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Cosmetics — Microbiology — Evaluation of the antimicrobial protection of a cosmetic product

Cosmétiques — Microbiologie — Évaluation de la protection antimicrobienne d'un produit cosmétique

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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.

This second edition cancels and replaces the first edition (ISO 11930:2012), which has been technically revised. The main changes compared to the previous edition are as follows.

— Two types of diluents, composition 1 and composition 2 can be used as the diluents for bacteria and *Candida albicans* on the revised version (5.2.3).

— 5.6.2 Paragraph 2 has been changed to "When counts of surviving microorganisms obtained in 5.6.1.4 c) are less than 30 for bacteria and *C. albicans* or less than 15 for *A. brasiliensis* at the dilution where neutralization has been checked, record the number of colonies on Petri dishes and express results by multiplying by the dilution factor. If no colonies are observed at the dilution where neutralization has been checked, note the result as <1 and multiply by the dilution factor."

Introduction

This document is designed to be used in the overall evaluation of the antimicrobial protection of a cosmetic product.

The antimicrobial protection of a product can come from many sources:

— chemical preservation;

— inherent characteristics of the formulation;

— package design;

— manufacturing process.

This document defines a series of steps to be taken when assessing the overall antimicrobial protection of a cosmetic product. A reference method for a preservation efficacy test (challenge test) along with evaluation criteria is also described in this document.

The test described in this document involves, for each test microorganism, placing the formulation in contact with a calibrated inoculum, and then measuring the changes in the microorganism count at set time intervals for a set period and at a set temperature.

The data generated by the risk assessment (see ISO 29621) or by the preservation efficacy test, or both, are used to establish the level of antimicrobial protection required to minimize user risk.

Cosmetics — Microbiology — Evaluation of the antimicrobial protection of a cosmetic product

1 Scope

This document specifies a procedure for the interpretation of data generated by the preservation efficacy test or by the microbiological risk assessment, or both, when evaluating the overall antimicrobial protection of a cosmetic product.

It comprises:

— a preservation efficacy test;

— a procedure for evaluating the overall antimicrobial protection of a cosmetic product that is not considered low risk, based on a risk assessment described in ISO 29621.

The preservation efficacy test is a reference method to evaluate the preservation of a cosmetic formulation. It is applicable to cosmetic products in the marketplace.

This test does not apply to those cosmetic products for which the microbiological risk has been determined to be low according to Annex A and ISO 29621.

This test is primarily designed for water-soluble or water-miscible cosmetic products and can be used with modification to test products in which water is the internal (discontinuous) phase.

NOTE This test can be used as a guideline to establish a development method during the development cycle of cosmetic products. In this case, the test can be modified or extended, or both, for example, to make allowance for prior data and different variables (microbial strains, media, incubation conditions exposure time, etc.). Compliance criteria can be adapted to specific objectives. During the development stage of cosmetic products, other methods, where relevant, can be used to determine the preservation efficacy of formulations.

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

ISO 18415, *Cosmetics — Microbiology — Detection of specified and non-specified microorganisms*

ISO 21148:2017, *Cosmetics — Microbiology — General instructions for microbiological examination*

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

ISO 29621, *Cosmetics — Microbiology — Guidelines for the risk assessment and identification of microbiologically low-risk products*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 21148 and the following 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 https://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

3.3
antimicrobial protection of a cosmetic product
ability of a *cosmetic product* (3.2) to overcome microbial contamination that might present a potential risk to the user or to the aesthetic and functional integrity of the product, during intended use

Note 1 to entry: The overall antimicrobial protection includes preservation of the formulation, the specific manufacturing process and protective packaging.

3.4
preservation of a cosmetic formulation
set of means used to avoid microbial proliferation in a *cosmetic formulation* (3.1)

EXAMPLE Preservatives, multifunctional compounds, hostile raw materials, extreme pH, low water-activity values.

3.5
reference method
method applied by interested parties to assess a product on the market and in case of dispute

3.6
development method
in-house method
method used during the development stage of a product before the product is put on the market

3.7
consumer
end user of a *cosmetic product* (3.2)

4 Principle

The evaluation of the antimicrobial protection of a cosmetic product combines the following elements (see Annex A).

a) The characteristics of its formulation (see ISO 29621) or the results of the preservation efficacy test (if performed), or both.

   The preservation efficacy test is described in Clause 5.

b) The characteristics of the cosmetic product in conjunction with the production conditions (see ISO 22716 and ISO 29621), the packaging materials and, if justified, recommendations for use of the product (see ISO 29621) and, when relevant, the area of application and the targeted user population (see Annex D).

This document describes a procedure for the interpretation of data generated by the preservation efficacy test (if appropriate) and by the microbiological risk assessment.

5 Preservation efficacy test

5.1 General

The evaluation of the preservation of a cosmetic formulation is based on inoculation of the formulation with calibrated inocula (prepared from relevant strains of microorganisms). The number of surviving microorganisms is measured at defined intervals during a period of 28 days. For each time and each strain, the log reduction value is calculated and compared to the minimum values required for evaluation criteria A or B (see Annex B).

When used as a reference method, procedures shall be strictly followed in order to avoid variability in results. To determine the preservation efficacy of a formulation during product development, other suitable development methods may be used.

Prior to the test, neutralizer efficacy shall be established (see 5.5), and the microbiological quality of the product shall be determined (in accordance with ISO 21149 and ISO 16212, or with ISO 18415) to ensure that any microorganisms present in the test sample do not interfere with recovery of test organisms.

5.2 Materials, apparatus, reagents and culture media

5.2.1 General

When water is used in diluents, neutralizers or culture media preparation, use distilled water or purified water as specified in ISO 21148:2017, 8.2.

5.2.2 Materials

In addition to the microbiology laboratory equipment described in ISO 21148, the following materials should be used

5.2.2.1 Glass beads, 3 mm to 4 mm in diameter.

5.2.2.2 Sintered glass filter, of porosity 2 (40 µm to 100 µm).

5.2.2.3 Sterile glass containers with closures, of suitable volumes.

5.2.2.4 Centrifuge, capable of a centrifugal force of 2 000*g*.

5.2.3 Diluents

5.2.3.1 General

Unless otherwise specified, all reagents shall be equilibrated at ambient temperature before use. When available, ready-to-use reagents and media may be used.

5.2.3.2 Diluents for bacteria and *Candida albicans*

5.2.3.2.1 Composition 1

Sodium chloride	8,5 g
Water	1 000 ml

5.2.3.2.2 Preparation

Dissolve sodium chloride in the water by mixing. Dispense into suitable containers. Sterilize in the autoclave at 121 °C for 15 min.

5.2.3.2.3 Composition 2

Tryptone pancreatic digest of casein	1,0 g
Sodium chloride	8,5 g
Water	1 000 ml

5.2.3.2.4 Preparation

Dissolve the components in the water by mixing while heating. Dispense into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization, the pH shall be equivalent to 7,0 ± 0,2, when measured at room temperature.

5.2.3.3 Diluent for preparation of *Aspergillus brasiliensis*: polysorbate solution

Prepare a solution of polysorbate 80 (0,5 g/l). Dissolve by mixing while heating until complete dissolution is achieved. Dispense the solution into suitable containers. Sterilize in the autoclave at 121 °C for 15 min.

5.2.4 Neutralizer

5.2.4.1 General

The suitability and effectiveness of the neutralizing agent with respect to the test strains used and to the tested formulation shall be demonstrated as specified in 5.5.

The neutralizer described in 5.2.4.2 is frequently used. Examples of other suitable neutralizers are given in Annex C (see Table C.1).

5.2.4.2 Eugon LT 100 liquid broth

5.2.4.2.1 General

This medium contains ingredients that neutralize inhibitory substances present in the sample (lecithin and polysorbate 80) and dispersing agent octoxynol 9 (Triton X100®[1]). It may be prepared as described in 5.2.4.2.2, or from dehydrated culture medium, according to the manufacturer's instructions. A ready-to-use medium may also be used.

5.2.4.2.2 Composition

Pancreatic digest of casein	15 g
Papaic digest of soybean meal	5 g
Sodium chloride	4 g
L-cystine	0,7 g

1) Triton X100® 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 ISO of this product.

Sodium sulphite	0,2 g
Glucose	5,5 g
Egg lecithin	1 g
Polysorbate 80	5 g
Octoxynol 9	1 g
Water	1 000 ml

5.2.4.2.3 Preparation

Dissolve successively into boiling water polysorbate 80, octoxynol 9 and egg lecithin until they are completely dissolved. Dissolve the other components by mixing while heating. Dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. Mix well after sterilization while the liquid is still hot to redissolve settled substances. After sterilization, the pH shall be equivalent to 7,0 ± 0,2 when measured at room temperature.

5.2.5 Culture media

5.2.5.1 General

Culture media may be prepared as in 5.2.5.2, 5.2.5.3 and 5.2.5.4, or from dehydrated culture media according to the manufacturer's instructions. Ready-to-use media may be used when their composition and/or growth yields are comparable to those of the formulae given in 5.2.5.2.1, 5.2.5.3.1 and 5.2.5.4.1.

5.2.5.2 Culture medium for bacteria: tryptic soy agar (TSA) or soybean casein digest agar medium

5.2.5.2.1 Composition

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

5.2.5.2.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by mixing while heating. Dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. Mix well after sterilization while the liquid is still hot to redissolve settled substances. After sterilization and cooling down, the pH shall be equivalent to 7,3 ± 0,2 when measured at room temperature.

5.2.5.3 Culture medium for *C. albicans*: Sabouraud dextrose agar medium (SDA)

5.2.5.3.1 Composition

Dextrose	40,0 g
Peptic digest of animal tissue	5,0 g
Pancreatic digest of casein	5,0 g
Agar	15,0 g
Water	1 000 ml

5.2.5.3.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by mixing while heating. Dispense the medium into suitable containers. Sterilize in an autoclave at 121 °C for 15 min. After sterilization, the pH shall be equivalent to 5,6 ± 0,2 when measured at room temperature.

5.2.5.4 Culture medium for *A. brasiliensis*: potato dextrose agar (PDA)

5.2.5.4.1 Composition

Potato infusion	200,0 g
Dextrose	20,0 g
Agar (see 5.2.5.4.2, Note 1)	20,0 g
Water	1 000 ml

5.2.5.4.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by heating. Dispense the medium into suitable containers. Sterilize in an autoclave at 121 °C for 15 min. After sterilization, the pH shall be equivalent to 5,6 ± 0,2 when measured at room temperature.

NOTE Commercially available dehydrated medium powders that contain less than 20 g/l of agar can be supplemented with extra agar to the final concentration of 20 g/l if necessary.

5.3 Microbial strains

The test shall be run using the following strains as test microorganisms[2)]:

— *Pseudomonas aeruginosa* ATCC®9027™[3)] (equivalent strain: CIP®82.118™[4)] or NCIMB®8626™[5)] or NBRC®13275™[6)] or KCTC®2513™[7)] or other equivalent national collection strain);

2) These are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

3) ATCC®: American Type Culture Collection

4) CIP®: Collection de l'Institut Pasteur

5) NCIMB®: National Collection of Industrial Marine Bacteria

6) NBRC®: NITE Biological Resource Center, JP

7) KCTC®: Korean Collection for Type Cultures

— *Staphylococcus aureus* ATCC®6538™ (equivalent strain: CIP®4.83™ or NCIMB®9518™ or NBRC®13276™ or KCTC®3881™ or NCTC®10788™[8]) or other equivalent national collection strain);

— *Escherichia coli* ATCC®8739™ (equivalent strain: CIP®53.126™ or NCIMB®8545™ or NBRC®3972™ or KCTC®2571™ or NCTC®12923™ or other equivalent national collection strain);

— *Candida albicans* ATCC®10231™ (equivalent strain: IP 48.72™[9]) or NCPF® 3179™[10]) or NBRC®1594™ or KCTC®7965™ or other equivalent national collection strain);

— *Aspergillus brasiliensis* ATCC®16404™ (equivalent strain: IP 1431 or IMI®149007™[11]) or NBRC®9455™ or KCTC®6196™ or other equivalent national collection strain).

The culture can be acquired frozen, freeze-dried, on slants or in ready-to-use formats and should be prepared according to the procedures provided by the supplier of the reference strain. The strains should be stored in a laboratory conforming to EN 12353 or according to another suitable method.

5.4 Preparation and enumeration of inocula

5.4.1 General

To perform the tests, use the strains stored in the laboratory (see 5.3) to obtain the stock cultures and the working cultures.

The stock culture is a confluent culture obtained by streaking slant tubes or plates with the stored strain (single-use vial or bead). After incubation, the stock culture can be kept between 2 °C and 8 °C for two months and is used to obtain the working cultures.

The working culture, prepared when needed to perform a test, is used to obtain the calibrated suspension (inoculum).

The same growth conditions (agar media and incubation) are used for both stock cultures and working cultures (see 5.4.2 and 5.4.3).

NOTE 1 A limited number of serial subcultures and the use of confluent cultures instead of isolated colonies lower the risk of change in the susceptibility of strains. The standardization of growth conditions and of inoculum preparation improves the reproducibility of the test.

NOTE 2 Avoid thawing when multi-dose containers are used (for example, containers with several beads brought out of the freezer to take one bead, then replaced in the freezer).

5.4.2 Preparation of bacterial and *Candida albicans* suspensions

5.4.2.1 To prepare the working culture of the test microorganism, prepare a subculture from the stock culture by streaking slant tubes or plates (TSA for bacteria, SDA for *C.albicans*) in order to obtain a confluent culture. Incubate at (32,5 ± 2,5) °C for 18 h to 24 h.

Prepare in the same way a second subculture, starting from the first subculture, and incubate at (32,5 ± 2,5) °C for 18 h to 24 h. A third subculture can be grown in the same way, starting from the second. The second subculture and the third one (if it was carried out) form the working cultures.

If the second subculture cannot be carried out in a timely manner, then the first subculture can be kept for up to 48 h in the incubator (32,5 ± 2,5) °C and used to prepare the second subculture. In this case, prepare the third 18 h to 24 h subculture and use this in the test.

8) NCTC®: National Collection of Type Cultures

9) IP: Institut Pasteur

10) NCPF®: National Collection of Pathogenic Fungi

11) IMI: International Mycological Institute, UK

It is recommended that a fourth subculture not be prepared from the initial stock culture.

5.4.2.2 Take 10 ml of diluent (5.2.3.2) and place in a suitable sterile container with approximately 5 g of sterile glass beads. Transfer loopfuls of the cells harvested from the agar medium into the diluent; the cells should be suspended in the diluent by rubbing the loop in a small amount of the diluent against the side of the container to dislodge the cells.

5.4.2.3 Shake the container manually or mechanically, for a maximum of 3 min, to homogenize the suspension. Aspirate the upper part of the suspension (avoiding any contact with the glass beads) and transfer the obtained suspension to a sterile container.

5.4.2.4 Adjust the number of cells in the suspension to 1×10^7 cfu/ml to 1×10^8 cfu/ml (bacteria) or 1×10^6 cfu/ml to 1×10^7 cfu/ml (*C. albicans*) using the diluent (5.2.3.2) and in accordance with calibration data produced in the laboratory (e.g. using a spectrophotometer, see ISO 21148:2017, Annex C).

Use this calibrated inoculum within 2 h.

5.4.2.5 At the time of the test, check the initial capacity of the suspension, *N*. Make successive tenfold dilutions of the calibrated suspension in the diluent (5.2.3.2). Perform the enumeration by duplicating 1 ml of the suitable dilutions (see 5.6.2) into TSA for bacteria and into SDA for *C. albicans*. Incubate the dishes at (32,5 ± 2,5) °C for 24 h to 48 h.

5.4.3 Preparation of *Aspergillus brasiliensis* spore suspension

5.4.3.1 To obtain the working culture of the test microorganism, use a stock culture (on PDA) aged not more than 2 months and prepare a suspension in the diluent (5.2.3.3). Inoculate by flooding the surface of PDA (use an appropriate number of Petri dishes), so as to obtain a confluent culture. Incubate at (22,5 ± 2,5) °C for 7 days to 11 days.

5.4.3.2 After incubation, transfer 10 ml of the polysorbate solution (5.2.3.3) to the surface of PDA. Gently detach the spores from the culture surface, for example, using a spatula or glass beads.

Transfer the suspension to an appropriate flask and stir gently for about 1 min in the presence of glass beads. Filter the suspension through a sintered glass filter of porosity 2 (i.e. 40 µm to 100 µm).

5.4.3.3 Carry out a microscopic examination (magnification × 400) to detect the presence of germinated spores or mycelium fragments.

— If germinated spores are present, the suspension shall be discarded.

— If mycelium is present in more than one field out of 10, wash the filtered suspension by centrifuging at 2 000*g* for 20 min. Wash the spores at least twice by resuspending them in the polysorbate solution (5.2.3.3) and centrifuging.

5.4.3.4 Adjust the number of spores in the suspension to a value of about 1×10^6 spores/ml to 1×10^7 spores/ml using the diluent (5.2.3.3) and any appropriate means.

The use of a cell enumeration device (e.g. a haemocytometer) is recommended to adjust the number of spores. If an appropriate cell count chamber is used, follow the instructions accurately.

The suspension should be used during the same working day. It can be used on the following day if stored between 2 °C and 8 °C, but, at the time of the test, the absence of germinated spores shall be checked.

5.4.3.5 At the time of the test, check the initial number of microorganisms, *N*. Make successive tenfold dilutions of the calibrated suspension in the diluent (5.2.3.3). Perform the enumeration by duplicating

1 ml of the suitable dilutions (see 5.6.2) into PDA plates (using an appropriate number of Petri dishes). Incubate the dishes at (22,5 ± 2,5) °C for 3 days to 5 days.

5.5 Demonstration of the neutralizer efficacy

5.5.1 Principle

Neutralization efficacy is the verification that the test method protocol sufficiently neutralizes the antimicrobial aspects of a formulation, ensuring that microorganisms can be detected in the product matrix, without inhibiting the test microorganisms.

A calibrated suspension of microorganisms (about 10^3 cfu/ml) is inoculated in the neutralizer in the presence (test) and in the absence (control) of the formulation. The neutralizer efficacy is demonstrated if the counts performed on the inoculum, N_v, and on the control, N_{vn} (mixture of the neutralizer and diluent), are equivalent and if the count in the test, N_{vf} (mixture of the neutralizer and the formulation), is at least 50 % of N_{vn} (see 5.5.4).

5.5.2 Procedure

Run the test separately for each strain.

a) Prepare a dilution of the calibrated suspension of microorganisms [N is between 1×10^7 cfu/ml and 1×10^8 cfu/ml for bacteria, and between 1×10^6 cfu/ml and 1×10^7 cfu/ml for *C. albicans* and *A. brasiliensis* (see 5.4.2 and 5.4.3)] in order to obtain a suspension containing about 10^3 cfu/ml (inoculum).

b) Transfer 1 g or 1 ml of the formulation to be tested into 9 ml of neutralizer (5.2.4). Shake to disperse the formulation. If the initial dilution conditions/neutralizer prove insufficient for neutralization efficacy, repeat the procedure following guidance in 5.5.4, paragraph 4. Other test conditions are acceptable provided that at least 1 g or 1 ml of formulation is used and a minimal tenfold dilution is performed.

c) Leave the "test" tubes for (30 ± 15) min at room temperature. Run a control in parallel with the same neutralizer, replacing the tested formulation with 1 ml of diluent (5.2.3).

d) Inoculate the "test" tubes [tenfold and, if necessary, the additional dilution in 5.5.2 b)] and "control" tubes with 1 ml of inoculum [5.5.2 a)] (the final volume is 11 ml). Mix.

e) Prepare the "inoculum control". Add 1 ml of the inoculum [5.5.2 a)] to 10 ml of diluent (the final volume is 11 ml). Mix.

f) Enumerate in duplicate by inclusion of 1 ml of each mixture ("test", "control" and "inoculum control") into appropriate agar medium (TSA for bacteria, SDA for *C. albicans* and PDA for *A. brasiliensis*).

   The use of a 1 ml volume of the calibrated suspension is recommended to improve the precision of the counts ("test", "control" and "inoculum control" mixtures).

g) Incubate at (32,5 ± 2,5) °C for 48 h to 72 h for the bacteria and *C. albicans* and at (22,5 ± 2,5) °C for 3 days to 5 days for *A. brasiliensis*.

5.5.3 Calculations

Calculate the number, N_v, of microorganisms, in colony-forming units per millilitre, present in the inoculum control [see 5.5.2 e)].

$\overline{N}$ is the mean number of colonies counted in duplicate over the plates in a 1 ml sample.

N_v shall be about 100.

Calculate the number of microorganisms, in colony-forming units per millilitre, present in the "test" mixture with the neutralizer in the presence of the formulation, N_{vf}, and in the "control" mixture with the neutralizer in the absence of the formulation, N_{vn}.

N_{vf} or N_{vn} is the mean number of colonies counted in duplicate over the plates in a 1 ml sample of "test" or "control" mixture.

5.5.4 Interpretation of results and conclusion on neutralizer efficacy

The efficacy of the neutralizer is demonstrated if $N_{vf} \geq 0{,}5N_{vn}$ and if N_{vn} is close to N_v. If N_{vn} is not close to N_v, the neutralizer is considered toxic for microorganisms.

The inherent variability in enumeration on agar plates shall be taken into account. Two counts are usually considered different only if their difference exceeds 50 %.

Take note of the test conditions (neutralizer, volume, etc.) and in particular the dilution of the formulation (1/10, 1/100 or other) for which the efficacy of the neutralizer was demonstrated.

If the results do not comply with the requirements, it is necessary to

— either modify the neutralizer (see Annex C) or make a further dilution of the sample,

— or carry out a membrane filtration, if possible.

If the results still do not comply with the requirements, it is unlikely that the formulation can be contaminated by the strain concerned. It is possible, even in this case, to issue a test report [see 5.7 and 5.8 f)].

5.6 Determination of the preservation efficacy of the formulation

5.6.1 Procedure

Run the test separately for each strain.

5.6.1.1 Sampling of test product

For each strain, dispense 20 g or 20 ml of the test formulation into a sterile container (5.2.2.3).

5.6.1.2 Inoculation of test microorganisms

Add to each container 0,2 ml of calibrated inoculum (see 5.4.2 and 5.4.3) to obtain between 1×10^5 cfu/ml and 1×10^6 cfu/ml or g for bacteria, and between 1×10^4 cfu/ml and 1×10^5 cfu/ml or g for *C. albicans* and *A. brasiliensis* in the formulation (final concentration). Mix thoroughly to ensure a homogeneous distribution of the inoculum.

The initial concentration of microorganisms present in the inoculated product, N_0, is calculated using the results of the enumeration of the calibrated inoculum, N [see 5.6.3.2 b)].

5.6.1.3 Incubation of the inoculated formulation

Store the containers holding the inoculated formulation at (22,5 ± 2, 5) °C.

5.6.1.4 Sampling and enumeration

a) At each specified sampling interval: 7 days (T7), 14 days (T14) and 28 days (T28), according to the test strain (see Annex B), sample 1 g of inoculated formulation and prepare the dilution for

which neutralization efficacy has been demonstrated (5.5). Ensure the correct dilution factor is used when calculating N_x (see 5.6.3.3).

Leave in contact for (30 ± 15) min at room temperature.

b) Starting from dilution with demonstrated neutralization efficacy, make successive tenfold dilutions in the diluent (see 5.2.3).

c) Carry out microbial enumeration in duplicate using a suitable agar medium (TSA for bacteria, SDA for *C. albicans* or PDA for *A. brasiliensis*) for all dilutions at T7. At T14 and T28, the dilution series may be adjusted based on results from T7.

d) In Petri dishes of 85 mm to 100 mm in diameter, place 1 ml of each dilution and pour 15 ml to 20 ml of melted agar medium kept in a water bath at no more than 48 °C. If larger Petri dishes are used, the amount of agar medium is increased accordingly. Mix the dilution with the medium, carefully rotating or tilting the plates sufficiently to disperse the microorganisms. Allow the mixture to solidify with the Petri dishes starting on a horizontal surface at the room temperature. Other methods of counting (e.g. spread-plating and membrane filtration) can be used in accordance with the parameters indicated above. The use of a 1 ml volume of the counted dilution is recommended to improve the precision in the counts.

e) Incubate at (32,5 ± 2,5) °C for 48 h to 72 h for the bacteria and *C. albicans* and at (22,5 ± 2,5) °C for 3 days to 5 days for *A. brasiliensis*.

5.6.2 Counting of colonies

After incubation, enumerate the colonies on the incubated dishes. For all the enumerations [see 5.4.2.5, 5.4.3.5 and 5.6.1.4 c)], retain the dishes containing between 30 and 300 colonies for bacteria and *C. albicans* and between 15 and 150 colonies for *A. brasiliensis*.

When counts of surviving microorganisms obtained in 5.6.1.4 c) are more than 300 (150 for *A. brasiliensis*), note the result as > 300 (>150 for *A. brasiliensis*).

When counts of surviving microorganisms obtained in 5.6.1.4 c) are less than 30 for bacteria and *C. albicans* or less than 15 for *A. brasiliensis* at the dilution where neutralization has been checked, record the number of colonies on Petri dishes and express results by multiplying by the dilution factor.

If no colonies are observed at the dilution where neutralization has been checked, note the result as < 1 and multiply by the dilution factor.

Determine the number of microorganisms present in the sample at time t_0 ($N_0 = N/100$) in accordance with 5.6.3.2, and the number of survivors at each sampling time, N_x, in accordance with 5.6.3.3.

5.6.3 Calculations

5.6.3.1 General

Check that the neutralizer efficacy is verified (see 5.5.4) and that the experimental data are in accordance with the rules indicated in 5.6.2.

5.6.3.2 Determination of the initial numbers of microorganisms, N and N_0

a) Calculate N, the number of microorganisms present in the calibrated suspensions (see 5.4.2 and 5.4.3), in colony-forming units per millilitre, using Formula (1):

$$N = \bar{C} / (V \times d) \quad (1)$$

where

$\bar{C}$ is the mean number of colonies (see 5.6.2) counted in duplicate over the plates;

V is the volume of inoculum applied to each dish, in millilitres (1 ml, in accordance with 5.4.2 and 5.4.3);

d is the dilution factor of the counted dilution;

N shall be between 1×10^7 cfu/ml and 1×10^8 cfu/ml for bacteria, and between 1×10^6 cfu/ml and 1×10^7 cfu/ml for *C. albicans* and *A. brasiliensis*.

b) Determine N_0, the number of microorganisms inoculated in the formulation at time t_0 using Formula (2):

$$N_0 = N/100 \quad (2)$$

where N_0 shall be between 1×10^5 cfu/ml and 1×10^6 cfu/ml or g for the bacteria, and between 1×10^4 cfu/ml and 1×10^5 cfu/ml or g for *C. albicans* and *A. brasiliensis*.

5.6.3.3 Enumeration of the microorganisms at each sampling time, N_x

Calculate, N_x, the number of surviving microorganisms in the contaminated formulation, in colony-forming units per millilitre or grams, at each sampling time, t_x (T7, T14 or T28), using Formula (3):

$$N_x = C / (V \times d) \quad (3)$$

where

C is the mean number of colonies (see 5.6.2) counted in duplicate over the plates;

V is the volume of inoculum applied to each dish, in millilitres [1 ml, in accordance with 5.6.1.4 a)];

d is the dilution factor corresponding to the retained and counted dilution [see 5.6.1.4 c), taking into account the second tenfold dilution in the neutralizer as in 5.6.1.4 a)];

5.6.3.4 Reduction in microbial counts

Calculate the reduction values, R_x, expressed in log units, obtained at each sampling time using Formula (4):

$$R_x = \lg N_0 - \lg N_x \quad (4)$$

where

N_0 is the number of microorganisms inoculated at time t_0 [see 5.6.3.2 b)];

N_x is the number of surviving microorganisms at each sampling time, t_x (see 5.6.3.3).

There may be no reduction, and there may even be an increase in the microorganism count.

5.7 Interpretation of test results and conclusions

5.7.1 Criteria

The obtained log reduction values, R_x (see 5.6.3.4), are compared to the minimum values required for evaluation criterion A or B reported in Annex B.

The criteria representing the protection capacities of a cosmetic formulation are:

— criterion A, whereby the formulation is protected against microbial proliferation that may present a potential risk for the user and no additional factors are considered [see 6.2 a)];

— criterion B, whereby the level of protection is acceptable if the risk analysis demonstrates the existence of control factors not related to the formulation indicating that the microbiological risk is tolerable for the cosmetic product [see 6.2 b)].

The criteria are expressed either by a minimum log reduction value or by "NI" when the requirement is that there be no increase in the microbial population.

The inherent variability in microbial counts that are used to determine R_X values shall be taken into consideration when comparing the obtained R_X values and the preset criteria A or B. In this document, a deviation of 0,5 log units from the preset criteria is considered acceptable.

5.7.2 General case (efficacy of the neutralizer is demonstrated for all strains)

For each microorganism, compare the values of R_X to criterion A or B (as described in Annex B and 5.7).

a) If all the reduction values comply with criterion A, the formulation satisfies requirements A of the preservation efficacy test and, in accordance with 6.2 a), meets the requirements of this document.

b) If all the reduction values comply only with criterion B, the formulation satisfies requirement B of the preservation efficacy test. Additional justification should be provided to show that the product satisfies the requirements of this document [see 6.2 b)].

c) If one or more of the reduction values do not comply with criterion A or B, then the formulation does not satisfy the requirements of the preservation efficacy test. The status of the product shall be evaluated solely according to the microbiological risk assessment [see 6.2 c)].

5.7.3 Case of formulations for which the efficacy of the neutralizer is not demonstrated for some strains

If the efficacy of the neutralizer has not been demonstrated for some strains despite additional tests (see 5.5.4), the formulation can be considered not susceptible to contamination from these microbial strains. Note the result as "not susceptible to contamination". This result is deemed equivalent to R_X given by the preset criterion of logarithmic reduction given in Annex B.

Interpret the results for the strains for which the efficacy of the neutralizer is demonstrated and compare the values of R_X to the preset criterion A or B (as described in Annex B and 5.7).

If all the reduction values comply with criterion A (or B), the formulation satisfies by extrapolation the requirements A (or B) of the preservation efficacy test.

If one or more of the reduction values do not comply with criterion A (or B), the formulation does not satisfy the requirements of the preservation efficacy test.

5.8 Test report

The test report shall contain the following information:

a) a reference to this document, i.e. ISO 11930:2018;

b) the identification of the testing laboratory;

c) the identification of the cosmetic product (or formulation):

   1) name of product;

   2) batch number or lot date code;

3) name of the entity responsible for marketing the product and name of the manufacturer, if known;

4) date of reception at the laboratory;

5) conditions of storage at the laboratory;

d) the enumeration method used;

e) the experimental conditions:

1) analysis period;

2) conditions of incubation of the inoculated formulation;

3) composition of the neutralizer;

4) incubation temperature of Petri dishes;

5) culture media used;

6) test strains (origin and modes of storage);

7) modes of contamination of the formulation with the test microorganisms (mass/volume of product formulation, volume of calibrated suspension);

f) the test results:

1) quantity of the initial numbers of microorganisms, N and N_0 [see 5.6.3.2 a) and 5.6.3.2 b)];

2) results of the demonstration of the neutralizer efficacy for each test strain and concentration of the formulation for which the neutralization is demonstrated (in the case of non-demonstration for some strains, indicate the test results obtained and the tests carried out to achieve neutralization);

3) test results (enumerations and logarithmic reductions) for each of the test strains and each sampling time (see 5.6.3.3 and 5.6.3.4);

g) conclusions.

6 Overall evaluation of the antimicrobial protection of the cosmetic product

6.1 General

Antimicrobial protection is based on a combination of formulation characteristics, production conditions and final packaging. The overall evaluation takes into account the microbiological risk assessment together with the preservation efficacy test results, if relevant, as set out in the decision diagram (see Annex A). It is the manufacturer's responsibility to provide information to demonstrate that safety has been satisfactorily demonstrated and that the level of risk is tolerable.

6.2 Case 1 — Preservation efficacy test has been performed on the formulation

a) If the formulation meets criterion A, the microbiological risk is considered to be tolerable (the cosmetic product is protected against microbial proliferation that may present a potential risk for the user) and the cosmetic product is deemed to meet the requirements of this document without additional rationale.

b) If the formulation meets criterion B, the microbiological risk analysis shall demonstrate the existence of control factors not related to the formulation; for example, a protective package such

as a pump provides a higher level of protection than a jar (see Annex D). This would be considered a protective device for risk reduction.

Hence, if the risk analysis demonstrates the existence of control factors, the cosmetic product is deemed to meet the requirements of this document on the basis of criterion B plus additional characteristics indicating that the microbiological risk is tolerable.

c) If the formulation does not meet the requirements of either criterion A or B, then the status of the product shall be evaluated solely according to the microbiological risk assessment. For example, a product in single-dose units can be considered a tolerable microbiological risk, even if the formulation does not meet the requirements of either criterion A or B, provided that the microbiological quality of the finished product is ensured at the time of release.

NOTE Other data such as consumer in-use data can be used to substantiate the microbiological risk of a product.

Hence, if the risk analysis demonstrates the existence of strengthened control factors (risk reduction), then the cosmetic product is deemed to meet the requirements of this document.

d) If the cosmetic product does not comply with any of the three previous situations, it does not satisfy the requirements of this document.

6.3 Case 2 — Preservation efficacy test has not been performed on the formulation

In accordance with ISO 29621, the entity responsible for the production and/or the marketing of the cosmetic product shall identify those characteristics of the formulation or the finished cosmetic product, and the control factors, that ensure that the microbiological risk is low.

Those characteristics, and any supporting documentation, that demonstrates that the microbiological risk is tolerable, shall be reported.

Hence, if the results of the microbiological risk analysis show that the formulation or the finished product would be considered low risk, then the cosmetic product is deemed to meet the requirements of this document on the basis of a microbiological risk evaluation, provided that the cosmetic product is produced in compliance with good manufacturing practice.

If the results of the microbiological risk assessment show that the formula or the finished product would not be considered a tolerable risk, then the cosmetic product does not meet the requirements of this document. Risk reduction shall be performed to bring the product into compliance.

Annex A
(normative)

Decision diagram

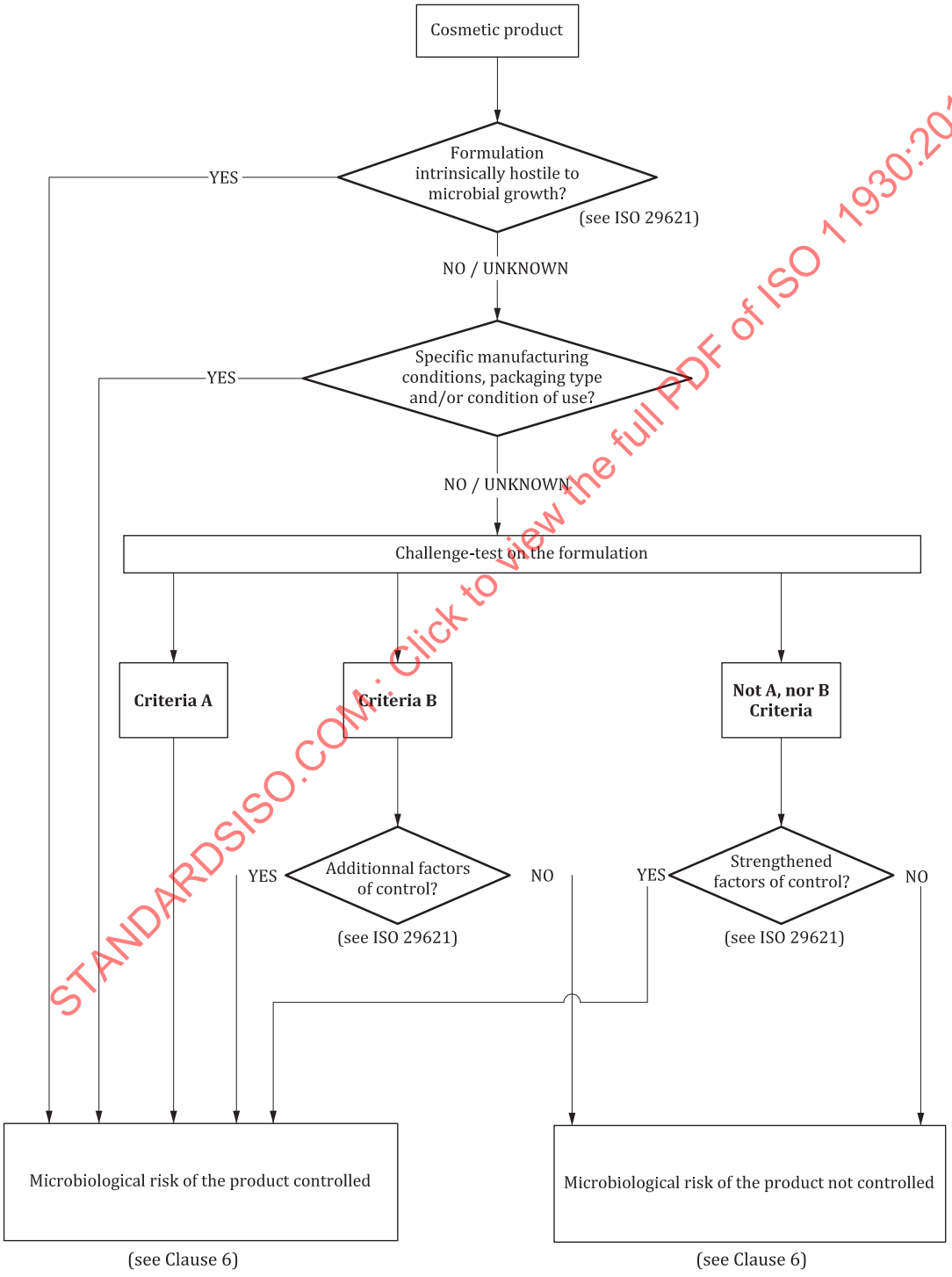

Figure A.1 — Decision diagram

Annex B
(normative)

Evaluation criteria for the preservation efficacy test

NOTE See 5.7.

Table B.1 — Evaluation criteria

Log reduction values ($R_x = \lg N_0 - \lg N_x$) required[a]								
Micro organisms	**Bacteria**			***C. albicans***			***A. brasiliensis***	
Sampling time	T7	T14	T28	T7	T14	T28	T14	T28
Criteria A	≥ 3	≥ 3 and NI[b]	≥ 3 and NI	≥ 1	≥ 1 and NI	≥ 1 and NI	≥ 0[c]	≥ 1 and NI
Criteria B	Not performed	≥ 3	≥ 3 and NI	Not performed	≥ 1	≥ 1 and NI	≥ 0	≥ 0 and NI

a In this test, an acceptable range of deviation of 0,5 log is accepted (see 5.7).

b NI: no increase in the count from the previous contact time.

c $R_x = 0$ when $\lg N_0 = \lg N_x$ (no increase from the initial count).

Annex C
(informative)

Examples of neutralizers for the antimicrobial activity of preservatives and washing liquids

Table C.1 — Examples of neutralizers for the antimicrobial activity of preservatives and washing liquids

Preservative	Chemical compounds able to neutralize the antimicrobial activity of preservatives	Suitable neutralizers and washing liquids (for membrane filtration methods)
Phenolic substances: Parabens, phenoxyethanol, phenylethanol, etc. Anilides	Lecithin Polysorbate 80 Fatty alcohol ethylene oxide condensate Non-ionic surfactants	Polysorbate 80, 30 g/l + lecithin, 3 g/l Fatty alcohol ethylene oxide condensate, 7 g/l + lecithin, 20 g/l + polysorbate 80, 4 g/l D/E neutralizing broth[a]; SDCLP broth[b] Washing liquid: distilled water; tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l
Quaternary ammonium salts Cationic surfactants	Lecithin, saponin, polysorbate 80, sodium dodecylsulphate Fatty alcohol ethylene oxide condensate	Polysorbate 80, 30 g/l + sodium dodecylsulphate, 4 g/l + lecithin, 3 g/l Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l D/E neutralizing broth[a]; SDCLP broth[b] Washing liquid: distilled water; tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l
Aldehydes Formaldehyde-generating agents	Glycine, histidine	Lecithin, 3 g/l + polysorbate 80, 30 g/l + L-histidine, 1 g/l Polysorbate 80, 30 g/l + saponin, 30 g/l + L-histidine, 1 g/l + L-cysteine, 1 g/l D/E neutralizing broth[a]; SDCLP broth[b] Washing liquid: polysorbate 80, 3 g/l + L-histidine 0,5 g/l
Oxidizing agents	Sodium thiosulphate	Sodium thiosulphate, 5 g/l Washing liquid: sodium thiosulphate, 3 g/l

[a] Dey/Engley neutralizing broth.

[b] Soybean-casein digest broth with lecithin and polysorbate 80.