INTERNATIONAL STANDARD

ISO 21150

Second edition
2015-12-01

Cosmetics — Microbiology — Detection of *Escherichia coli*

*Cosmétiques — Microbiologie — Détection d'*Escherichia coli

Reference number
ISO 21150:2015(E)

ISO copyright office
Ch. de Blandonnet 8 • CP 401
CH-1214 Vernier, Geneva, Switzerland
Tel. +41 22 749 01 11
Fax +41 22 749 09 47
copyright@iso.org
www.iso.org

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.

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).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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 21150:2006), which has been technically revised.

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.

Cosmetics — Microbiology — Detection of *Escherichia coli*

1 Scope

This International Standard gives general guidelines for the detection and identification of the specified microorganism *Escherichia coli* 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, so as to determine the types of cosmetic products 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 *Escherichia coli* 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 *Escherichia coli*, 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 might not be suited to some products in every detail (e.g. certain water-immiscible products). Other International Standards (ISO 18415) may be appropriate. Other methods (e.g. automated) can be substituted for the test 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
Escherichia coli
gram-negative rod, motile, smooth colonies

Note 1 to entry: The main characteristics for identification are catalase positive, oxidase negative, fermentation of lactose, production of indole, growth on selective medium containing bile salts with characteristic colonies.

Note 2 to entry: *Escherichia coli* can be isolated from the moist environmental sources (air, water, soil) and is a faecal contamination indicator.

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) of the test 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.[1] 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 *Escherichia coli* in accordance with this International Standard provided that they have 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 ± 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 from 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

— papaic 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 ± 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 which 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 ± 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 *Escherichia coli*

5.3.4.1 MacConkey agar medium

5.3.4.1.1 Composition

— pancreatic digest of gelatin	17,0 g
— pancreatic digest of casein	1,5 g
— peptic digest of animal tissue	1,5 g
— lactose	10,0 g
— bile salts mixture	1,5 g

— sodium chloride 5,0 g

— agar 13,5 g

— neutral red 30,0 mg

— crystal violet 1,0 mg

— water 1 000 ml

5.3.4.1.2 Preparation

Dissolve all solid components in the water and boil for 1 min to effect solution.

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

The pH, after sterilization and cooling down, shall be equivalent to 7,1 ± 0,2 when measured at room temperature.

5.3.5 Selective agar medium for confirmation of *Escherichia coli*

5.3.5.1 Levine eosin-methylene blue agar medium

5.3.5.1.1 Composition

— pancreatic digest of gelatin 10,0 g

— potassium hydrogen phosphate (K_2HPO_4) 2,0 g

— agar 15,0 g

— lactose 10,0 g

— eosin Y 400 mg

— methylene blue 65 mg

— water 1 000 ml

5.3.5.1.2 Preparation

Dissolve the pancreatic digest of gelatin, the potassium hydrogen phosphate, and the agar in the water, with warming, and allow to cool. Just prior to use, liquefy the gelled agar solution, add the remaining ingredients, as solutions in the following amounts, and mix; for each 100 ml of the liquefied agar solution

— 5 ml of 20 % lactose solution,

— 2 ml of 2 % eosin Y solution, and

— 2 ml of 0,033 % methylene blue solution.

The finished medium may not be clear.

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

The pH, after sterilization and cooling down, shall be equivalent to 7,1 ± 0,2 when measured at room temperature.

6 Apparatus and glassware

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

7 Strains of microorganisms

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

Escherichia coli ATCC[1] 8739 [equivalent strain: CIP[2] 53.126 or NCIMB[3] 8545 or NBRC[4] 3972 or KCTC[5] 2571 or other equivalent national collection strain].

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

The strain can be kept in the laboratory according to EN 12353.[12]

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 = Collection de l'Institut Pasteur.

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 *Escherichia coli*

9.4.1 Isolation

Using a sterile loop, streak an aliquot of the incubated enrichment broth (9.3) onto the surface of MacConkey agar medium (5.3.4.1) 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 *Escherichia coli* on MacConkey agar medium

Selective medium	Characteristic colonial morphology of *Escherichia coli*
MacConkey agar medium	Brick-red; may have surrounding zone of precipitated bile

9.4.2 Identification of *Escherichia coli*

9.4.2.1 General

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

9.4.2.2 Gram's stain

Perform the test specified in ISO 21148. Check for Gram-negative rods (bacilli).

9.4.2.3 Culture on levine eosin-methylene blue agar medium (EMB agar medium)

Inoculate the surface of the levine eosin-methylene blue agar medium with suspect isolated colonies grown on MacConkey agar medium, so that isolated colonies develop. 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 as specified in Table 2.

Table 2 — Morphological characteristics of *Escherichia coli* on levine eosin-methylene blue agar medium

Selective medium	Characteristic colonial morphology of *Escherichia coli*
Levine eosin-methylene blue agar medium	Metallic sheen under reflected light and a blue-black appearance under transmitted light

10 Expression of the results (detection of *Escherichia coli*)

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

— "Presence of *Escherichia coli* 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 *Escherichia coli* in the sample *S.*"

11 Neutralization of the antimicrobial properties of the product

11.1 General

The different tests in 11.2 to 11.3 demonstrate that the microorganism can grow under the conditions of analysis.

11.2 Preparation of inoculum

Prior to the test, inoculate the surface of soybean-casein digest agar (SCDA) or tryptic soy agar (TSA) (5.3.2) or other suitable (non-selective, non-neutralizing) medium with *Escherichia coli*. 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 it into the diluent (5.2) 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 (5.2), prepare a dilution of the calibrated suspension (11.2) in order to obtain a final count between 100 CFU/ml and 500 CFU/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 15 ml to 20 ml of the melted agar medium (5.3.2) kept in a water bath at no more than 48 °C. Let solidify and then incubate at 32,5 °C ± 2,5 °C for 20 h to 24 h.

11.3.1.2 Prepare in duplicate the initial suspension (9.2) 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 (9.2.4), 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 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 °C ± 2,5 °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 onto the surface of the MacConkey agar medium (approximately 15 ml to 20 ml) in the Petri dish (diameter 85 mm to 100 mm). Incubate the plates at 32,5 °C ± 2,5 °C for 24 h to 48 h.

11.3.2 Interpretation of suitability test results

Check that the dilution of the calibrated suspension of bacteria contains between 100 CFU/ml and 500 CFU/ml.

The neutralization is verified and the detection method is satisfactory if a growth characteristic of *Escherichia coli* 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 *Escherichia coli* is recovered on the suitability test plates.

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 *Escherichia coli*.

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

12 Test report

The test report shall specify the following information:

a) a reference to this International Standard, i.e. ISO 21150:2015;

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 document, or regarded as optional, together with details of any incidents which may have influenced the results.

Annex A
(informative)

Other enrichment broths

A.1 Fluid lactose medium with neutralizing and dispersing agents

This medium contains ingredients

— which neutralize inhibitory substances present in the sample: lecithin and polysorbate 80, and

— dispersing agent: octoxynol 9.

A.1.1 Composition

— beef extract	3,0 g
— pancreatic digest of gelatin	5,0 g
— lactose	5,0 g
— egg lecithin	1,0 g
— polysorbate 80	5,0 g
— octoxynol 9	1,0 g
— water	1 000 ml

A.1.2 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. Cool the medium as quickly as possible after sterilization. The pH shall be equivalent to 6,9 ± 0,2 when measured at room temperature.

A.2 Fluid lactose medium

A.2.1 Composition

— beef extract	3,0 g
— pancreatic digest of gelatin	5,0 g
— lactose	5,0 g
— water	1 000 ml

A.2.2 Preparation

Dissolve the components in water. Dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. Cool the medium as quickly as possible after sterilization. The pH shall be equivalent to 6,9 ± 0,2 when measured at room temperature.

A.3 Soybean-casein-digest-lecithin-polysorbate 80 medium (SCDLP 80 broth)

A.3.1 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.3.2 Preparation

Dissolve all of these components or dehydrated complete medium successively into 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 ± 0,2 when measured at room temperature.

A.4 D/E neutralizing broth (Dey/Engley neutralizing broth)[7]

A.4.1 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
— bromocresol purple	0,02 g
— water	1 000 ml

A.4.2 Preparation

Dissolve all of these components or dehydrated complete medium successively into 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 7,6 ± 0,2 when measured at room temperature.