INTERNATIONAL STANDARD

ISO 21322

First edition
2020-06

Cosmetics — Microbiology — Testing of impregnated or coated wipes and masks

Cosmétiques — Microbiologie — Essais sur lingettes et masques imprégnés ou enduits

Reference number
ISO 21322:2020(E)

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Published in Switzerland

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 217, *Cosmetics*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

For technical reasons, current standards in cosmetics microbiology may not be applicable to impregnated or coated cosmetic products, such as wipes and masks, in which there is no direct access to the formulation.

Based on their product form or delivery there is a need to adapt these standards to assess the microbiological quality of these products.

Cosmetics — Microbiology — Testing of impregnated or coated wipes and masks

1 Scope

This document gives guidance for the enumeration and/or detection of microorganisms present in a cosmetic product that is impregnated or coated onto a substrate (i.e. wipes and masks) where sampling and microbiological influence of the manufactured product presents particular challenges in terms of microbiological sampling and testing.

The principle of this document can also be applied to test similar products (e.g. cushion, impregnated sponge, etc.) or applicators (e.g. brush, puff, sponge, etc.) with modification of the procedure as appropriate.

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.

ISO 11930, *Cosmetics — Microbiology — Evaluation of the antimicrobial protection of a cosmetic product*

ISO 16212, *Cosmetics — Microbiology — Enumeration of yeast and mould*

ISO 18416, *Cosmetics — Microbiology — Detection of Candida albicans*

ISO 21148, *Cosmetics — Microbiology — General instructions for microbiological examination*

ISO 21149, *Cosmetics — Microbiology — Enumeration and detection of aerobic mesophilic bacteria*

ISO 21150, *Cosmetics — Microbiology — Detection of Escherichia coli*

ISO 22717, *Cosmetics — Microbiology — Detection of Pseudomonas aeruginosa*

ISO 22718, *Cosmetics — Microbiology — Detection of Staphylococcus aureus*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

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

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
cosmetic formulation
preparation of raw materials with a qualitatively and quantitatively defined composition

3.2
cosmetic product
cosmetic formulation (3.1) that has undergone all stages of production, including packaging in its final container, for shipment

3.3
impregnated product
product absorbed on the support

3.4
coated product
product adsorbed on the support

3.5
test sample
representative unit of the entire *cosmetic product* (3.2) for testing

4 Principle

4.1 General information

The method determines the population of viable microorganisms by enumeration of colonies on a non-selective agar medium and by the presence or absence of specified microorganisms growth after enrichment.

The method involves the following steps:

— selection of the test sample;

— selection of an appropriate method;

— recovery of microorganisms;

— enumeration of the population of viable microorganisms by filtration or plate count method;

— tests for specified microorganisms by enrichment method.

The experimental conditions shall be evaluated to ensure that the method should not affect the viability of microorganisms and the recovery of bioburden from the sample and should include the verification of the efficacy of the neutralization (see Clause 11).

In order to ensure product quality and safety for the consumer, an appropriate microbiological risk assessment should be performed to determine the types of cosmetic products to which this document is applicable (see ISO 29621:2017, Table 2).

Other methods may be substituted provided that their equivalence has been demonstrated.

4.2 Selection of the test sample

— Whenever practical, the entire unit should be used for testing with a minimum weight of 1 g. If for technical reasons the entire unit cannot be tested, a defined Unit Item Portion (UIP) is used for testing. A “UIP” is a microbiologically-representative subunit of the test sample and is referenced throughout the document.

— If the unit is < 1 g per unit then the appropriate number of units should be sampled to achieve the appropriate weight or volume.

— The weight of the test sample shall be recorded even if the results are expressed by unit.

Selection of the test sample shall be according to A.1.

4.3 Selection of the method

The method should be conducted according to an appropriate procedure based on the specifics of the product (size, volume, single unit/multi–unit package, level of bioburden. etc.) and should ensure that a representative sample is evaluated.

Selection of the method shall be according to A.1 and A.2.

4.4 Recovery of microorganisms from the test sample

The degree to which microorganisms adhere to the test sample surface is dependent on the wipe or mask in which the liquid portion of the formulation has been either impregnated or coated. Preliminary treatments may be necessary to separate microorganisms from the test sample.

Regardless of the treatment, the verification of recovery method should be performed in order to demonstrate that the method can release microorganisms from the test sample without having an adverse effect on their viability (see Clause 11).

4.5 Enumeration of aerobic mesophilic microorganisms

4.5.1 General

The enumeration of aerobic mesophilic microorganisms includes bacteria, yeasts and moulds.

Based on the nature of the test sample, the volume of diluent used to immerse the test sample and the expected level of bioburden, two types of counting methods may be used:

— plate count method;

— membrane filtration method.

4.5.2 Plate count method overview

Plate count method consists of either using a pour plate or spread plate method.

Each method consists of the following steps.

— Prepare the agar plates and diluent using a non-selective agar medium for plating the diluent in which the sample was immersed.

— Incubate the plates for enumeration and/or detection.

— Count the number of colonies forming units (CFU) based on the number of aerobic mesophilic microorganisms recovered per unit or g.

4.5.3 Membrane filtration method overview

Membrane filtration consists of the following steps.

— Transfer the diluent or a defined quantity of diluent in which the test sample was immersed to a filtration apparatus wetted with a small volume of an appropriate sterile diluent.

— After filtration and rinsing, transfer the membrane filter onto the surface of plates with non-selective agar medium.

— Aerobic incubation of the plates.

— Count the number of colony forming units (CFU) and calculate of the number of aerobic mesophilic microorganisms per g or unit.

4.6 Detection of specified microorganisms by enrichment method

The objective of the enrichment method is to incubate a test sample in a non-selective broth medium to increase the number of microorganisms that are present in a test sample.

— The first step of an enrichment method is to incubate the test sample in a non-selective broth medium to increase the number of microorganisms present in the test sample.

— The second step of an enrichment method is to isolate specified microorganisms that may be present on a test sample through the use of selective agar media followed by confirmatory identification tests for characteristic colonies. See ISO 18416, ISO 21150, ISO 22717 and ISO 22718.

5 Diluents, neutralizers and culture media

5.1 General

The diluents, neutralizers and culture media suitable for enumeration and detection of aerobic mesophilic microorganisms are described in ISO 11930, ISO 16212 and ISO 21149. Other diluents, neutralizers and culture media may be used if they have been demonstrated to be suitable for use.

Use the general instructions given in ISO 21148. When water is mentioned in this document, use distilled water or purified water as specified in ISO 21148.

5.2 Diluents and neutralizers

The diluent is used to disperse the sample. It is required that it contain neutralizers if the sample to be tested has antimicrobial properties or contains a preservative. The efficacy of the neutralization shall be demonstrated before the determination of the count (see Clause 11). Diluents and neutralizers shall be in accordance with ISO 11930, ISO 16212, ISO 18416, ISO 21149, ISO 21150, ISO 22717 and ISO 22718.

5.3 Culture media

5.3.1 Media for enumeration and detection

Culture media for enumeration and/or detection shall be in accordance with ISO 11930, ISO 16212, ISO 18416, ISO 21149, ISO 21150, ISO 22717 and ISO 22718.

5.3.2 Media for preparation of spores of *Bacillus subtilis*

See C.1.3.1.

6 Apparatus and glassware

The laboratory equipment, apparatus and glassware are described in ISO 21148.

7 Strains of microorganisms

The culture should be reconstituted according to the procedures provided by the supplier of the reference strain. The strains may be stored in the laboratory conforming to EN 12353 or according to another suitable method.

For testing the recovery of microorganisms on the test samples, spores of *Bacillus subtilis* ATCC 6633 (equivalent strain CIP 52.62 or NCIMB 8054 or NBRC 3134 or other equivalent national collection strain) are used.

For testing the efficacy of neutralizers, two strains representative of both Gram negative and Gram positive bacteria and a yeast are used:

— *Staphylococcus aureus* ATCC 6538 (equivalent strain: CIP 4.83 or NCIMB 9518 or NBRC 13276 or KCTC 1916 or other equivalent national collection strain);

— *Pseudomonas aeruginosa* ATCC 9027 (equivalent strain: CIP 82.118 or NCIMB 8626 or NBRC 13275 or KCTC 2513 or other equivalent national collection strain).

An alternative Gram negative strain may be *Escherichia coli* ATCC 8739 (equivalent strain: CIP 53.126 or NCIMB 8545 or NBRC 3972 or KCTC 2571 or other equivalent national collection strain).

— *Candida albicans* ATCC 10231 (equivalent strain: IP 48.72 or NCPF 3179 or NBRC 1594 or KCTC 17205, or other equivalent national collection strain).

The strains may be kept in the laboratory according to the EN 12353.

8 Handling of cosmetic products and laboratory samples

If storage is necessary, keep the products to be tested at room temperature. Do not incubate, refrigerate or freeze products and samples before or after analysis. Sampling and test procedures should follow the guidelines specified in ISO 21148 and in accordance with the procedure outlined in Clause 9.

9 Procedure

9.1 General recommendation

Use sterile equipment and aseptic technique whenever preparing the test sample and diluent.

For the preparation of an initial suspension, the time which elapses between the end of the preparation of the test sample and the moment the diluent of the initial suspension comes into contact with the culture medium shall not exceed (30 ± 15) min, unless specifically mentioned in the established protocols or documents.

The method should follow the procedure developed during the suitability test, to ensure neutralization of potential inhibitory effects (see Clause 11) and to guarantee the recovery of microorganisms.

9.2 Selection and preparation of the test sample

9.2.1 Selection of the test sample

The test sample shall weigh at least 1 g.

The test sample can be either the entire unit, or multiple units if the weight of one unit is less than 1 g, or the UIP (see A.1).

Record the exact weight of the test sample, *S*, and, the number of units, *n*.

If a UIP is used for testing, record the UIP value of the test sample (see 4.2).

9.2.2 Preparation of the initial suspension

Place the test sample (see 9.2.1) into an appropriate container, with a known volume of diluent, defined in the suitability test (see Clause 11). The test sample should be completely immersed in the diluent.

Record the value for “V”, the volume of diluent used.

9.3 Recovery of microorganisms

9.3.1 General

After immersion, the following treatments may be used to remove microorganisms from the test sample:

— stomaching;

— shaking/stirring;

— vortexing (see A.3).

NOTE If necessary, record the volume of the diluent after stomaching or shaking of test sample.

9.3.2 Stomaching

Prepare the initial suspension utilizing a sterile stomacher bag and then place it into the stomacher.

Proceed according to the parameters (time and speed) outlined in the suitability test (see Clause 11).

Record the time and the speed at which the stomaching took place.

9.3.3 Shaking/Stirring

Prepare the initial suspension in the appropriate closed container and mix according to the parameters (duration and frequency) applied in the suitability test (see Clause 11).

Sterile glass beads may be added to enhance product mixing and organism recovery.

Record the time, frequency and the speed of shaking/stirring (if applicable) and whether glass beads are added or not.

9.4 Enumeration of aerobic mesophilic microorganisms

9.4.1 General

The enumeration of aerobic mesophilic microorganisms includes bacteria, yeasts and moulds.

The choice of the method depends on the volume of diluent used for the preparation of the initial suspension (see A.2).

Based on the test sample size, the level of bioburden and the sensitivity of the method, all of the diluent in which the test sample is immersed (V) or a fraction of V (Vd) is used for enumeration.

Usually, the volume V or Vd of the initial suspension diluent is the dilution used for enumeration. No further diluting of the initial preparation is required.

The minimal volume of diluent shall be equivalent to at least 1 g of the test sample.

9.4.2 Pour plate method

Use the appropriate number of Petri dishes to properly evaluate the volume of diluent needed to properly immerse and transfer the product to be plated (V or Vd).

The diluent (V) is divided into two work streams: one half (V/2) is for the enumeration of bacteria and the other half (V/2) for enumeration of yeasts and moulds.

If the enumeration is conducted on a fraction of the total diluent (Vd), two equal fractions (Vd/2) shall be plated: one for bacteria and the other for yeasts and moulds.

If Petri dishes, 85 mm to 100 mm in diameter are used, add 1 ml of the diluent and pour 15 ml to 20 ml of the melted agar medium (as specified in ISO 21149 and ISO 16212) kept in a water bath not to exceed 48 °C.

If larger Petri dishes (140 mm in diameter) are used, add no more than 10 ml of diluent in each plate and the appropriate amount of agar medium based on the volume of diluent dispensed.

Slowly mix the transferred diluent with the medium, carefully rotating or tilting the plates to sufficiently disperse the agar. Allow the mixture in the Petri dishes to solidify on a horizontal surface at room temperature. Record the volume of diluent plated for each medium V/2 or Vd/2.

9.4.3 Surface spread method

Use the appropriate number of Petri dishes to properly evaluate the volume of diluent needed to properly immerse and transfer the product to be plated (V or Vd).

In Petri dishes 85 mm to 100 mm in diameter, put 15 ml to 20 ml of the melted agar medium kept in a water bath at no more than 48 °C. If larger Petri dishes are used, the volume of the agar is increased accordingly. Allow plates to cool and solidify on a horizontal surface at room temperature.

The diluent (V) is divided into two work streams: one half (V/2) is for the enumeration of bacteria and the other half (V/2) for enumeration of yeasts and moulds.

If the enumeration is conducted on a fraction of the total diluent (Vd), two equal fractions (Vd/2) shall be plated: one for bacteria and the other for yeasts and moulds.

Spread over the surface of the medium a measured volume (V or Vd) of not less than 0,1 ml of the initial suspension and/or sample dilution prepared as described in Clause 11.

Record the volume of diluent plated for each medium V/2 or Vd/2.

9.4.4 Membrane filtration method

Use membranes having a nominal pore size no greater than 0,45 µm.

If all of the diluent (V) is filtered, half (V/2) is for the enumeration of bacteria and the other half (V/2) for enumeration of yeasts and moulds.

If the enumeration is conducted on a fraction of the diluent (Vd), two equal fractions (Vd/2) shall be filtered: one for bacteria and the other for yeasts and moulds.

Filter the volume V/2 of diluent in which the test sample was immersed or the fraction Vd/2 through each of two sterile filter membranes and wash according to the results of the suitability test (See Clause 11).

Transfer the membrane filters onto the surface of the agar media as specified in ISO 21149 and ISO 16212.

Record the volume of diluent filtered for each medium V/2 or Vd/2.

9.4.5 Incubation

Unless otherwise stated, place the inoculated dishes in the incubator set at 32,5 °C ± 2,5 °C for 72 h ± 6 h (bacteria) or at 25 °C ± 2,5 °C for 3 d to 5 d (yeasts and moulds) if antibiotic is added to the medium. If a culture medium without antibiotic is used, incubate at 22,5 °C ± 2,5 °C for 5 days to 7 days.

9.4.6 Counting of colonies

After incubation, count the colonies on each culture medium (N_b for bacteria or N_{ym} for yeasts and moulds) within the recommended countable ranges (see Annex B).

If several plates have been used, take the sum of the colonies counted on each plate for each culture medium.

Calculate the number of bacteria, $N_{b,}$ and the number of yeasts and moulds, $N_{ym,}$ present in the volume of tested diluent plated or filtered for each medium (V/2 or Vd/2) as follows.

N_b or N_{ym} is equal to the sum of the number of colonies obtained from the plates (plate-count method) or to the number of colonies counted on a single plate (filtration method).

Check that the counts are obtained from experimental conditions applied during the suitability test procedure (see Clause 11).

9.5 Detection of specified microorganisms by enrichment method

9.5.1 General

The test for specified microorganisms includes the detection of *P. aeruginosa*, *S. aureus*, *E. coli*, and *C. albicans*.

The principle of the test is the enrichment in a non-selective broth medium, followed by an isolation on selective agar media and the identification of characteristics colonies.

The test may be conducted on the test sample or on 1 g of the test sample.

9.5.2 Test for specified microorganisms

9.5.2.1 Preparation of the initial suspension in the enrichment broth

The initial suspension is prepared by transferring the test sample (see 9.2.) or 1 g of the test sample in a closed container with an appropriate volume of enrichment broth chosen following the procedure developed during the suitability test (see Clause 11). The test sample shall be totally covered and the minimal quantity to be tested should be equivalent to at least 1 g of the test sample. Record the volume of enrichment broth, V, the exact weight of the test sample, S, and the number of units tested, n, if applicable.

9.5.2.2 Incubation of the initial suspension

After recovery of microorganisms (9.3), incubate the initial suspension prepared in broth (see 9.5.2.1) at 32,5 °C ± 2,5 °C for a minimum of 20 h and a maximum of 72 h max.

9.5.2.3 Isolation

After incubation, using a sterile loop streak an aliquot of the incubated enrichment broth onto the surface of suitable detection agar media.

Invert the Petri dishes and incubate at 32,5 °C ± 2,5 °C for 48 h to 72 h in order to obtain isolated colonies, according to ISO 21150, ISO 22717, ISO 22718 and ISO 18416.

9.5.2.4 Detection of specified microorganisms

Growth of characteristic colonies indicates the possible presence of specified microorganisms to confirm by identification tests according to ISO 21150, ISO 22717, ISO 22718 and ISO 18416.

10 Expression of results

10.1 Enumeration of aerobic mesophilic microorganisms

Calculate NS_b the total number of bacteria and NS_{ym} the total number of yeasts and moulds present in the unit. Expression of the results is given in Annex B.

The total number of aerobic mesophilic microorganisms can be expressed in CFU per unit ($NS_b + NS_{ym}$) or in CFU per g ($NS_b + NS_{ym}$)/S.

10.2 Detection of specified microorganisms

If the identification of the colonies confirms the presence of specified microorganism, express the result as:

— "Presence of *P. aeruginosa*, *S. aureus*, *E. coli* or *C. albicans* in the test sample S *or* 1 g of the test sample".

If no growth after enrichment is observed and/or if the identification of the colonies does not confirm the presence of this species, express the result as:

— "Absence of *P. aeruginosa*, *S. aureus*, *E. coli* or *C. albicans* in the test sample S *or* in 1 g of the test sample".

11 Suitability test

The suitability of the test method consists of demonstrating that the method allows for the detection of viable microorganisms and requires:

— The demonstration of the ability of the method for removing the microorganisms from the test sample.

— The evaluation of the effect of the test sample processing on microorganisms.

— The verification of the efficacy of the neutralization.

The method shall be as described in Annex C.

12 Test report

The test report shall specify the following:

a) all information necessary for the complete identification of the product;

b) description of the method used, suitability test included or standard operating procedure reference;

c) results obtained;

d) any point not specified in this document, or regarded as optional, together with details of any incidents that may have influenced the results.

Annex A
(normative)

Guidance on methods for microbiological testing of impregnated or coated products — Wipes and masks

A.1 Selection of the test sample

The choice of an adequate test sample may depend on many factors such as size, volume, nature (wipes or masks) packaging (unitary units or identical units packaged together).

For wipes or masks in a single package, the test sample is the entire unit.

For wipes or masks in a multi-use package, the test sample is one identical unit.

A minimum of 1 unit (wipe or mask) shall be tested except for the following cases.

— If for technical reasons the entire unit cannot be used, a defined Unit Item Portion (UIP) may be used for testing. A UIP is a microbiologically representative subunit of the test sample. In this case attention should be paid to the representativeness of the UIP.

— In the case where the unit is less than 1 g, then a sufficient number of units representing at least 1 g shall be tested.

For example:

— in the case of a wipes pack, the unit is one wipe;

— for a single dose face mask, the unit is the whole mask;

— when the unit is 0,4 g, a minimum of 3 units shall be tested.

A.2 Selection of the enumeration method for aerobic mesophilic microorganisms

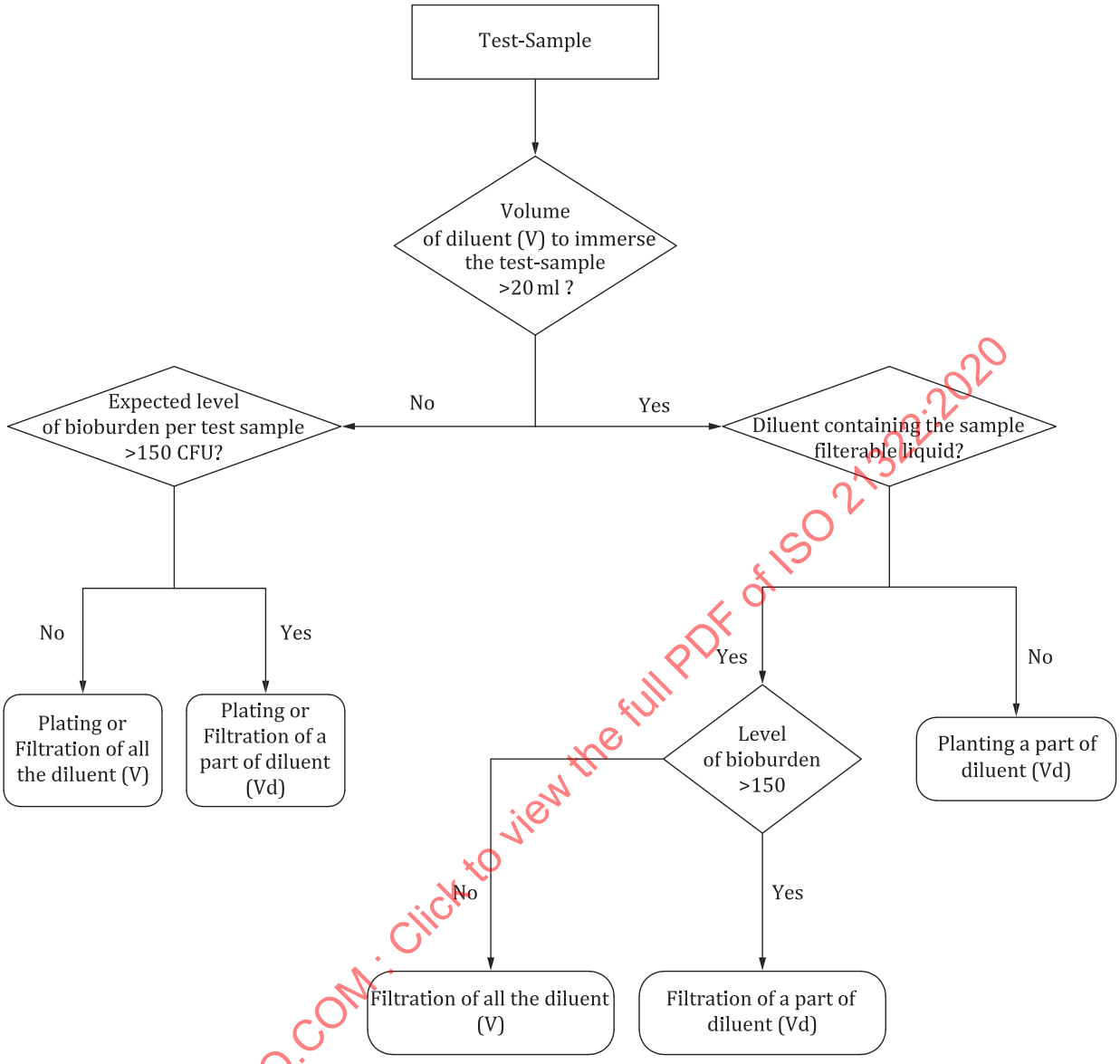
The choice of an adequate enumeration method may depend on the volume of diluent necessary to immerse the test sample and the level of expected bioburden.

For a small volume of diluent (not more than 20 ml) use the plate count method and for larger volume of diluent, the membrane filtration method is preferable (see decision tree).

If high levels of bioburden are expected, the enumeration may be conducted on a fraction of the diluent (Vd).

The sensitivity of the method should provide sufficient margin below the threshold of the product specification.

See Figure A.1.



NOTE In case the test volume is Vd, the dilution factor, the threshold of the method and the specifications are taken into account.

Figure A.1 — Flow chart for the choice of plate count method

A.3 Recovery of microorganisms

Considering that the degree of adhesion of microorganisms may depend on the nature of the product, preliminary treatments may be necessary to remove microorganisms. Treatments should not affect the viability of microorganisms.

Treatment may consist of stomaching, shaking, stirring and/or vortexing.

— **Stomaching**

Stomaching consists of the action of twin reciprocating paddles on the sample contained inside a sterile bag forcing the eluent throughout the sample to ensure proper mixing.

Stomaching is suitable for soft, fibrous and/or absorbent materials such as wipes, facial masks, sponges, puffs, etc. It would be unsuitable for any materials which would puncture the bag.

The duration and the speed of stomaching shall be sufficient to allow the separation of microorganisms from the substrate (see C.4).

— **Shaking/stirring**

Shaking or stirring, consists of an initial suspension mixed with a mechanical shaker by reciprocating or orbital action. Vortex mixing may also be used for small samples with regular surfaces.

Whatever the treatment, the recovery verification should be performed, demonstrating that the method can remove the microorganisms from the test sample (see Clause 11).

Sterile glass beads may be added to enhance recovery.

The duration of mechanical shaking shall be sufficient to allow for the separation of the microorganisms from the substrate (see C.4).

A.4 Summary of procedure for microbiological control of wipes and masks

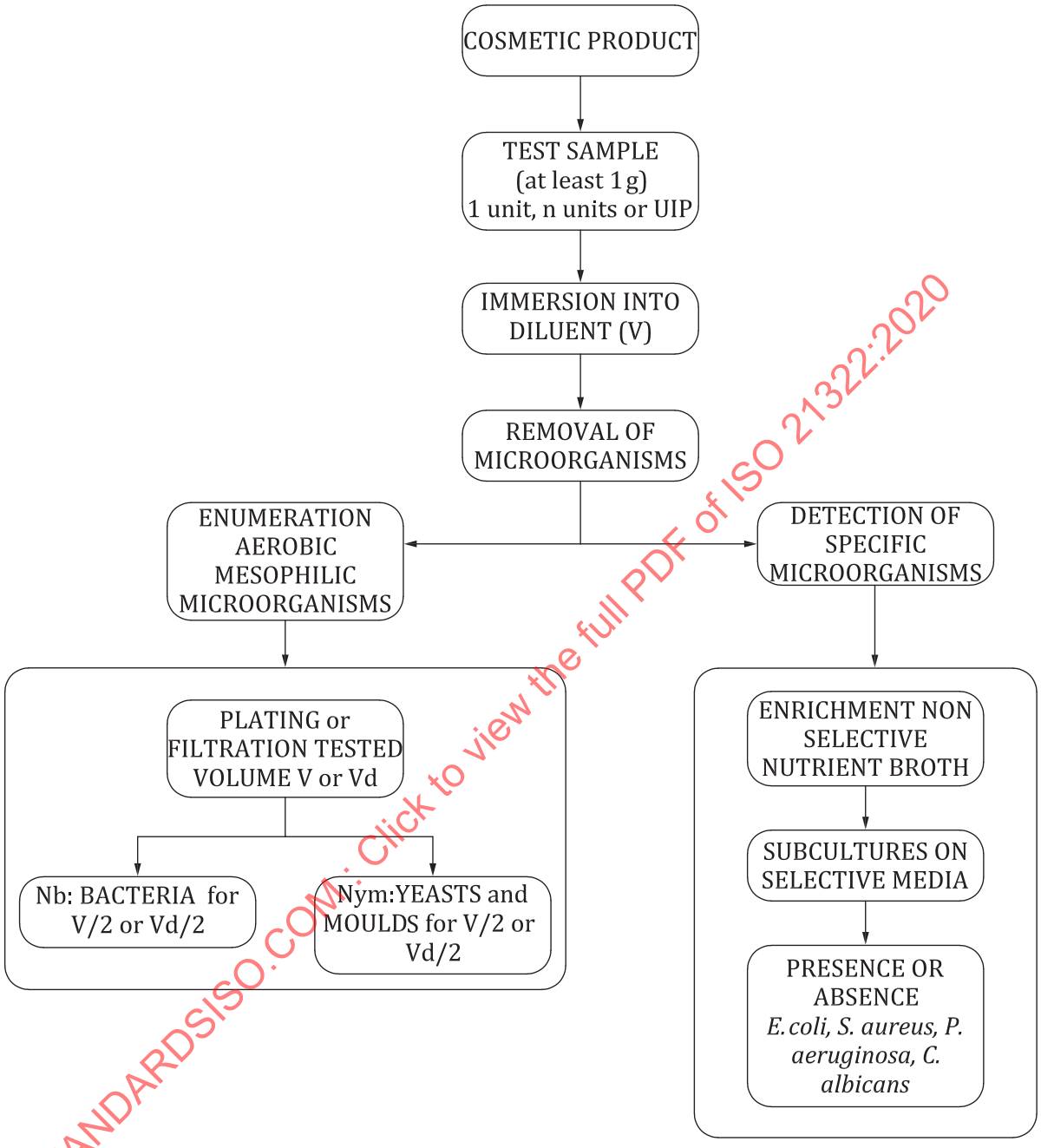


Figure A.2 — Testing flow

Annex B
(informative)

Expression and interpretation of results

B.1 Counting and expression of results

After incubation, the dishes shall, if possible, be examined immediately. Otherwise, they may be stored, unless otherwise specified, for up to a maximum of 24 h in the refrigerator (5 °C ± 3 °C).

NOTE In certain cases, where there is a potential for confusing particles from the product with counted colonies, it can be useful to prepare duplicate dishes containing the same sample dilutions and agar medium which are stored in the refrigerator for comparison with incubated dishes.

B.1.1 Where the number of CFU is between 1 and 300 on bacteria plates or between 1 and 150 on yeasts and moulds plates or membranes, record the number of colonies on Petri dishes and express the result as follow (CFU/unit).

— If one unit has been used for the initial suspension and if all the volume (V) has been plated:

NS_b is equal to $N_b \times 2$ and NS_{ym} is equal to $N_{ym} \times 2$;

— If one unit has been used for the initial suspension and if a fraction of the volume (Vd) has been plated:

NS_b is equal to $(N_b \times 2) \times (V/Vd)$ and NS_{ym} is equal to $(N_{ym} \times 2) \times (V/Vd)$;

— If a portion of unit has been used for the initial suspension and if all the volume (V) has been plated:

NS_b is equal to $(N_b \times 2) \times (1/UIP)$ and NS_{ym} is equal to $(N_{ym} \times 2) \times (1/UIP)$;

— If a portion of unit has been used for the initial suspension and if a fraction of the volume (Vd) has been plated:

NS_b is equal to $(N_b \times 2) \times (V/Vd) \times (1/UIP)$ and NS_{ym} is equal to $(N_{ym} \times 2) \times (V/Vd) \times (1/UIP)$;

— If multiple units (n) have been used for the initial suspension and if all the volume (V) has been plated:

NS_b is equal to $(N_b \times 2) \times (1/n)$ and NS_{ym} is equal to $(N_{ym} \times 2) \times (1/n)$;

— If multiple units (n) have been used for the initial suspension and if a fraction of the volume (Vd) has been plated:

NS_b is equal to $(N_b \times 2) \times (V/Vd) \times (1/n)$ and NS_{ym} is equal to $(N_{ym} \times 2) \times (V/Vd) \times (1/n)$.

The inherent variability of plate counting should be taken into account particularly when the plates contain less than 30 CFU for bacteria, 15 for yeasts and moulds and membrane filtration method.

B.1.2 Where the number of bacteria (N_b) is more than 300 on plates or more than 150 on membranes and the number of yeasts and moulds (N_{ym}) is more than 150 on plates or membranes express the result as follow (CFU/unit).

— If one unit has been used for the initial suspension and if all the volume (V) has been plated:

NS_b is more than 300×2 or more than 150×2 and NS_{ym} is more than 150×2;

— If one unit has been used for the initial suspension and if a fraction of the volume (Vd) has been plated:

NS_b is more than (300 × 2) × (V/Vd) or (150 × 2) × (V/Vd) and NS_{ym} is more than (150 × 2) × (V/Vd);

— If a portion of unit has been used for the initial suspension and if all the volume (V) has been plated:

NS_b is more than (300 × 2) × 1/UIP or (150 × 2) × 1/UIP and NS_{ym} is more than (150 × 2) × (1/UIP);

— If a portion of unit has been used for the initial suspension and if a fraction of the volume (Vd) has been plated:

NS_b is more than (300 × 2) × (V /Vd) × (1/UIP) or (150 × 2) × (V /Vd) × (1/UIP) and NS_{ym} is more than (150 × 2) × (V /Vd) × (1/UIP);

— If multiple units (n) have been used for the initial suspension and if all the volume (V) has been plated:

NS_b is more than (300 × 2) × (1/n) or (150 × 2) × (1/n) and NS_{ym} is more than (150 × 2) × (1/n);

— If multiple units (n) have been used for the initial suspension and if a fraction of the volume (Vd) has been plated:

NS_b is more than (300 × 2) × (V/Vd) × (1/n) or (150 × 2) × (V/ Vd) × (1/n) and NS_{ym} is more than (150 × 2) × (V/Vd) × (1/n).

B.1.3 Where no colony is observed, the result is reported as follows (CFU/unit).

— If one unit has been used for the initial suspension and if all the volume (V) has been plated:

NS_b or NS_{ym} is less than 2;

— If one unit has been used for the initial suspension and if a fraction of the volume (Vd) has been plated:

NS_b or NS_{ym} is less than 2 × (V/Vd);

— If a portion of unit has been used for the initial suspension and if all the volume (V) has been plated:

NS_b or NS_{ym} is less than 2 × (1/UIP);

— If a portion of unit has been used for the initial suspension and if a fraction of the volume (Vd) has been plated:

NS_b or NS_{ym} is less than 2 × (V /Vd) × (1/UIP);

— If multiple units (n) have been used for the initial suspension and if all the volume (V) has been plated:

NS_b or NS_{ym} is less than 2 × (1/n);

— If multiple units (n) have been used for the initial suspension and if a fraction of the volume (Vd) has been plated

NS_b or NS_{ym} is less than 2 × (V/Vd) × (1/n).

NOTE The total number of aerobic mesophilic microorganisms can be also expressed in CFU per g of unit $(NS_b + NS_{ym})/S$.

B.2 Examples of expression of results — Plate count: Pour plate method

B.2.1 Treatment of the test sample

The test sample is transferred to a sterile stomacher bag containing diluent.

The test sample should be totally covered by the diluent.

The bag is placed into a stomacher for 3 min at a speed 5.

After stomaching the initial suspension is shaken at room temperature for approximately 10 min.

B.2.2 Single dose eye masks — Test sample 1 unit

UIP = 1

S = 1,5 g

V = 20 ml

V for bacteria = 10 ml and V for yeasts and moulds = 10 ml

10 plates of 90 mm were used for each medium.

Number of bacteria (per 10 plates):	N_b = 34 CFU
Number of yeasts and moulds (per 10 plates):	N_{ym} < 1 CFU
Total number of bacteria:	NS_b = 34 × 2 = 68 CFU/Unit
Total number of yeasts and mould:	NS_{ym} < 1 × 2
	NS_{ym} < 2 CFU/Unit

The number of aerobic mesophilic microorganisms is 68 CFU/Unit or 45 CFU/g.

B.2.3 Pack of wipes — Test sample 1/5 of a unit (test sample was a large towelette and therefore 1/5 of the towelette was weighed and tested)

UIP = 0,2

S = 6 g

V = 20 ml

V for bacteria = 10 ml and V for yeasts and moulds = 10 ml

Number of bacteria:	N_b < 1 CFU
Number of yeasts and moulds:	N_{ym} < 1 CFU
Total number of bacteria:	NS_b < (1 × 2) × (1/ 0,2)
	NS_b < 10 CFU/Unit
Total number of yeasts and moulds NS_{ym}:	< (1 × 2) × (1/ 0,2)
	NS_{ym} < 10 CFU/Unit

The number of aerobic mesophilic microorganisms is < 10 CFU/Unit or < 2 CFU/g.

B.2.4 Single dose face mask — Test sample 1/2 unit

UIP = 0,5

S = 8 g (formula and support)

V = 50 ml

Vd = 20 ml

V/Vd = 50/20

Vd for bacteria = 10 ml and Vd for yeasts and moulds = 10 ml

Number of bacteria: N_b = 6 CFU

Number of yeasts and moulds: N_{ym} < 1 CFU

Total number of bacteria: NS_b (6 × 2) × (50/20) × (1/0,5) = 60 CFU / Unit

Total number of yeasts and moulds: NS_{ym} < (1 × 2) × (50/20) × (1/0,5)

NS_{ym} < 10 CFU/Unit

The number of aerobic mesophilic microorganisms is 60 CFU/Unit or 8 CFU/g.

B.2.5 Single dose eyes mask — Test sample 2 units (1 unit is less than 1 g)

n = 2

S = 1,5 g

V = 20 ml

V for bacteria = 10 ml and V for yeasts and moulds = 10 ml

Number of bacteria: N_b = 30 CFU

Number of yeasts and moulds: N_{ym} < 1 CFU

Total number of bacteria: NS_b (30 × 2) × (1/ 2) = 30 CFU / unit

Total number of yeasts and moulds: NS_{ym} < (1 × 2) × (1/ 2)

NS_{ym} < 1 CFU / Unit

The number of aerobic mesophilic microorganisms is 30 CFU/Unit or 40 CFU/g.

B.2.6 Single dose face mask — Test sample 1 unit

UIP = 1

S = 5,42 g

V = 100 ml

Vd = 20 ml

V/Vd = 100/20

Vd for bacteria = 10 ml and Vd for yeasts and mould = 10 ml

Number of bacteria: N_b = 15 CFU

Number of yeasts and moulds: N_{ym} < 1 CFU

Total number of bacteria: NS_b (15 × 2) × (100/20) = 150 CFU/Unit

Total number of yeasts and moulds: NS_{ym} < (1 × 2) × (100/20)

NS_{ym} < 10 CFU/Unit

The number of aerobic mesophilic microorganisms is < 150 CFU/Unit or 28 CFU/g.

B.3 Examples of expression of results — Plate count membrane filtration method

B.3.1 Treatment of the test sample

The test sample is transferred to a sterile stomacher bag containing diluent.

The test sample should be totally covered by the diluent. The bag is placed into stomacher for 3 min at a speed of 5.

After stomaching the initial suspension is shaken at room temperature for 10 min.

B.3.2 Pack of wipes — Test sample 1 unit

UIP = 1

S = 5,42 g (formula and support)

V = 100 ml

V for bacteria = 50 ml and V for yeasts and moulds = 50 ml

Number of bacteria: N_b = 108 CFU

Number of yeasts and moulds: N_{ym} < 1 CFU

Total number of bacteria: NS_b 108 × 2 = 216 CFU/Unit

Total number of yeasts and moulds: NS_{ym} < 1 × 2

NS_{ym} < 2 CFU / Unit

The number of aerobic mesophilic microorganisms is 216 CFU/Unit or 40 CFU/g.

B.3.3 Single dose face mask — Test sample — 1/2 unit

UIP = 0,5

S = 20 g (formula and support)

V = 100 ml

V for bacteria = 50 ml and V for yeasts and moulds = 50 ml

Number of bacteria: N_b < 1 CFU

Number of yeasts and moulds: N_{ym} < 1 CFU

Total number of bacteria: NS_b < (1 × 2) × (1/0,5)

NS_b < 4 CFU/Unit

Total number of yeasts and moulds: NS_{ym} < (1 × 2) × (1/0,5)

NS_{ym} < 4 CFU/Unit

The number of aerobic mesophilic microorganisms is < 4 CFU/Unit or < 0,2 CFU/g.

B.3.4 Single dose eyes mask — Test sample 2 units — Each unit is less than 1 g

n = 2

S = 1,5 g (formula and support)

V = 50 ml

Vd = 20 ml (for this example 20 ml was used as the representative aliquot)

Vd for bacteria = 10 ml and Vd for yeasts and moulds = 10 ml

V/Vd = 50/20

Number of bacteria: N_b = 30 CFU

Number of yeasts and moulds: N_{ym} < 1 CFU

Total number of bacteria: NS_b (30 × 2) × (50/20) / 2 = 75 CFU / unit

Total number of yeasts and moulds: N_{sym} < (1 × 2) × (50/20) / 2

Ns 2,5 CFU / unit

The number of aerobic mesophilic microorganisms is 75 CFU/Unit or 100 CFU / g based on Formula (B.1):

(75 CFU /unit) × (2 units) / 1,5 g = 100 CFU /g (B.1)

B.3.5 Single dose face mask — Test sample 1 unit

UIP = 1

S = 20 g (formula and support)

V = 100 ml

V for bacteria = 50 ml and V for yeasts and moulds = 50 ml

Number of bacteria: N_b < 1 CFU

Number of yeasts and moulds: N_{ym} < 1 CFU

Total number of bacteria: NS_b < 1 × 2

NS_b < 2 CFU/Unit

Total number of yeasts and moulds: NS_{ym} < 1 × 2

NS_{ym} < 2 CFU/Unit

The number of aerobic mesophilic microorganisms is < 2 CFU/Unit or < 0,1 CFU/g based on Formula (B.2):

(<2 CFU / unit) × (1 unit) / 20 g = <0,1 CFU / g (B.2)

B.3.6 Single dose face mask — Test sample 1/2 unit

UIP = 0,5

S = 28 g

V = 100 ml

Vd = 50 ml - (for this example 50 ml was used as the representative aliquot)

Vd for bacteria = 25 ml and Vd for yeasts and moulds = 25 ml

V/Vd = 100/50

Number of bacteria:	N_b = 38 CFU
Number of yeasts and moulds:	N_{ym} < 1 CFU
Total number of bacteria:	NS_b (38 × 2) × (100/50) × (1/0,5) = 304 CFU/Unit
Total number of yeasts and moulds:	NS_{ym} < (1 × 2) × (100/50) × (1/0,5)
	NS_{ym} < 8 CFU/Unit

The number of aerobic mesophilic microorganisms is 304 CFU/Unit or 11 CFU/g.

Annex C
(normative)

Suitability test method

C.1 Preparation of inoculum

C.1.1 Bacteria

Prior to the test, and for each strain, inoculate the surface of soybean casein digest agar (SCDA) or other suitable medium. Incubate at 32,5 °C ± 2,5 °C for 18 h to 24 h. To harvest the bacterial culture, use a sterile loop, streak the surface of the culture and re-suspend into the diluent for bacterial suspensions (see ISO 21149) to obtain a calibrated suspension of about 1×10^8 CFU/ml (e.g. using a spectrophotometer, see ISO 21148:2017, Annex C).

Use this suspension and its dilutions within 2 h.

C.1.2 *Candida albicans*

Prior to the test, inoculate the *surface* of Sabouraud dextrose agar (SDA) or other suitable medium.

Incubate the plate at 32,5 °C ± 2,5 °C for 18 h to 24 h.

To harvest the culture, use a sterile loop, streak the surface of the culture and suspend into the diluent (see ISO 18416) to obtain a calibrated suspension of about 1×10^6 CFU per ml (e.g. using a spectrophotometer, see ISO 21148:2017, Annex C). Use this calibrated suspension and its dilutions within 2 h.

C.1.3 Preparation of spores of *Bacillus subtilis*

C.1.3.1 Culture media

C.1.3.1.1 Tryptic soy agar (TSA) or soybean casein digest agar (SCDA)

Pancreatic digest of casein 15,0 g

Papaic digest of soybean meal 5,0 g

Sodium chloride 5,0 g

Agar 15,0 g

Water 1 000 ml

Distribute in suitable flasks. After sterilization the pH of the medium shall be equivalent to (7,3 ± 0,2) when measured at room temperature.

C.1.3.1.2 Peptone glucose agar with meat and yeast extract

Glucose 1,0 g

Meat peptone 6,0 g

Casein peptone 4,0 g