
**Cosmetics — Microbiology —
Detection of *Pseudomonas aeruginosa***

*Cosmétiques — Microbiologie — Détection de Pseudomonas
aeruginosa*

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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.

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 on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: [Foreword - Supplementary information](#)

The committee responsible for this document is ISO/TC 217, *Cosmetics*.

This second edition cancels and replaces the first edition (ISO 22717:2006), of which it constitutes a minor revision.

Introduction

Microbiological examinations of cosmetic products are carried out according to an appropriate microbiological risk analysis in order to ensure their quality and safety for consumers.

Microbiological risk analysis depends on several parameters such as the following:

- potential alteration of cosmetic products;
- pathogenicity of microorganisms;
- site of application of the cosmetic product (hair, skin, eyes, mucous membranes);
- type of users (adults, children under 3 years).

For cosmetics and other topical products, the detection of skin pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* may be relevant because they can cause skin or eye infections. The detection of other kinds of microorganism might be of interest since these microorganisms (including indicators of faecal contamination e.g. *Escherichia coli*) suggest hygienic failure during the manufacturing process.

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Cosmetics — Microbiology — Detection of *Pseudomonas aeruginosa*

1 Scope

This International Standard gives general guidelines for the detection and identification of the specified microorganism *Pseudomonas aeruginosa* in cosmetic products. Microorganisms considered as specified in this International Standard might differ from country to country according to national practices or regulations.

In order to ensure product quality and safety for consumers, it is advisable to perform an appropriate microbiological risk analysis to determine the types of cosmetic product to which this International Standard is applicable. Products considered to present a low microbiological (see ISO 29621) risk include those with low water activity, hydro-alcoholic products, extreme pH values, etc.

The method described in this International Standard is based on the detection of *Pseudomonas aeruginosa* in a non-selective liquid medium (enrichment broth), followed by isolation on a selective agar medium. Other methods may be appropriate, depending on the level of detection required.

NOTE For the detection of *Pseudomonas aeruginosa*, subcultures can be performed on non-selective culture media followed by suitable identification steps (e.g. using identification kits).

Because of the large variety of cosmetic products within this field of application, this method may not be appropriate in every detail for some products (e.g. certain water immiscible products). Other International Standards (ISO 18415) may be appropriate. Other methods (e.g. automated) may be substituted for the tests presented here provided that their equivalence has been demonstrated or the method has been otherwise shown to be suitable.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 21148:2005, *Cosmetics — Microbiology — General instructions for microbiological examination*

EN 12353, *Chemical disinfectants and antiseptics — Preservation of test organisms used for the determination of bactericidal (including Legionella), mycobactericidal, sporicidal, fungicidal and virucidal (including bacteriophages) activity*

3 Terms and definitions

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

3.1

product

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

3.2

sample

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

3.3 initial suspension

suspension (or solution) of the sample in a defined volume of an appropriate enrichment broth

3.4 sample dilution

dilution of the initial suspension

3.5 specified microorganism

aerobic mesophilic bacterium or yeast that is undesirable in a cosmetic product and is recognized as a skin pathogen species that may be harmful for human health or as an indication of hygienic failure in the manufacturing process

3.6 *Pseudomonas aeruginosa*

gram-negative rod, motile; smooth colonies pigmented brown or greenish

Note 1 to entry: The main characteristics for identification are: growth on selective cetrimide agar medium, oxidase positive, production of diffusible fluorescent pigments and production of a soluble phenazine pigment (pyocyanin) in suitable media.

Note 2 to entry: *Pseudomonas aeruginosa* may be isolated from a wide variety of environmental sources, especially in water and has a very high potential to spoil many different substrates. It may produce infections of human skin or eye area. It is undesirable in cosmetic products for its potential pathogenicity and its capacity to affect the physico-chemical properties of the cosmetic formula.

3.7 enrichment broth

non-selective liquid medium containing suitable neutralizers and/or dispersing agents and demonstrated to be suitable for the product under test

4 Principle

The first step of the procedure is to perform an enrichment by using a non-selective broth medium to increase the number of microorganisms without the risk of inhibition by the selective ingredients that are present in selective/differential growth media.

The second step of the test (isolation) is performed on a selective medium followed by identification tests.

The possible inhibition of microbial growth by the sample shall be neutralized to allow the detection of viable microorganisms. In all cases and whatever the methodology, the neutralization of the antimicrobial properties of the product shall be checked and demonstrated (see [Clause 11](#)).

5 Diluents and culture media

5.1 General

General instructions are given in ISO 21148. When water is mentioned in this International Standard, use distilled water or purified water as specified in ISO 21148.

The enrichment broth is used to disperse the sample and to increase the initial microbial population. It may contain neutralizers if the specimen to be tested has antimicrobial properties. The efficacy of the neutralization shall be demonstrated (see [Clause 11](#)). Information relative to suitable neutralizers is given in [Annex B](#).

The enrichment broth ([5.3.3.1](#)), or any of the ones listed in [Annex A](#), is suitable for checking the presence of *Pseudomonas aeruginosa* in accordance with this International Standard provided that it has been demonstrated to be suitable in accordance with [Clause 11](#).

Other diluents and culture media may be used if it has been demonstrated that they are suitable for use.

5.2 Diluent for the bacterial suspension (tryptone sodium chloride solution)

5.2.1 General

The diluent is used for the preparation of bacterial suspension used for the suitability test procedure (see [Clause 11](#)).

5.2.2 Composition

— tryptone, pancreatic digest of casein	1,0 g
— sodium chloride	8,5 g
— water	1 000 ml

5.2.3 Preparation

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

After sterilization and cooling down, the pH shall be equivalent to $7,0 \pm 0,2$ when measured at room temperature.

5.3 Culture media

5.3.1 General

Culture media may be prepared using the descriptions provided below or from dehydrated culture media according to the instructions of the manufacturer. The instructions provided by the supplier of the media should be followed.

NOTE Ready-to-use media can be used when their composition and/or growth yields are comparable to those of the formulae given herein.

5.3.2 Agar medium for the suitability test (see Clause 11) [soybean–casein digest agar medium (SCDA) or tryptic soy agar (TSA)]

5.3.2.1 Composition

— pancreatic digest of casein	15,0 g
— papain digest of soybean meal	5,0 g
— sodium chloride	5,0 g
— agar	15,0 g
— water	1 000 ml

5.3.2.2 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.

After sterilization and cooling down, the pH shall be equivalent to $7,3 \pm 0,2$ when measured at room temperature.

5.3.3 Enrichment broth

5.3.3.1 Eugon LT 100 broth

5.3.3.1.1 General

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

5.3.3.1.2 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

5.3.3.1.3 Preparation

Dissolve the components polysorbate 80, octoxynol 9 and egg lecithin successively into boiling water until their complete dissolution. 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 and cooling down, the pH shall be equivalent to $7,0 \pm 0,2$ when measured at room temperature.

5.3.3.2 Other enrichment broths

Other enrichment broths may be used as appropriate (see [Annex A](#)).

5.3.4 Selective agar medium for isolation of *Pseudomonas aeruginosa*

5.3.4.1 Cetrimide agar medium

5.3.4.1.1 Composition

— pancreatic digest of gelatin	20,0 g
— magnesium chloride	1,4 g
— potassium sulfate	10,0 g
— cetrimide (cetyltrimethylammonium bromide)	0,3 g
— agar	13,6 g
— glycerol	10,0 ml
— water	1 000 ml

5.3.4.1.2 Preparation

Dissolve all solid components in the water and add the glycerol. Heat, with frequent agitation, and boil for 1 min to effect dissolution.

Dispense in suitable flasks and sterilize at 121 °C for 15 min.

After sterilization and cooling down, the pH shall be equivalent to $7,2 \pm 0,2$ when measured at room temperature.

5.3.5 Selective agar medium for confirmation of *Pseudomonas aeruginosa*

5.3.5.1 *Pseudomonas* agar medium for detection of pyocyanin (*Pseudomonas* agar P)

5.3.5.1.1 Composition

— pancreatic digest of gelatin	20,0 g
— anhydrous magnesium chloride	1,4 g
— anhydrous potassium sulfate	10,0 g
— agar	15,0 g
— glycerol	10,0 ml
— water	1 000 ml

5.3.5.1.2 Preparation

Dissolve all solid components in the water, and add the glycerol. Heat, with frequent agitation, and boil for 1 min to effect dissolution.

Dispense in suitable flasks and sterilize at 121 °C for 15 min.

After sterilization and cooling down, the pH shall be equivalent to $7,2 \pm 0,2$ when measured at room temperature.

6 Apparatus and glassware

Use the laboratory equipment, apparatus and glassware as described in ISO 21148.

7 Strains of microorganisms

For the verification of the suitability test conditions, the following representative strain is used:

Pseudomonas aeruginosa ATCC¹⁾ 9027 (equivalent strain: CIP²⁾ 82118 or NCIMB³⁾ 8626 or NBRC⁴⁾ 13275 or KCTC⁵⁾ 2513 or other equivalent national collection strain).

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

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

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 and samples 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 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 in an appropriate solubilizing agent, the time which elapses between the end of preparation and the moment the inoculum comes into contact with the enrichment broth shall not exceed 45 min, unless specifically mentioned in the established protocols or documents.

9.2 Preparation of the initial suspension in the enrichment broth

9.2.1 General

The enrichment is prepared from a sample ([3.2](#)) of at least 1 g or 1 ml of the well-mixed product under test, which is dispersed in at least 9 ml of enrichment broth.

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

The method shall be checked to ensure that the composition (neutralizer eventually added) and the volume of the broth perform satisfactorily (see [11.3](#)).

NOTE In some cases, and when possible, filtration of the cosmetic product through a membrane that is afterwards immersed in the enrichment broth, facilitates the neutralization of the antimicrobial properties of the product (see [11.3](#)).

1) ATCC = American Type Culture Collection.

2) CIP = Institut Pasteur Collection.

3) NCIMB = National Collection of Industrial and Marine Bacteria.

4) NBRC = National Biological Resource center.

5) KCTC = Korean Collection for type culture.

9.2.2 Water-miscible products

Transfer the sample, *S*, of product to a suitable container containing an appropriate volume of broth.

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).

Disperse the sample within the solubilizing agent and add an appropriate volume of broth.

9.2.4 Filterable products

Use a membrane filter having a nominal pore size of not greater than 0,45 µm.

Transfer the sample, *S*, on to the membrane in a filtration apparatus (see ISO 21148). Filter immediately and wash the membrane using defined volumes of water and/or diluent.

Transfer and immerse the membrane into a tube or flask of suitable size containing an appropriate volume of broth.

9.3 Incubation of the inoculated enrichment broth

Incubate the initial suspension prepared in broth (see 9.2) at 32,5 °C ± 2,5 °C for at least 20 h (maximum 72 h).

9.4 Detection and Identification of *Pseudomonas aeruginosa*

9.4.1 Isolation

Using a sterile loop, streak an aliquot of the incubated enrichment broth on the surface of cetrimide agar medium in order to obtain isolated colonies.

Invert the Petri dish and then incubate at 32,5 °C ± 2,5 °C for at least 24 h (maximum 48 h).

Check for characteristic colonies (see Table 1).

Table 1 — Morphological characteristics of *Pseudomonas aeruginosa* on selective medium

Selective medium	Characteristic colonial morphology of <i>Pseudomonas aeruginosa</i>
Cetrimide agar medium	Yellow-green pigment (pyocyanin), which fluoresces under UV light.

9.4.2 Identification of *Pseudomonas aeruginosa*

9.4.2.1 General

Proceed to the following tests, for the suspect colonies isolated on the cetrimide agar medium. The presence of *Pseudomonas aeruginosa* may be confirmed by other suitable, cultural and biochemical tests.

9.4.2.2 Gram's stain

This test is described in ISO 21148.

Check for Gram-negative rods.

9.4.2.3 Oxidase test

This test is described in ISO 21148.

Check for oxidase positive test.

9.4.2.4 Culture on *Pseudomonas* agar medium for detection of pyocyanin

Inoculate the surface of the *Pseudomonas* agar medium for detection of pyocyanin with suspect isolated colonies grown on ceftrimide agar medium, so that individual colonies develop. Incubate at $32,5\text{ }^{\circ}\text{C} \pm 2,5\text{ }^{\circ}\text{C}$.

Check for bacterial growth after 24 h, 48 h and 72 h. *Pseudomonas aeruginosa* forms colonies surrounded by a blue to green zone due to pyocyanin formation or with a red to dark brown zone due to pyorubin production.

10 Expression of results (detection of *Pseudomonas aeruginosa*)

If the identification of the colonies confirms the presence of this species, express the result as:

— presence of *Pseudomonas aeruginosa* in the sample, *S*.

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 *Pseudomonas aeruginosa* in the sample, *S*.

11 Neutralization of the antimicrobial properties of the product

11.1 General

The different tests described below demonstrate that the microorganism can grow in analysis conditions.

11.2 Preparation of the inoculum

Prior to the test, inoculate the surface of soybean casein digest agar (SCDA) or other suitable (non-selective, non-neutralizing) medium with *Pseudomonas aeruginosa*. Incubate the plate at $32,5\text{ }^{\circ}\text{C} \pm 2,5\text{ }^{\circ}\text{C}$ 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 to obtain a calibrated suspension of about 1×10^8 CFU per ml (e.g. using spectrophotometer, ISO 21148:2005, Annex C).

Use this calibrated suspension and its dilutions within 2 h.

11.3 Suitability of the detection method

11.3.1 Procedure

11.3.1.1 In tubes of 9 ml of diluent, prepare a dilution of the calibrated suspension in order to obtain a final count between 100 CFU and 500 CFU per ml. To count the final concentration of viable microorganisms in the diluted calibrated suspension, transfer 1 ml of the suspension into a Petri dish and pour on 15 ml to 20 ml of the melted agar medium kept in a water bath at no more than 48°C. Let solidify and then incubate at $32,5\text{ }^{\circ}\text{C} \pm 2,5\text{ }^{\circ}\text{C}$ for 20 h to 24 h.

11.3.1.2 Prepare in duplicate, the initial suspension in the conditions chosen for the test (at least 1 g or 1 ml of product under test, defined volume of enrichment broth) in a tube or flask. When using the membrane filtration method filter in duplicate at least 1 ml of product under test and transfer each membrane into a tube or flask containing the enrichment broth in the conditions chosen for the test.

11.3.1.3 Introduce aseptically, 0,1 ml of the diluted calibrated suspension (11.3.1.1) of microorganisms into one tube or flask (suitability test). Mix, then incubate both tubes or flasks (suitability test and non-inoculated control) at $32,5\text{ }^{\circ}\text{C} \pm 2,5\text{ }^{\circ}\text{C}$ for 20 h to 24 h.

11.3.1.4 Perform an isolation for each tube or flask (suitability test and non-inoculated control). Using a sterile loop, streak an aliquot (same conditions as in the test) of the incubated mixture on to the surface of a Petri dish (diameter 85 mm to 100 mm) containing approximately 15 ml to 20 ml of cetrimide agar medium. Incubate the plates at $32,5\text{ }^{\circ}\text{C} \pm 2,5\text{ }^{\circ}\text{C}$ for 24 h to 48 h.

11.3.2 Interpretation of suitability test results

Check that the diluted calibrated suspension (11.3.1.1) of bacteria contains between 100 CFU and 500 CFU per ml.

The neutralization is verified and the detection method is satisfactory if a growth characteristic of *Pseudomonas aeruginosa* occurs on the suitability test plate and no growth occurs on the control plate.

When growth is detected on the control plate (contaminated products), the neutralization is verified and the detection method is satisfactory if *Pseudomonas aeruginosa* is recovered on the suitability test plate.

Failure of growth on the suitability test plates indicates that antimicrobial activity is still present and necessitates a modification of the conditions of the method by an increase in the volume of nutrient broth, the quantity of product remaining the same, or by incorporation of a sufficient quantity of inactivating agent in the enrichment broth, or by an appropriate combination of modifications so as to permit the growth of *Pseudomonas aeruginosa*.

If, in spite of the incorporation of suitable inactivating agents and a substantial increase in the volume of broth, it is still not possible to recover viable cultures as described above, indicate that the article is not likely to be contaminated with *Pseudomonas aeruginosa*.

12 Test report

The test report shall contain the following information:

- a) a reference to this International Standard, i.e. ISO 22717:2015;
- b) all information necessary for the complete identification of the product;
- c) the method used;
- d) the results obtained;
- e) all operating details for the preparation of the initial suspension;
- f) the description of the method with the neutralizers and media used;
- g) the demonstration of the suitability of the method, even if the test has been performed separately;
- h) 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 enrichment broths

A.1 Soybean-casein-digest-lecithin-polysorbate 80 medium (SCDLP 80 broth)

A.1.1 Composition

— casein peptone	17,0 g
— soybean peptone	3,0 g
— sodium chloride	5,0 g
— potassium hydrogen phosphate	2,5 g
— glucose	2,5 g
— lecithin	1,0 g
— polysorbate 80	7,0 g
— water	1 000 ml

A.1.2 Preparation

Dissolve all of these components (or dehydrated complete medium) one after another in boiling water until their complete dissolution. Dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min.

After sterilization and cooling down, the pH shall be equivalent to $7,2 \pm 0,2$ when measured at room temperature.

A.2 D/E neutralizing broth (Dey/Engley neutralizing broth)^[7]

A.2.1 Composition

— glucose	10,0 g
— soybean lecithin	7,0 g
— sodium thiosulfate pentahydrate	6,0 g
— polysorbate 80	5,0 g
— pancreatic digest of casein	5,0 g
— sodium bisulfite	2,5 g
— yeast extract	2,5 g
— sodium thioglycolate	1,0 g