  for cell culture and virus incubation.

**5.3.2.4 pH-meter** accurate to  $\pm 0,1$  pH units at  $(20 \pm 1)^\circ\text{C}$ , 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,  $0,45\ \mu\text{m}$  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  $2^\circ\text{C}$  and  $8^\circ\text{C}$

**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  $40\ \mu\text{m}$  to  $100\ \mu\text{m}$  (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 sporocidal 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, spores, fungal spores, yeasts, mycobacteria)

#### 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  $(37 \pm 1)^\circ\text{C}$ . 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  $(37 \pm 1)^\circ\text{C}$ .

##### 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 5.2.2.2.4) and incubate them in an incubator at  $(36 \pm 1)^\circ\text{C}$  or  $(37 \pm 1)^\circ\text{C}$ , to prevent the plates from drying out, use a  $\text{CO}_2$  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  $(36 \pm 1)^\circ\text{C}$ .

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  $(30 \pm 1)^\circ\text{C}$ . 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  $(30 \pm 1)^\circ\text{C}$  for seven to nine days.

### 5.4.1.3 Preparation of suspensions in interfering substance

#### 5.4.1.3.1 Bacteria (see 5.2.1.1 to 5.2.1.6)

Take 10 ml of diluent (5.2.2.2.7) and place in a 100 ml flask with 5 g of glass beads  $\text{A}_1$  (see 5.3.2.11)  $\text{A}_1$ .

Take a loopful of working culture  $\text{A}_1$  (refer to 5.4.1.2.2)  $\text{A}_1$  and transfer it into the diluent (refer to 5.2.2.2.7).

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  $\text{A}_1$  (see 5.3.2.6)  $\text{A}_1$ .

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 5.2.2.2.7), estimating the number of colony forming units (cfu) either spectrophotometrically or by any other suitable means between  $5 \times 10^7$  and  $2 \times 10^9$  cfu/ml.

Then add the interfering substance in order to obtain a 1/10 dilution  $\text{A}_1$  (e.g. add 1ml of interfering substance to 9 ml of suspension)  $\text{A}_1$ .

Keep this test suspension in the water bath at  $(20 \pm 1)^\circ\text{C}$  and use within 2 h after preparation.

For protecting test organisms sensitive to drying (refer to 3.7), 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.

1<sup>st</sup> adaptation step<sup>2</sup>:

$\text{A}_1$  Adding skimmed milk (refer to 5.2.2.2.3) to the interfering substance (refer to 5.2.2.5) as indicated in Annex C  $\text{A}_1$ .

2<sup>nd</sup> adaptation step<sup>2</sup>:

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

Increase the initial inoculum up to  $5 \times 10^9$  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).

3<sup>rd</sup> adaptation step:

Allowed if standard conditions cannot be achieved when applying the 2<sup>nd</sup> adaptation step.

Increase the initial inoculum up to  $\text{A}_1$   $5 \times 10^9$   $\text{A}_1$  and add to the interfering substance a 1/20 dilution rate of skimmed milk  $\text{A}_1$  (refer to 5.2.2.2.3)  $\text{A}_1$ .

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