INTERNATIONAL STANDARD

ISO 16212

First edition
2008-10-15

Cosmetics — Microbiology — Enumeration of yeast and mould

Cosmétiques — Microbiologie — Dénombrement des levures et des moisissures

Reference number
ISO 16212:2008(E)

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

Published in Switzerland

Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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

ISO 16212 was prepared by Technical Committee ISO/TC 217, *Comestics*.

Cosmetics — Microbiology — Enumeration of yeast and mould

1 Scope

This International Standard gives general guidelines for enumeration of yeast and mould present in cosmetics by counting the colonies on selective agar medium after aerobic incubation.

In order to ensure product quality and safety for consumers, it is advisable to perform an appropriate microbiological risk analysis so as to determine the types of cosmetic products to which this International Standard is applicable. Products considered to present a low microbiological risk include those with low water activity, hydro-alcoholic products, products with extreme pH values, etc.

Because of the large variety of cosmetic products within this field of application, this method might not be suited to some products in every detail (e.g. certain water-immiscible products). Other methods (e.g. automated) can be used for the test presented here provided that their equivalence has been demonstrated or the method has been otherwise validated.

Yeast enumerated can be identified using suitable identification tests, for example tests described in the standards listed in the Bibliography. Mould enumerated can be identified by other appropriate methods, if necessary.

2 Normative references

The following referenced documents are indispensable for the application 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 21148, *Cosmetics — Microbiology — General instructions for microbiological examination*

EN 12353, *Chemical disinfectants and antiseptics — Preservation of test organisms used for the determination of bactericidal, mycobactericidal, sporicidal and fungicidal activity*

3 Terms and definitions

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

3.1
yeast
single-cell fungus, which multiplies mainly vegetatively by budding, able to grow under the test conditions specified in this International Standard

3.2
mould
mycelium forming microfungus, including spores and conidia, able to grow under the test conditions specified in this International Standard

3.3
product
portion of an identified cosmetic product received in the laboratory for testing

3.4
sample
portion of the product (at least 1 g or 1 ml) which is used in the test to prepare the initial suspension

3.5
initial suspension
suspension (or solution) of a sample in a defined volume of an appropriate liquid (diluent, neutralizer, broth or combination thereof)

3.6
sample dilution(s)
dilution(s) of the initial suspension

4 Principles

4.1 General

This method involves enumeration of colonies on a selective agar medium. The possible inhibition of fungal growth by the sample shall be neutralized to allow the detection of viable microorganism (see Reference [2]). In all cases and whatever the methodology, the neutralization of the antifungicidal properties of the product shall be checked and validated (see References [3], [5], [6]).

4.2 Plate count

Plate count consists of the following steps.

a) Preparation of poured plates or spread plates, using a specified culture medium, and inoculation of the plates using a defined quantity of the initial suspension or dilution of the product.

b) Aerobic incubation of the plates at 25 °C ± 2,5 °C for 3 d to 5 d.

c) Counting of the number of colony-forming units (CFU) and calculation of the amount of yeast and mould per millilitre or per gram of product.

NOTE An alternative condition for incubation is 22,5 °C ± 2,5 °C for 5 d to 7 d using the culture medium without antibiotic.

4.3 Membrane filtration

Membrane filtration consists of the following steps.

a) Transfer of a suitable amount of the sample, prepared by a valid method, in the filtration apparatus, wetted with a small volume of an appropriate sterile diluent. Immediate filtration and washing according to the validated procedure. Transfer of the membrane filter on to the surface of the specified agar medium as specified in ISO 21148.

b) Aerobic incubation of the membranes at 25 °C ± 2,5 °C for 3 d to 5 d.

c) Counting of the number of colony-forming units (CFU) and calculation of the amount of yeast and mould per millilitre or per gram of product.

NOTE An alternative condition for incubation is 22,5 °C ± 2,5 °C for 5 d to 7 d using the culture medium without antibiotic.

5 Diluents, neutralizers and culture media

5.1 General

General specifications are given in ISO 21148. When water is mentioned in a formula, use distilled water or purified water as specified in ISO 21148.

The following diluents, neutralizers and culture media are suitable for enumeration of yeasts and moulds. Other diluents, neutralizers and culture media may be used if they have been demonstrated to be suitable for use.

5.2 Neutralizing diluents and diluents

5.2.1 General

The diluent is used to disperse the sample. It may contain neutralizers if the sample to be tested has antifungicidal properties. The efficacy of the neutralization shall be demonstrated before the determination of the count (see Clause 12). Information relative to suitable neutralizers is given in Annex D.

5.2.2 Neutralizing diluent

5.2.2.1 Fluid casein digest-soy lecithin-polysorbate 20 medium (SCDLP 20 broth)

5.2.2.1.1 Composition

— pancreatic digest of casein 20,0 g

— soy lecithin 5,0 g

— polysorbate 20 40 ml

— water 960 ml

5.2.2.1.2 Preparation

Dissolve the polysorbate 20 in 960 ml of water by mixing while heating in a water bath at 49 °C ± 2 °C. Add pancreatic digest of casein and soy lecithin. Heat for about 30 min to effect solution. Mix and dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization, the pH shall be equivalent to 7,3 ± 0,2 when measured at room temperature.

5.2.2.2 Other neutralizing diluents

Other neutralizing diluents may be used as appropriate (see Annex A and Annex D).

5.2.3 Diluent

5.2.3.1 Fluid A

5.2.3.1.1 Composition

— peptic digest of animal tissue 1,0 g

— water 1 000 ml

5.2.3.1.2 Preparation

Dissolve 1 g of peptone in water to make 1 l. Heat with frequent agitation. Dispense into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization the pH shall be equivalent to 7,1 ± 0,2 when measured at room temperature.

5.2.3.2 Other diluents

Other diluents may be used as appropriate (see Annex B).

5.3 Diluent for yeast suspension (tryptone sodium chloride solution)

5.3.1 Composition

— tryptone, pancreatic digest of casein 1,00 g

— sodium chloride 8,50 g

— water 1 000 ml

5.3.2 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.

5.4 Culture media

5.4.1 General

Culture media may be prepared as follows, 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 herein.

5.4.2 Sabouraud dextrose chloramphenicol agar medium (SDCA)

5.4.2.1 Composition

— dextrose 40,0 g

— peptic digest of animal tissue 5,0 g

— pancreatic digest of casein 5,0 g

— chloramphenicol 0,050 g

— agar 15,0 g

— water 1 000 ml

5.4.2.2 Preparation

Dissolve the components (including the chloramphenicol) 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.

NOTE For known and non-contaminated products (with bacteria), the media are used without chloramphenicol.

5.4.3 Other media

Other media may be used as appropriate (see Annex C).

5.4.4 Agar medium for cultivation of reference strain: Sabouraud dextrose agar medium (SDA)

5.4.4.1 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

5.4.4.2 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.

6 Apparatus and glassware

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

7 Strain of microorganisms

For testing the efficacy of neutralizers, one yeast reference strain is used.

Candida albicans ATCC[1)] 10231 or equivalent strain (IP[2)] 48.72 or NCPF[3)] 3179 or NBRC[4)] 1594 or KCTC[5)] 17205 or TISTR[6)] 5779) or other equivalent national collection strain.

The selected yeast strain being considered more susceptible to antifungicidal activity than moulds may be accepted as representative of fungi (yeast and mould) for the validation of the methodology. However, in case of specific needs, the test for the efficacy of neutralizers may be performed with an additional mould reference strain, using a suitable protocol for the preparation of a calibrated inoculum (e.g. see EN 13624:2003[11], 5.4.1.4).

The culture should be reconstituted according to the procedures provided by the supplier of reference strain.

The strain may be kept in the laboratory in accordance with EN 12353.

---

1) ATCC = American Type Culture Collection.

2) IP = Institut Pasteur.

3) NCPF = National Collection of Pathogenic Fungi.

4) NBRC = National Biological Resource Center.

5) KCTC = Korean Collection for Type Culture.

6) TISTR = Thailand Institute of Scientific and Technological Research.

8 Handling of cosmetic products and laboratory samples

If necessary, store products to be tested at room temperature.

Do not incubate, refrigerate or freeze products (3.3) and samples (3.4) before or after analysis.

Sampling of cosmetic products to be analysed should be carried out as described in ISO 21148. Analyse samples as described in ISO 21148 and according to the procedure described in Clause 9.

9 Procedure

9.1 General recommendation

Use sterile material, equipment and aseptic techniques to prepare the sample, initial suspension and dilutions. In the case of the preparation of the initial suspension, the time that elapses between the end of the preparation and the moment the inoculum comes into contact with the culture medium shall not exceed 45 min, unless specifically mentioned in the established protocols or documents.

9.2 Preparation of the initial suspension

9.2.1 General

The initial suspension is prepared from a sample of at least 1 g or 1 ml of the well-mixed product under test.

Note S, the exact mass or volume of the sample.

The initial suspension is usually a 1:10 dilution. Larger volumes of diluent may be required if high levels of contamination are expected and/or if antifungicidal properties are still present in the 1:10 dilution.

9.2.2 Water-miscible products

Transfer the sample, S, of product to an appropriate volume (e.g. 9 ml) of neutralizing diluent (see 5.2.2) or diluent (see 5.2.3).

Note d, the dilution factor.

9.2.3 Water-immiscible products

Transfer the sample, S, of product to a suitable container containing a suitable quantity of solubilizing agent (e.g. polysorbate 80 solution). Disperse the sample within the solubilizing agent and add an appropriate volume (e.g. 9 ml) of neutralizing diluent (see 5.2.2) or diluent (see 5.2.3).

Note d, the dilution factor.

9.3 Counting methods

9.3.1 Dilutions for counting methods

Usually, the initial suspension is the first counted dilution. If needed, additional serial dilutions (e.g. 1:10 dilution) may be performed from the initial suspension using the same diluent (according to the expected level of contamination of the product).

Generally, counting is performed using at least two Petri dishes, but it is possible to use only one Petri dish in case of routine testing, or if counts are performed on successive dilutions of the same sample or according to previous results.

9.3.2 Plate-count methods

9.3.2.1 Pour-plate method

In Petri dishes of 85 mm to 100 mm in diameter, add 1 ml of the initial suspension and/or sample dilution prepared as validated (see Clause 12) and pour 15 ml to 20 ml of the melted agar medium (see 5.4.2) 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 initial suspension and/or sample dilution with the medium, carefully rotating or tilting the plates sufficiently to disperse them. Allow the mixture in the Petri dishes to solidify on a horizontal surface at room temperature.

9.3.2.2 Surface spread method

In Petri dishes of 85 mm to 100 mm in diameter, put 15 ml to 20 ml of the melted agar medium (see 5.4.2) 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, for example in a microbiological cabinet or in an incubator. Spread over the surface of the medium a measured volume of not less than 0,1 ml of the initial suspension and/or sample dilution prepared as validated (see Clause 12).

9.3.2.3 Membrane filtration method

Use a membrane having a nominal pore size $\leqslant$ 0,45 µm.

Transfer a suitable amount of the initial suspension or of the sample dilution prepared as validated (preferably representing at least 1 g or 1 ml of the product) on to the membrane.

Filter immediately and wash the membrane (follow the procedure developed during the validation, see Clause 12).

Transfer the membrane on to the surface of the agar medium (see 5.4.2).

9.3.2.4 Incubation

Unless otherwise stated, invert the inoculated dishes and place them in the incubator set at 25 °C $\pm$ 2,5 °C for 3 d to 5 d or use the alternative condition (see Notes in 4.2 and 4.3). After incubation, the dishes shall, if possible, be examined immediately. Alternatively, they may be stored, unless otherwise specified, for up to a maximum of 24 h in the refrigerator at 5 °C $\pm$ 3 °C.

NOTE 1 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.

NOTE 2 An intermediate check can be performed where both yeast and mould are suspected.

10 Counting of colonies (plate counts and membrane filtration methods)

After incubation, count the colonies:

— in Petri dishes containing 15 colonies to 150 colonies; if less than 15 colonies are counted, see 11.2.3;

— on membranes containing 15 colonies to 150 colonies; if less than 15 colonies are counted, see 11.2.3.

11 Expression of results

11.1 Method of calculation for plate count

Calculate the number, N, of microorganisms present in the sample, S, using:

— m, the arithmetic mean of the counts obtained from the duplicates (1),

— c, the number of colonies counted on a single plate (2) or

— wm, the weighted mean of the counts obtained from two successive dilutions (3),

according to the following formulae:

$$N = m/(V \times d) \quad (1)$$

$$N = c/(V \times d) \quad (2)$$

$$N = wm/(V \times d) \quad (3)$$

where:

m is the arithmetic mean of the counts obtained from the duplicates;

V is the volume of inoculum applied to each dish, in millilitres;

d is the dilution factor corresponding to the dilution made for the preparation of the initial suspension (see 9.2) or for the first counted dilution;

c is the number of colonies counted on a single plate.

The weighted mean of the colonies counted from two successive dilutions, $\overline{x}_c$, is given by:

$$\overline{x}_c = \frac{\Sigma c}{(n_1 + 0{,}1 n_2)}$$

where

Σc is the sum of colonies counted on all the dishes retained from two successive dilutions;

n_1 is the number of dishes counted for the initial suspension (or for the first counted dilution);

n_2 is the number of dishes counted for the 1:10 dilution of the initial suspension (or for the second counted dilution).

Round off the result calculated to two significant figures. For this, if the last figure is below 5, the preceding figure is not modified; if the last figure is 5 or more, the preceding figure is increased by one unit. Proceed stepwise until two significant figures are obtained. Note the number, N, obtained.

11.2 Interpretation

11.2.1 The inherent variability of plate count should be taken into account. Two results should only be considered different if the difference exceeds 50 % or, when expressed logarithmically, the difference exceeds 0,3 log.

For a count to be precise, only plates or membranes with more than 15 colonies and less than 150 colonies shall be taken into account. Check that the counts are obtained from dilutions validated according to the chosen method (see Clause 12).

11.2.2 Where the number of CFU is more than 15 and less than 150 on plates or membranes, express the result as follows:

— if S is at least 1 g or 1 ml, and V is at least 1 ml, the number of yeast and mould per millilitre or per gram of the sample = N/S;

— if S is less than 1 g or 1 ml, and/or V is less than 1 ml, the number of yeast and mould in the sample (note the tested quantity of sample taking into account S and V) = N;

where S is the mass or the volume of the sample (see 9.2).

Express the result as a number between 1,0 and 9,9 multiplied by the appropriate power of 10 (see examples).

11.2.3 Where the number of CFU is less than 15 on plates or membranes, express the result as follows:

— if S is at least 1 g or 1 ml, and V is at least 1 ml, the estimated number of yeast and mould per millilitre or per gram of the sample = N/S;

— if S is less than 1 g or 1 ml, and/or V is less than 1 ml, the estimated number of yeast and mould in the sample = N;

where S is the mass or the volume of the sample (see 9.2).

Express the result as a number between 1,0 and 9,9 multiplied by the appropriate power of 10 (see examples).

11.2.4 Where no colony is observed, the result is reported as follows:

— there is less than $1/d \times V \times S$ of yeast and mold per gram or millilitre of the product (S is at least 1 g or 1 ml);

— there is less than $1/d \times V$ of yeast and mould in the sample S (note the tested quantity of sample taking into account S and V) (S is less than 1 g or 1 ml);

where

d is the dilution factor of the initial suspension (see 9.2);

V is 1 (for counting with the pour-plate method and for membrane filtration) or 0,1 (for the spread plate method) (see example).

EXAMPLE 1 Two dishes for one dilution

S = 1 g; V = 1; counts obtained for the dilution 10^{-1}: 38 and 42

(1) $N = ml/(V \times d)$ = 40/(1 × $10^{-1)}$ = 40/0,1= 400 or 4 × 10^2 yeast and mould per millilitre or per gram of the sample.

EXAMPLE 2 One dish for one dilution

S = 1 g; V = 1; count obtained for the dilution 10^{-1}: 60

(2) $N = cl/(V \times d)$ = 60/(1 × $10^{-1)}$ = 60/0,1 = 600 or 6 × 10^2 yeast and mould per millilitre or per gram of the sample.

EXAMPLE 3 Two dishes for two dilutions

S = 1 g; V = 1; counts obtained for the dilution 10^{-2}: 235 and 282; for the dilution 10^{-3}: 31 and 39

(3) $N = wm/(V \times d)$ = 235 + 282 + 31 + 39/1(2 + 0,1 × 2) × 10^{-2} = 587/0,022 = 26 682.

Rounding the result as specified above gives 27 000 or 2,7 × 10^4 yeast and mould per millilitre or per gram of the sample.

EXAMPLE 4 Two membrane filters for one dilution

S = 1 g; V = 1; counts obtained for the dilution 10^{-1}: 18 and 22

(1) $N = m/(V \times d)$ = 20/(1 × $10^{-1)}$ = 20/0,1= 200 or 2 × 10^2 yeast and mould per millilitre or per gram of the sample.

EXAMPLE 5 One membrane filter for one dilution

S = 1 g; V = 1; count obtained for the dilution 10^{-1}: 65

(2) $N = c/(V \times d)$ = 65/(1 × $10^{-1)}$ = 65/0,1 = 650 or 6,5 × 10^2 aerobic yeast and mould per millilitre or per gram of the sample.

EXAMPLE 6 Two membrane filters for two dilutions

S = 1 g; V = 1; counts obtained for the dilution 10^{-1}: 121 and 105; for the dilution 10^{-2}: 15 and 25

(3) $N = wm/(V \times d)$ = 121 + 105 + 15 + 25/1(2 + 0,1 × 2) × 10^{-1} = 266/0,22 = 1 209.

Rounding the result as specified above gives 1 200 or 1,2 × 10^3 yeast and mould per millilitre or per gram of the sample.

EXAMPLE 7 Two dishes for one dilution

S = 1 g; V = 1; estimated counts obtained for the dilution 10^{-1}: 28 and 22

(1) $N = m/(V \times d)$ = 25/(1 × $10^{-1)}$ = 25/0,1= 250 or 2,5 × 10^2 yeast and mould per millilitre or per gram of the sample.

The estimated number is 250 or 2,5 × 10^2 yeast and mould per millilitre or per gram of the sample.

EXAMPLE 8

S = 1 g; V = 1; estimated counts obtained for the dilution 10^{-1}: 0 and 0

(1) $N = \leqslant 1/d \times V \times S, \leqslant (1/0,1) \times 1 \times 1 \leqslant 10$ yeast and mould per millilitre or per gram of the sample.

The estimated number is less than 10 yeast and mould per millilitre or per gram of the sample.

12 Neutralization of the antifungicidal properties of the product

12.1 General

The different tests described below demonstrate that the microorganisms can grow under the conditions of analysis.

12.2 Preparation of inoculum

Prior to the test, inoculate the surface of the non-selective agar medium, Sabouraud dextrose agar (SDA), with *Candida albicans*. 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 re-suspend in the diluent (see 5.2) to obtain a calibrated suspension of about 1×10^6 CFU/ml (e.g. determined using spectrophotometer); see ISO 21148.

Use this calibrated suspension and its dilutions within 2 h.

12.3 Validation of counting methods

12.3.1 Principle

Mix the neutralized sample (initial suspension or sample dilution according to the antifungicidal activity or the low solubility of the product) with a dilution of the reference strain. Plate on a Petri dish or filter on a membrane. After incubation, check the nature of the colonies and compare the count with a control (without the sample).

If the count is less than 50 % (0,3 log) of the control, modify the procedure (diluents, neutralization agents or a combination of both, see Annex D). Failure of the inoculum to grow invalidates the test unless possible contamination of the product with this microorganism is unlikely.

12.3.2 Validation of the pour-plate method

Mix 9 ml of the initial suspension and/or the sample dilution(s) in neutralizing diluent (or other, see 5.2) with 1 ml of a suspension of microorganisms containing 1 000 CFU/ml to 3 000 CFU/ml. Transfer 1 ml in a Petri dish (preferably in duplicate) and pour 15 ml to 20 ml of the melted agar medium (see 5.4.2) kept in a water bath at no more than 48 °C. In parallel, prepare and plate a control using the same diluent and the same suspension of microorganisms, but without the sample.

After incubation for 3 d to 5 d at 25 °C $\pm$ 2,5 °C or using the alternative condition (see Notes in 4.2 and 4.3), count the colonies on the plates and compare the counts obtained for the test and for the control. The diluent and the counting method are validated at the 1:10 dilution (when 1 ml of the initial suspension is used) if the validation count is at least 50 % (0,3 log) of the control count.

12.3.3 Validation of the surface spread method

Mix 9 ml of the initial suspension in neutralizing diluent (or other, see 5.2) with 1 ml of a suspension of microorganisms containing 10 000 CFU/ml to 30 000 CFU/ml (or less if 0,5 ml or 1 ml are spread). Spread at least 0,1 ml on a solidified agar plate (see 5.4.2), preferably in duplicate. In parallel, prepare and plate a control using the same diluent and the same suspension of microorganisms, but without the sample.

After incubation for 3 d to 5 d at 25 °C $\pm$ 2,5 °C or using the alternative condition (see Notes in 4.2 and 4.3), count the colonies on the plates and compare the counts obtained for the test and for the control. The diluent and the counting method are validated at the 1:10 dilution (when 1 ml of the initial suspension is used) if the validation count is at least 50 % (0,3 log) of the control count.

12.3.4 Validation of the membrane filtration method

Mix to the volume of initial suspension or of the sample dilution used in the test (see 9.3.2.3) a suitable amount of a calibrated suspension of microorganisms corresponding to approximately 100 CFU.

Filter immediately the entire volume and wash the membrane using defined volumes of water (see 5.1), diluent (see 5.2.3) or neutralizing diluent (see 5.2.2). Transfer the membrane on to the surface of a suitable agar medium (see 5.4.2).

In parallel, prepare a control under the same conditions as above, but without the product. Filter and wash the control under the same conditions.

After incubation for 3 d to 5 d at 25 °C $\pm$ 2,5 °C or using the alternative condition (see Notes in 4.2 and 4.3), count the colonies on the membranes and compare the counts obtained for the test and for the control. The membrane filtration method and the diluent are validated if the validation count is at least 50 % (0,3 log) of the control count.

13 Test report

The test report shall contain the following:

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

b) the method used;

c) the results obtained;

d) all operating details for the preparation of the initial suspension;

e) the description of the method with the neutralizers and media used;

f) the validation of the method, even if the test has been performed separately;

g) any point not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the results.

Annex A
(informative)

Other neutralizing diluents

A.1 General

Any neutralizing diluent may be used to prepare the initial suspension if it has been checked and validated. The following neutralizing diluents are examples of suitable formulae. General information on neutralization is given in Annex D.

A.2 Eugon LT100 liquid broth

A.2.1 General

This medium contains ingredients that neutralize inhibitory substances present in the sample (lecithin and polysorbate 80); and dispersing agent (octoxynol 9).

A.2.2 Composition

— pancreatic digest of casein 15,0 g

— papaic digest of soybean meal 5,0 g

— L-cystine 0,7 g

— sodium chloride 4,0 g

— sodium sulfite 0,2 g

— glucose 5,5 g

— egg lecithin 1,0 g

— polysorbate 80 5,0 g

— octoxynol 9 1,0 g

— water 1 000 ml

A.2.3 Preparation

Dissolve successively in hot water: polysorbate 80, octoxynol 9 and egg lecithin until their complete dilution. Dissolve the other components by mixing while heating. Dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization, the pH shall be equivalent to 7,0 $\pm$ 0,2 when measured at room temperature.

A.3 Lecithin polysorbate (LP) diluent

A.3.1 Composition

— polypeptone 1,0 g

— egg lecithin 0,7 g

— polysorbate 80 20,0 g

— water 980 ml

A.3.2 Preparation

Mix and dissolve the ingredients by heating. Cool to 25 °C before dispensing the solution into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization the pH shall be equivalent to 7,2 ± 0,2 when measured at room temperature.

Annex B
(informative)

Other diluents

B.1 General

Any diluent may be used to prepare the initial suspension if it has been checked and validated. The following diluent is an example of a suitable formula.

B.2 Buffered peptone solution (pH 7)

B.2.1 Composition

— meat peptone 1,0 g

— sodium chloride 4,3 g

— monopotassium phosphate 3,6 g

— disodium phosphate dihydrate 7,2 g

— water 1 000 ml

B.2.2 Preparation

Dissolve the ingredients in hot water. Mix and cool to 25 °C before dispensing the solution into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization the pH shall be equivalent to 7,1 ± 0,2 when measured at room temperature.

B.3 Phosphate buffer (pH 7,2)

B.3.1 Composition

— potassium dihydrogen phosphate 34 g

— water 500 ml

B.3.2 Preparation

Dissolve the ingredients in water in a 1 000 ml volumetric flask. Adjust the pH value to 7,2 ± 0,1 with about 175 ml of sodium hydroxide (4,3 g/100 ml). Then water is added to a final volume of 1 000 ml. The final concentration of the solution is 0,05 mol/l. It is the stock solution. Store it under refrigeration.

Before use, dilute the stock solution with water at a ratio of 1:800 and sterilize it in the autoclave at 121 °C for 15 min.

Annex C
(informative)

Other culture media

C.1 General

Any culture medium may be used if it has been checked and validated. The following media are examples of suitable formulae.

C.2 Agar for counting

C.2.1 Potato dextrose agar medium with antibiotics

C.2.1.1 Composition

— potato extract 4,0 g

— glucose 20,0 g

— agar 15,0 g

— chloramphenicol 0,05 g

— water 1 000 ml

C.2.1.2 Preparation

Mix all the components and dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization and cooling, the pH shall be equivalent to 5,6 ± 0,2 when measured at room temperature.

Alternatively, chloramphenicol may be replaced by use of 0,10 g of benzylpenicillin potassium and 0,10 g of tetracycline per litre of medium, added as sterile solution, just prior to use.

C.2.2 Glucose-peptone (GP) agar medium with antibiotics

C.2.2.1 Composition

— glucose 20,0 g

— yeast extract 2,0 g

— magnesium sulfate 0,5 g

— peptone 5,0 g

— monobasic potassium phosphate 1,0 g

— agar 15,0 g

— chloramphenicol 0,05 g

— water 1 000 ml

C.2.2.2 Preparation

Mix all the components and dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization and cooling, the pH shall be equivalent to 5,7 ± 0,1 when measured at room temperature.

Alternatively, chloramphenicol may be replaced by use of 0,10 g of benzylpenicillin potassium and 0,10 g of tetracycline per litre of medium, added as sterile solution, just prior to use.

C.3 Malt extract medium

C.3.1 Composition

— malt extract 30,0 g

— soya peptone, papaic digest of soybean meal 3,0 g

— agar 15,0 g

— chloramphenicol 0,05 g

— water 1 000 ml

C.3.2 Preparation

Dissolve the components (including the chloramphenicol) or the dehydrated complete medium in the water by heating. Dispense the medium into suitable containers. Sterilize in an autoclave at 115 °C for 10 min. After sterilization the pH shall be equivalent to 5,6 ± 0,2 when measured at room temperature.

Annex D
(informative)

Neutralizers of antifungicidal activity of preservatives and rinsing liquids

Preservative	Chemical compounds able to neutralize preservative's antifungicidal activity	Examples of suitable neutralizers and of rinsing liquids (for membrane filtration methods)
Phenolic compounds: parabens, phenoxyethanol, phenylethanol, anilides	Lecithin Polysorbate 80 Ethylene oxide condensate of fatty alcohol Non-ionic surfactants	Polysorbate 80, 30 g/l + lecithin, 3 g/l. Ethylene oxide condensate of fatty alcohol, 7 g/l + lecithin, 20 g/l + polysorbate 80, 4 g/l. D/E neutralizing broth Rinsing liquid: distilled water; tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.
Quaternary ammonium compounds Cationic surfactants	Lecithin, saponin polysorbate 80, sodium dodecyl sulphate Ethylene oxide condensate of fatty alcohol	Polysorbate 80, 30 g/l + sodium dodecyl sulphate, 4 g/l + lecithin, 3 g/l. Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. D/E neutralizing broth Rinsing liquid: distilled water; tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.
Aldehydes Formaldehyde-release 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 Rinsing liquid: polysorbate 80, 3 g/l + L-histidine 0,5 g/l.
Oxidizing compounds	Sodium thiosulphate	Sodium thiosulphate, 5 g/l. Rinsing liquid: sodium thiosulphate, 3 g/l.
Isothiazolinones, imidazoles	Lecithin, saponin, amines, sulfates, mercaptans, sodium bisulfite, sodium thioglycollate	Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. Rinsing liquid: tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.
Biguanides	Lecithin, saponin, polysorbate 80	Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. Rinsing liquid: tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.
Metallic salts (Cu, Zn, Hg) Organo-mercuric compounds	Sodium bisulphate, L-cysteine Sulfhydryl compounds, thioglycollic acid	Sodium thioglycollate, 0,5 g/l or 5 g/l. L-cysteine, 0,8 g/l or 1,5 g/l. D/E neutralizing broth Rinsing liquid: sodium thioglycollate, 0,5 g/l.
NOTE According to the pH of the cosmetic product, the pH of the neutralizer can be adjusted at a suitable value.		

Bibliography

[1] COLIPA, *Guidelines on Microbial Quality Management,* European Cosmetic, Toiletry and Perfumery Association (COLIPA), 1997

[2] CTFA, *Microbiology Guidelines*, Toiletry and Fragrance Association, ISBN 1-882621-32-8, 2001

[3] EP, *Microbiological Examination of Non-Sterile Products*, 4th edition, European Pharmacopoeia, 2002

[4] FDA, *Bacteriological Analytical Manual*, 8th edition, U.S. Food and Drug Administration, 1995, http://www.cfsan.fda.gov/~ebam/bam-23.html

[5] JP 14, *General Tests — Microbial Limit Test*, Japanese Pharmacopoeia, 2001

[6] USP 28, *Microbial Limit Test* ⟨61⟩, U.S. Pharmacopoeia, 2005

[7] ATLAS, R.M., *Handbook of Microbiological Media*, CRC Press, ISBN 0-8493-2944-2, 1993

[8] SINGER, S., *The Use of Preservative Neutralizers in Diluents and Plating Media*, Cosmetics and Toiletries, **102**, p. 55, December 1987

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

[10] ISO 18416, *Cosmetics — Microbiology — Detection of Candida albicans*

[11] EN 13624:2003, *Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of fungicidal activity of chemical disinfectants for instruments used in the medical area — Test method and requirements (phase 2, step 1)*

ICS 71.100.70

Price based on 19 pages