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METHOD OF TEST FOR

THE DETECTION OF *Staphylococcus aureus* IN COSMETICS

(FIRST REVISION)

SRI LANKA STANDARDS INSTITUTION

Sri Lanka Standard

METHOD OF TEST FOR THE DETECTION OF *Staphylococcus aureus* IN COSMETICS

(First Revision)

SLS 1351 : 2016
ISO 22718 : 2015

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Sri Lanka Standards are subject to periodical revision in order to accommodate the progress made by industry. Suggestions for improvement will be recorded and brought to the notice of the Committees to which the revisions are entrusted.

This standard does not purport to include all the necessary provisions of a contract.

Sri Lanka Standard
METHOD OF TEST FOR THE DETECTION OF *Staphylococcus aureus* IN COSMETICS
(First Revision)

FOREWORD

This Sri Lanka Standard was approved by the Sectoral Committee on Chemical and Polymer Technology and was authorized for adoption and publication as a Sri Lanka Standard by the Council of the Sri Lanka Standards Institution on 2016-10-27.

This Sri Lanka Standard was first published in 2008 which was an adoption of ISO 22718 : 2006 Cosmetics – Microbiology – Detection of *Staphylococcus aureus*. The International Standard ISO 22718 : 2006 has been technically revised in 2015. ISO 22718 : 2015 which gives general guidelines for the detection and identification of *Staphylococcus aureus* in cosmetic products has been accepted to adopt as the first revision to **SLS 1351 : 2016**

This Standard is identical with ISO 22718 : 2015 Cosmetics – Microbiology – Detection of *Staphylococcus aureus*, published by the International Organization for Standardization (ISO).

TERMINOLOGY AND CONVENTIONS :

The text of the International Standard has been accepted as suitable for publication, without deviation, as a Sri Lanka Standard. However, certain terminology and conventions are not identical with those used in Sri Lanka Standards. Attention is therefore drawn to the following :

a) Wherever the words ‘International Standard’ appear referring to a particular standard, they should be interpreted as “Sri Lanka Standard”.

b) The comma has been used throughout as a decimal marker. In Sri Lanka Standards it is the current practice to use the full point at the base as the decimal marker.

c) Wherever page numbers are quoted, they are ISO page numbers.

Cross References

International Standard	Corresponding Sri Lanka Standard
ISO 21148 Cosmetics – Microbiology – General instructions for microbiological examination	No corresponding Sri Lanka Standard
EN 12353 Chemical disinfectants and antiseptics – Preservation of microbial stains used for the determination of bactericidal and fungicidal activity	No corresponding Sri Lanka Standard

SLS 1351:2016

INTERNATIONAL STANDARD

ISO 22718

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Cosmetics — Microbiology — Detection of *Staphylococcus aureus*

Cosmétiques — Microbiologie — Détection de Staphylococcus aureus

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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).

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 22718:2006), 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.

Cosmetics — Microbiology — Detection of *Staphylococcus aureus*

1 Scope

This International Standard gives general guidelines for the detection and identification of the specified microorganism *Staphylococcus aureus* 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 *Staphylococcus aureus* in a non-selective liquid medium (enrichment broth), followed by isolation on a selective agar medium. Other methods may be appropriate dependent on the level of detection required.

NOTE For the detection of *Staphylococcus aureus*, 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 for some products in every detail (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
Staphylococcus aureus
gram-positive cocci, mainly aggregated in grape-like clusters, smooth colonies generally pigmented in yellow

Note 1 to entry: The main characteristics for identification are: growth on specific selective medium, catalase positive, coagulase positive.

Note 2 to entry: *Staphylococcus aureus* is an opportunistic pathogen for humans that can also be present on the skin of healthy people without causing disorder for them. It is undesirable in cosmetic products due to its potential pathogenicity.

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[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 *Staphylococcus aureus* 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 ± 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 *Staphylococcus aureus*

5.3.4.1 Baird Parker agar medium

5.3.4.1.1 Base medium

5.3.4.1.1.1 Composition

—	pancreatic digest of casein	10,0 g
—	yeast extract	1,0 g
—	meat extract	5,0 g
—	sodium pyruvate	10,0 g
—	L-glycine	12,0 g
—	lithium chloride	5,0 g
—	agar	12 g to 22 g
—	water	to a final volume of 950 ml

5.3.4.1.1.2 Preparation

Dissolve the components or the complete dehydrated base in the water by boiling. Transfer the medium in quantities of 100 ml to flasks or bottles of appropriate capacity. Sterilize the medium 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.

5.3.4.1.2 Potassium tellurite solution

5.3.4.1.2.1 Composition

—	potassium tellurite (K_2TeO_3)	1,0 g
—	water	100 ml

5.3.4.1.2.2 Preparation

Dissolve the potassium tellurite completely in the water with minimal heating.

Sterilize by filtration using 0,22 µm pore size membranes. The solution may be stored at the maximum for one month at 3 °C ± 2 °C. Discard the solution if a white precipitate forms.

The solid should be readily soluble. If a white insoluble material is present in the water, the powder should be discarded.

5.3.4.1.3 Egg yolk emulsion (concentration approximately 20 % or according to the manufacturer's instructions)

If a commercial preparation is not available, prepare the medium as follows.

Use fresh hens' eggs, the shells being intact. Clean the eggs with a brush using a liquid detergent. Rinse them under running water, then disinfect the shells either by immersing them in 70 % (volume fraction) ethanol for 30 s and allow them to dry in the air, or by spraying them with alcohol followed by flame

sterilization. Proceeding under aseptic conditions, break each egg and separate the yolk from its white by repeated transfer of the yolk from one half of the shell to the other. Place the yolks in a sterile flask and add four times their volume of sterile water. Mix thoroughly. Heat the mixture at 47 °C for 2 h and leave for 18 h to 24 h at 3 °C ± 2 °C to allow a precipitate to form. Aseptically collect the supernatant liquid in a fresh sterile flask for use.

The emulsion may be stored at 3 °C ± 2 °C for a maximum of 72 h.

5.3.4.1.4 Complete medium

5.3.4.1.4.1 Composition

— base medium (5.3.4.1.1) 100 ml

— potassium tellurite solution (5.3.4.1.2) 1,0 ml

— egg yolk emulsion (5.3.4.1.3) 5,0 ml

5.3.4.1.4.2 Preparation

Melt the base medium (5.3.4.1.1) then cool it to approximately 47 °C. Add, under aseptic conditions, the two other solutions (5.3.4.1.2 and 5.3.4.1.3), each of them previously warmed at 47 °C, mixing well after each addition.

5.3.4.2 Other selective agar media

Other selective agar media may be used (see Annex A).

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:

Staphylococcus aureus ATCC[1)] 6538 (equivalent strain: CIP[2)] 4.83 or NCIMB[3)] 9518).

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 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.

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1) ATCC = American Type Culture Collection.

2) CIP = Institut Pasteur Collection.

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

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.)

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 *Staphylococcus aureus*

9.4.1 Isolation

Using a sterile loop, streak an aliquot of the incubated enrichment broth on the surface of Baird Parker 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 *Staphylococcus aureus* on selective medium

Selective medium	Aspect of the colonies of *Staphylococcus aureus*
Baird Parker agar medium	Black, shiny colonies, surrounded by clear zones (2 mm to 5 mm)

9.4.2 Identification of *Staphylococcus aureus*

9.4.2.1 General

Proceed to the following tests, for the suspect colonies isolated on the Baird Parker agar medium. The presence of *Staphylococcus aureus* 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-positive cocci in clusters.

9.4.2.3 Catalase test

This test is described in ISO 21148.

Check for a catalase positive test.

9.4.2.4 Coagulase test

With an inoculating loop, transfer representative suspected well isolated colonies from the agar surface of the Baird Parker agar medium to individual sterile tubes, each containing 0,5 ml of mammalians, preferably rabbit or horse, plasma with or without suitable additives.

Incubate at 37 °C ± 2°C and examine the tubes at 3 h, 4 h, 6 h and up to 24 h if no coagulation appears within 6 h, unless otherwise specified by the manufacturer. A positive coagulation only appearing at 24 h shall be confirmed.

Test controls simultaneously with the suspected colonies according to the manufacturer recommendations.

Check for a coagulase positive test.

10 Expression of the results (detection of *Staphylococcus aureus*)

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

— Presence of *Staphylococcus aureus* 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 *Staphylococcus aureus* 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 inoculum

Prior to the test, inoculate the surface of soybean-casein digest agar (SCDA) or other suitable (non-selective, non-neutralizing) medium with *Staphylococcus aureus*. 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 to obtain a calibrated suspension of about 1×10^8 CFU per ml (e.g. using spectrophotometer see 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 per ml 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 in 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 °C ± 2,5 °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 to 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 °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 a Petri dish (diameter 85 mm to 100 mm) containing approximately 15 ml to 20 ml of Baird Parker agar medium. 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 diluted calibrated suspension (11.3.1.1) of bacteria contains between 100 CFU per ml and 500 CFU per ml.

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

When growth is detected on the control plates (contaminated products), the neutralization is verified and the detection method is satisfactory if *Staphylococcus aureus* 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 *Staphylococcus aureus*.

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 *Staphylococcus aureus*.

12 Test report

The test report shall contain the following information:

a) a reference to this International Standard, i.e. ISO 22718:2015;

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 media

A.1 Other enrichment broths

A.1.1 Fluid soybean-casein digest medium

A.1.1.1 Composition

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

A.1.1.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, heating if necessary. Sterilize in an 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.

Dispense the medium into suitable containers.

A.1.2 D/E neutralizing broth (Dey/Engley neutralizing broth)[7]

A.1.2.1 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
—	bromocresol purple	0,02 g
—	water	1 000 ml

A.1.2.2 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 7,6 ± 0,2 when measured at room temperature.

A.1.3 Modified Letheen broth

A.1.3.1 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.1.3.2 Preparation

Dissolve, successively, in boiling water: polysorbate 80 and lecithin 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.

A.2 Other selective agar medium

A.2.1 Mannitol-salt agar medium (Chapman agar)

A.2.1.1 Composition

— beef extract	1,0 g
— pancreatic digest of casein	5,0 g
— pancreatic digest of beef	5,0 g
— sodium chloride	75,0 g
— D-mannitol	10,0 g
— agar	15,0 g
— phenol red	0,025 g
— water	1 000 ml

A.2.1.2 Preparation

Mix, then heat with frequent agitation, and boil for 1 min to effect dissolution. Dispense as desired, and sterilize.

After sterilization and cooling down, the pH shall be equivalent to 7,4 ± 0,2 when measured at room temperature.

A.2.2 Vogel-Johnson agar medium

A.2.2.1 Composition

— pancreatic digest of casein	10,0 g
— yeast extract	5,0 g
— mannitol	10,0 g
— potassium hydrogen phosphate	5,0 g
— lithium chloride	5,0 g
— glycine	10,0 g
— agar	16,0 g
— phenol red	0,025 g
— water	1 000 ml

A.2.2.2 Preparation

Boil the solution of solids for 1 min. Sterilize, cool to between 45 °C and 50 °C and add 20 ml of sterile potassium tellurite solution.

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

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 rinse liquids (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] Rinse 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 sulfate, Ethylene oxide condensate of fatty alcohol	Polysorbate 80, 30 g/l + sodium dodecyl sulfate, 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] Rinse 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] Rinse liquid: polysorbate 80, 3 g/l + L-histidine, 0,5 g/l.
Oxidizing compounds	Sodium thiosulfate	Sodium thiosulfate, 5 g/l. Rinse liquid: sodium thiosulfate, 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. Rinse 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. Rinse 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[a] Rinse liquid: sodium thioglycollate, 0,5 g/l.

NOTE See References [8] and [11].

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

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