INTERNATIONAL STANDARD

ISO 21150

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Cosmetics — Microbiology — Detection of *Escherichia coli*

*Cosmétiques — Microbiologie — Détection d'*Escherichia coli

Reference number
ISO 21150:2006(E)

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Foreword

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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 21150 was prepared by Technical Committee ISO/TC 217, *Cosmetics*.

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Introduction

Microbiological examinations of cosmetic products are to be 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:

— potential alteration of cosmetic products;

— pathogenicity of microorganisms;

— site of application of the cosmetic product (hair, skin, eyes, mucous membranes, etc.);

— type of users (adults, children under 3 years, etc.).

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

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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 risk include those with low water activity, hydro-alcoholic products, extreme pH values, etc.

This International Standard specifies a method that 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 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 validated.

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:—[1)], *Cosmetics — Microbiology — General instructions for microbiological examination*

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) which is used in the test to prepare the initial suspension

1) To be published.

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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 bacteria or yeast which is undesirable in a cosmetic product because it can cause skin or eye infection, or it can be recognized as an indicator of hygienic failure in the manufacturing process

3.6
Escherichia coli
Gram-negative rod, motile, smooth colonies

NOTE 1 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 *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 validated 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 (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 [5]. In all cases and whatever the methodology, the neutralization of the antimicrobial properties of the product shall be checked and validated [6] [7] [8].

5 Diluents and culture media

5.1 General

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

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 following enrichment broth is suitable for checking the presence of *Escherichia coli* according to this International Standard provided that it is validated according to Clause 11.

Other diluents and culture media may be used if they have been demonstrated to be suitable for use.

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

The diluent is used for the preparation of bacterial suspension used for the validation procedure (see Clause 11).

5.2.1 Composition

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

5.2.2 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 from the manufacturer. The instructions provided by the supplier of the media should be followed.

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

5.3.2 Agar medium for validation [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 $7,3 \pm 0,2$ when measured at room temperature.

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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,

— 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

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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 $7,1 \pm 0,2$ 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 dihydrogen phosphate (KH_2PO_4)	2,0 g
Agar	15,0 g
Lactose	10,0 g
Eosine Y	400 mg
Methylene blue	65 mg
Water	1 000 ml

5.3.5.1.2 Preparation

Dissolve the pancreatic digest of gelatin, the dibasic potassium 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.

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The pH, after sterilization and cooling down, shall be equivalent to $7,1 \pm 0,2$ 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 validation of the test conditions, use the following representative strain:

> *Escherichia coli* ATCC[2] 8739
> [equivalent strain: CIP[3] 53.126 or NCIMB[4] 8545 or NBRC[5] 3972 or KCTC[6] 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 may be kept in the laboratory according to EN 12353 [4].

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 (3.1) and samples (3.2) before or after analysis.

Carry out the sampling of cosmetic products in accordance with ISO 21148. Analyse the samples in accordance with ISO 21148, and with the procedure in Clause 9.

9 Procedure

9.1 General recommendations

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 the 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.

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2) ATCC = American Type Culture Collection.

3) CIP = Collection de l'Institut Pasteur.

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

5) NBRC = National Biological Resource Center.

6) KCTC = Korean Collection for Type Culture.

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Note S, the exact mass 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 which is afterwards immersed in the enrichment broth facilitates the neutralization of the antimicrobial properties of the product (see 11.3).

9.2.2 Water-miscible products

Transfer the sample S of product to a suitable container containing an appropriate volume of broth (5.3.3).

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 (5.3.3).

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 onto the membrane in a filtration apparatus (see ISO 21148). Filter immediately and wash the membrane using defined volumes of water (5.1) and/or diluent (5.2).

Transfer and immerse the membrane into a tube or flask of suitable size containing an appropriate volume of broth (5.3.3).

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).

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Check for characteristic colonies (see Table 1).

Table 1 — Morphologic 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 by performing 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 — Morphologic 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.

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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, Annex C).

Use this calibrated suspension and its dilutions within 2 h.

11.3 Validation 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 (validation test). Mix, then incubate both tubes or flasks (validation 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 (validation 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 validation results

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

The neutralization and the detection method are validated if a growth characteristic of *Escherichia coli* occurs on the validation plate and no growth occurs on the control plate.

When growth is detected on the control plate (contaminated products), the neutralization and the detection method are validated if *Escherichia coli* is recovered on the validation plate.

Failure of growth on the validation 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 enrichement 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*.

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12 Test report

The test report shall specify the following:

a) a reference to this International Standard, ISO 21150:2006;

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 validation 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.

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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

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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
Dipotassium 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)[11]

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 thioglycollate	1,0 g
Bromocresol purple	0,02 g
Water	1 000 ml

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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.

A.5 Modified letheen broth

A.5.1 Composition

Peptic digest of meat	20,0 g
Pancreatic digest of casein	5,0 g
Beef extract	5,0 g
Yeast extract	2,0 g
Lecithin	0,7 g
Polysorbate 80	5,0 g
Sodium chloride	5,0 g
Sodium bisulfite	0,1 g
Water	1 000 ml

A.5.2 Preparation

Dissolve successively polysorbate 80 and lecithin into boiling water until their complete dissolution. Dissolve the other components by mixing while heating. Mix gently to avoid foam. 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.

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Annex B
(informative)

Neutralizers of antimicrobial activity of preservatives and rinsing liquids

Preservative	Chemical compounds able to neutralize preservative's antimicrobial activity	Examples of suitable neutralizers and of *rinsing liquids*[3] [12] *(for membrane filtration methods)*
Phenolic compounds: parabens, phenoxyethanol, phenylethanol, etc. 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[a] *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[a] *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 [a] *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 bisulfite, 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[a] *Rinsing liquid: sodium thioglycollate, 0,5 g/l.*

[a] D/E neutralizing broth (Dey/Engley neutralizing broth), see Annex A.

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