Doc: PCD 19 (28490) WC
August 2025

BUREAU OF INDIAN STANDARDS

DRAFT FOR COMMENTS ONLY

भारतीय मानक मसौदा

परीक्षण के तरीके - सौंदर्य प्रसाधनों और सौंदर्य प्रसाधनों के कच्चे माल की सूक्ष्मजीवविज्ञानी जांच

(IS 14648 *का तीसरा पुनरीक्षण*)

Draft *Indian Standard*

MICROBIOLOGICAL EXAMINATION OF COSMETICS AND COSMETIC RAW MATERIALS — METHODS OF TEST

(*Third Revision of* IS 14648)

(ICS 07.100.40)

Cosmetics Sectional Committee,	Last date for Comments
PCD 19	**05 October 2025**

FOREWORD

(*Formal clauses to be added later*)

Cosmetics are used almost universally on a regular basis. The safety in cosmetics and toiletries is of prime importance, as only safe products should be used by consumers. As a result, awareness has grown both among the manufacturers and the health authorities of the hazards involved in using the cosmetics. The Committee responsible for formulating this standard agreed that bio-burden of non-objectionable micro-organisms beyond a specific limit and/or presence of pathogenic micro-organisms in cosmetic preparations pose a health hazard to the consumer; hence it is of great importance to have an appropriate micro-biological standard for the cosmetic products depending on nature of product and its end use.

This standard was originally published in 1999 and subsequently revised in 2005 and 2011. A panel for revision of IS 14648 Microbiological examination of cosmetics and cosmetic raw materials was formed and inputs taken from all members. The ISO documents for microbiological evaluation were referred to as were the IP, USP. EP and JP before proposing the modifications. A summary of the comparisons was prepared, modifications added and shared with the panel members. The limits for products limits in the individual standards were checked; the limits table was updated to cover various new categories of products and limits for henna powder were also introduced.

The first meeting of the Panel was held in CDTL Mumbai on December 17, 2018 wherein all the proposed modifications were deliberated upon in presence of Dr. N. Murugesan, Director, CDTL

Chennai and Dr. Raman Mohan Singh, Director CDTL Mumbai and Ms Nisha Bura Member Secretary, PCD 19. This revision for standard has been carried out to align it with international practices. The key changes and improvements, in line with global and ISO norms, in this revision are as follows:

a) Limits table updated to cover all product categories including henna powder and limits revised where necessary.

b) Inclusion of details on additional and/or alternate procedures for testing of water-soluble products (e.g. Spread plate technique and Membrane filtration).

c) Addition of new neutralizing diluent for enrichment and dilution purposes (TAT Broth base).

d) Change in sample size for microbial enumeration by plate count techniques.

e) Updated the counting range for colonies when tested by plate count and membrane filtration. (25-300 for plate count for TAMC, 15-150 for plate count for TYMC, 15-150 for membrane filtration).

f) Updated incubation conditions for plate count method (30-35°C for 3-5 days for bacterial enumeration and 20-27°C for 3-7 days for yeast and mould (enumeration).

g) Sterilizing cycle temperature for autoclave updated (121°C for 15-20 mins).

h) Inclusion of neutralization validation test method has been provided.

i) Inclusion of an alternate rapid test method (ATP) has been provided.

The effort of the Committee has been to adopt a risk assessment based approach to ensure consumer safety and quality.

The tests covered in this standard are designed for estimation of number of viable aerobic micro-organisms and presence of designated microbial species in cosmetic products. The general principle involved in microbiological testing of cosmetic products is preparation of sample, plating a known mass of sample on selected, validated culture media specifically suitable for recovery and growth of bacteria and fungi and incubating them under specified conditions (time and temperature) to permit development of visible colonies for counting.

Critical step involved in the process is isolation of micro-organisms from cosmetic products based on enrichment culture wherever necessary, direct dilution and plating. Products that are not water soluble shall be initially treated to render them miscible before enrichment or dilution procedures.

The methods given in this standard are invalid unless it is demonstrated that the test specimens to which they are applied do not, of themselves inhibit multiplication under the test conditions of micro-organisms that can be present. If inhibitory substances are present in the sample, use

validated culture media. While carrying out microbiological examination, precautions detailed in general recommendations shall be strictly followed.

Considerable assistance has been derived from international standards on the subject. The list of such references is given in Annex F.

In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, expressing the result of a test or analysis, shall be rounded off in accordance with IS 2 : 2022 'Rules for rounding off numerical values (*second revision*)'.

1 SCOPE

This standard prescribes methods for determination of microbial content of cosmetics and cosmetic raw materials. This standard lays down widely applicable methods but does not restrict the use of other validated methods. Microbial limits for finished cosmetic products are given in Annex A. Guideline for the neutralizers to be used with different preservatives is given in Annex B.

2 TERMS AND DEFINITIONS

For the purpose of this standard the following terms and definitions shall apply.

2.1 Product — Portion of an identified cosmetic product received in laboratory for testing.

2.2 Sample — Portion of the product (at least 1 g or 1 ml) that is used in the test to prepare initial suspension.

2.3 Diluent — This is a suitable liquid used for preparation of sample dilution that will be subjected to analysis/plating.

2.4 Neutralizing Diluent — Most cosmetics contain preservative which may interfere with estimation of micro-organisms. Neutralizing diluents inactivate preservative/anti-microbials and permit microorganisms in the sample to respond/show up appropriately during analysis.

2.5 Initial Suspension — Suspension (or solution) of the sample in a defined volume of an appropriate enrichment broth/medium.

2.6 Sample Dilution — Dilution of the initial suspension.

3 GENERAL RECOMMENDATIONS

3.1 A schematic flow chart shown in Annex C shall be followed for all samples that need to be analyzed.

All three analyses Total Microbial Count (*A*), Yeast and Mould Count (*B*) and Enrichment (*C*) shall be initiated simultaneously. Time which elapses between end of the preparation and the moment which the inoculum comes into contact with the culture medium should not be more than 45 mins.

Following precautions shall be taken by the analysts in order to avoid contaminating the samples and culture media; in addition this will protect them from acquiring infections:

a) Wear laboratory clothing that is light coloured, clean and in good condition; this clothing shall not be worn outside the work areas;

b) Keep nails perfectly clean and well groomed, and preferably short;

c) Wash and disinfect hands, before and after microbiological examinations and immediately after visiting the toilets or eating;

d) When inoculating, avoid speaking, coughing, etc;

e) Do not smoke, drink or eat in the test areas;

f) Do not put food for personal consumption in the laboratory refrigerators;

g) Special precautions shall be taken by persons having infections or illnesses that are likely to contaminate samples with microorganisms and may invalidate results;

h) Mouth pipetting shall not be allowed in the microbiology laboratory; and

j) All waste material containing live microorganisms must be autoclaved at 121°C for at least 30 min prior to discarding.

Use sterile material, equipment and aseptic techniques to prepare the sample, initial suspension and dilutions.

It is recommended that the samples process for dilutions be plated in to the media at the earliest preferably within 45 min of preparation.

3.2 Apparatus and Equipment — The apparatus recommended are as specified below or of standard dimensions generally used in microbiological labs.

3.2.1 *Test Tubes* — Of resistant glass, provided with closely fitting metal/plastic caps. Diameters 15 mm, length 95 mm or 150 mm are preferred.

3.2.2 *Petri Dishes* — Of 100 mm diameter and 15 mm depth are preferred. The bottom of the dishes shall be free from bubbles and scratches and shall be flat.

Disposable, single use plastic petri dishes (90 mm diameter and 15 mm depth) or reusable glass petri dishes may be used.

3.2.3 *Pipettes, Syringes, Spatulas and Other Suitable Sampling Devices*

3.2.4 *Glass Rods, Microscope*

3.2.5 *Autoclave* — The autoclave should be of sufficient capacity. The autoclave shall maintain uniform temperature within the chamber up to and including the sterilizing cycle of 121°C for 15-20 mins.

3.2.6 *Colony Counter* — Counting can be done manually. If required a suitable magnifying lens or a colony counter may be used.

3.2.7 *Incubators* — Should be capable of maintaining uniform temperatures in the range of 20°C to 35°C.

3.2.8 *Water Baths* — To maintain temperatures at 37°C and 44 to 48°C.

3.2.9 *Media* — Media may be prepared using individual ingredients in laboratory. Alternatively, commercially available dehydrated media may be used according to manufacturer's composition and instructions. See Annex D for details of compositions and instructions.

4 MICROBIOLOGICAL RISK ANALYSES

Cosmetic products can be categorized for microbial risk based on the following considerations:

a) Water activity (≤ 0.75);

b) *p*H ≤ 3 and ≥10);

c) Alcohol content (≥ 20 percent);

d) Filling temperature (≥ 65°C);

e) Anhydrous products

f) Raw materials which create a hostile environment (strong oxidizing agents like hydrogen peroxide or reducing agents, polar organic solvents such as ethyl acetate, butyl acetate, oxidizing dyes, aluminum chlorohydrate and related salts etc); and

g) Combined factors.

Cosmetic formulations falling into any of the above categories can be treated as microbiologically low risk products, hence can be exempted from microbiological testing.

5 ENUMERATION AND DETECTION (TOTAL VIABLE COUNT)

5.1 Principle

This method involves enumeration of colonies on a non-selective agar medium (Plate count). The possible inhibition of microbial growth by the sample shall be neutralized to allow detection of viable microorganisms.

5.2 Plate Count

Plate count consists of the following steps:

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

b) Aerobic incubation of the plates.

c) Counting the number of colony forming units (CFU) and calculation of the number of aerobic mesophilic bacteria per millilitre or per gram of product.

5.3 Diluents and Culture Media

5.3.1 *Neutralizing Diluents*

Fluid casein digest — soy lecithin — polysorbate 20 medium (SCDLP 20 broth)/Eugon LT 100 broth/D/E neutralizing broth/Modified Letheen broth/Inactivating Diluent. It is generally recommended to use a universal neutralizing agent, for example, D/E neutralizing broth. Any other neutralizing diluent may be used to prepare the initial suspension if it has been checked and validated.

5.3.2 *Diluent*

Peptone broth/Nutrient broth.

5.3.3 *Culture Media*

a) *For total microbial count* — Soyabean casein digest medium (Trypticase Soy agar/TSA).

b) *For yeast and mould count* — Sabourauds Dextrose agar/Potato Dextrose agar.

5.4 Validation

Validation of microbiological enumeration, detection and neutralization methods listed herewith may be carried out using appropriate standard non-pathogenic test bacterial and fungal strains.

5.4.1 Neutralizer Validation

This procedure ensures that the neutralising media chosen is appropriate to deliver adequate neutralisation of the preservative system within the product. If a neutralising media is found to be ineffective then repeat tests must be completed to find a suitable neutralising media.

Select inocula of one gram positive (*Staphylococcus. aureus* ATCC 6538) & gram negative (*Pseudomonas aeruginosa* ATCC 9027) organism for the product being tested. An alternative to the

gram-negative strain may be *Escherichia coli* ATCC 8739. If needed can also include *Candida albicans* ATCC 10231 / *Aspergillus niger* (*brasiliensis*) ATCC 16404 as reference test organisms as they are included to be one of the test organisms for enrichment. Prepare a dilution of the product in the neutralising diluent being evaluated following the procedure specific for the product. For example1:10 or 1:100 product to neutralising diluent ratio.
Allow to stand for 10- 15 mins minutes to achieve adequate neutralisation of preservative system.

Inoculate test samples to achieve approximately 10^2 cfu/ml (100 to 500 cfu/ml).

Pipette 1.0 ml of the first dilution of the test sample directly into a petri dish. Make further serial dilutions if required and plate out.
Follow same procedure as test product control for positive control (culture control) with neutralising diluent and water.

Pour plates, with 18-20 ml of appropriate agar, swirl gently to mix and allow to harden.
Invert and incubate plates at the temperature and time as 32.5ºC +/- 2.5ºC for 24-48 hours.

> NOTE —— Neutraliser toxicity can be determined by positive control (culture control) with neutraliser. Hence along with neutraliser effectiveness/validation its toxicity for respective cultures can be studied simultaneously. If fungal cultures are considered for Neutralisation validation, respective test temperatures & incubations conditions **(**26+/-2^0C /2 to 5 days) should be followed.

Acceptance Criteria: Organism counts recovered from the product in neutralising diluent should be within 50% of the positive control count for the results to be valid. If the results are not valid, the use of additional neutralisers or a larger volume of diluent should be tried to achieve adequate neutralisation.

If no suitable neutralizing method can be found, it can be assumed that the failure to isolate the inoculated organism is attributable to the microbicidal activity of the product. This information serves to indicate that the article is not likely to be contaminated with the given species of the microorganism. However, it is possible that the product inhibits only some of the microorganisms specified herein, but does not inhibit others not included among the test strains or those for which the latter are not representative. Then, perform the test with the highest dilution factor compatible with microbial growth and the specific acceptance criterion.

5.5 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.

5.6 Procedure — Preparation of the Initial Suspension

The initial suspension is prepared from a sample using a dilution ratio of 1:10 of the well-mixed product under test. Larger volumes may be allowed as and if needed.

Note the exact mass or volume of the sample. The initial suspension is usually 1:10 dilution. Larger volumes of diluents or enrichment broth may be required, if high levels of contamination are expected and/or if anti-microbial properties are still present in 1:10 dilution.

5.6.1 *Water-Miscible Products*

Transfer the sample of product to an appropriate volume (for example 90 ml) of neutralizing diluent. Note the dilution factor *d.*

5.6.2 *Water-Immiscible Products*

Transfer the sample of product to a suitable container containing a suitable quantity of solubilizing agent (for example polysorbate 80). Disperse the sample within the solubilizing agent and add an appropriate volume (for example 90 ml) of neutralizing diluent or diluent. Note the dilution factor *d*. In case of some immiscible products like lipsticks, warming of the sample and diluent up to 40°C may be required to facilitate dispersion. In case if a universal neutralizing agent (D/E) has been used, both water miscible and immiscible products shall be treated in the same manner as D/E contains emulsifiers.

5.6.3 *Dilutions for Counting Methods*

Additional serial dilutions (for example 1:100 dilution) should be performed from the initial suspension using the diluent (according to the expected level of contamination of the product or dilution validated for effective neutralization of anti-microbials present in the product).Counting shall be performed using at least two Petri dishes at each dilution.

5.7 Plate-Count Methods

5.7.1 *Pour-Plate Method*

In Petri dishes 90 mm to 100 mm in diameter, add 1 ml of the initial suspension and/or sample dilution prepared and pour 15 ml to 20 ml of sterile melted agar medium kept in a water bath at no more than 48°C. If larger Petri dishes are used, the amount of agar medium shall be increased accordingly. Mix the initial suspension and/or sample dilution with the medium carefully rotating or tilting the plates sufficiently to disperse them. Allow the mixture in the petri dishes to solidify on a horizontal surface at room temperature.

5.7.2 *Surface-Spread Method*

In Petri dishes 90 mm to 100 mm in diameter, pour 15 ml to 20 ml of sterile melted agar medium kept in a water bath at no more than 48°C and allow to solidify completely, if larger Petri dishes are used, the amount of agar medium shall be increased accordingly. Add not less than 0.1 ml of the initial suspension and/or sample dilution prepared. Spread the initial suspension and/or sample dilution on the medium carefully with a sterile surface spreader.

5.7.3 Membrane Filtration Method

Use membranes having a nominal pore size no greater than 0.45 µm. Transfer a suitable amount of the initial suspension or of the sample dilution (preferably representing at least 1 g or 1 ml of the product) onto the membrane. Filter immediately and wash the membrane. Transfer the membrane onto the surface of the agar medium.

5.7.4 *Incubation*

Invert the inoculated dishes and place them in the incubator set at 30°C to 35°C for 3 to 5 days in the case of bacterial enumeration and 20°C to 27°C for 3 to 7 days in the case of yeast and mould count. It is recommended that after incubation, the dishes shall, if possible, be examined immediately. Otherwise, they may be stored at less than 8°C (refrigerator) for maximum upto 48 hours.

5.7.5 *Colony Counts*

Select the plates corresponding to a given dilution and showing the highest number of colonies as follows:
TAMC: 25-300* colonies (Pour plate, spread plate), 15-150 colonies (Membrane filtration)
TYMC: 15-150 colonies (Pour plate, spread plate, Membrane filtration)

NOTE:
(i) However for individual colony counts of specific organisms e.g. moulds like *Aspergillus niger*, 8-80 colonies can be considered.
(ii) In case there are less number of colonies than mentioned above, then the result may be reported as < lower limit of colonies (e.g. <15) and other dilution to be tried.)
(iii) * If the plate has too many colonies showing matt growth then a range of 25-250 colonies can be considered for TAMC count.

5.8 Expression of Results

5.8.1 *Method of Calculation for Plate Count*

Calculate the number *N* of micro-organisms present in the sample using:

$$N = \frac{m}{V \times d} \quad \text{or}$$

$$N = \frac{c}{V \times d} \quad \text{or}$$

$$N = \frac{x}{V \times d}$$

where

m = arithmetic mean of the counts obtained from the duplicates;
C =number of colonies counted on a single plate;
x =weighted mean of the counts obtained from two successive dilutions;
V = volume of inoculums applied to each dish, in millilitre; and

d =dilution factor corresponding to the dilution made for the preparation of the initial suspension or for the first counted dilution.

5.8.2 Interpretation of results

The inherent variability of plate count should be taken into account. Two results should only be considered different if the difference exceeds 50 % or, when expressed logarithmically, the difference exceeds 0.3 log. For precise counts check plates having 25-250 colonies for bacterial enumeration and 15-150 colonies for yeast and mould enumeration and membrane filtration.

Where the number of CFU is more than 25 and less than 250 on plates or more than 15 and less than 150 on membranes, where S is the mass or the volume of the sample (see 9.2), express the result as follows: — if S is at least 1 g or 1 ml, and V is at least 1 ml: the number of aerobic mesophilic bacteria per millilitre or per gram of the sample = N/S; — if S is less than 1 g or 1 ml, and/or V is less than 1 ml: the number of aerobic mesophilic bacteria in the sample (note the tested quantity of sample, taking into account S and V) is = N.

Where the number of CFU is less than 25 on plates or 15 on membranes also follow the above procedure.

Where no colony count is observed, results can be reported as:

Less than 1/d×V×S of aerobic mesophilic bacteria / g or ml of product (S is atleast 1 g or 1ml)
Less than 1/d×V of aerobic mesophilic bacteria in sample S (note the tested quantity of sample, taking into account S and V) (S is less than 1g or 1ml) where d is the dilution factor of initial suspension and V is 1 (for pour plate and membrane filtration) and 0.1 (for spread plate) respectively.

5.8.3 Examples

Sr No.	Scenario	Counts obtained	Result
1	One dilution having 2 plates in countable range	10^{-1}: 38, 42	40 is the average plate count, multiplied by dilution factor 10 = 400 or 4×10^2 cfu/g or ml of sample
2	One dilution having 1 plate in countable range	10^{-1}: 60	60 is the average plate count, multiplied by dilution factor 10 = 600 or 6×10^2 cfu/g or ml of sample
3	Two dilutions having 2 plates in countable range	10^{-2}: 235, 282 10^{-3}: 31,39	235+282+31+39/1(2+0.1x2) × 10^{-2} = 587/0.022 = 26 682. Rounding off to 27000 gives 2.7×10^4 cfu/g or ml of sample

4	Two membrane filters for one dilution	10^{-1}: 18,22	20 is the average plate count, multiplied by dilution factor 10 = 200 or 2×10^2 cfu/g or ml of sample
5	One membrane filter for one dilution	10^{-1}: 65	65 is the average plate count, multiplied by dilution factor 10 = 650 or 6.5×10^2 cfu/g or ml of sample

Using the above table as a guide scenarios other than the ones mentioned can also be addressed.

6 METHODS FOR DETECTION OF SPECIFIC ORGANISMS

The first step of the procedure is to perform enrichment by using a non-selective broth medium to increase the number of micro-organisms 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. Other validated identification tests e.g. MSID or DNA sequencing may also be used wherever appropriate.

6.1 Preparation of the Initial Suspension in the Enrichment Broth

The enrichment shall be prepared from a sample of at least 1 g or 1 ml of the well-mixed product under test, which shall be dispersed in at least 9 ml of enrichment broth. (Dilution ratio 1: 10 is recommended, larger volumes may be allowed as and if needed)

6.2 Water-Miscible Products

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

6.3 Water-Immiscible Products

Transfer the sample of product to a suitable container containing a suitable quantity of solubilizing agent (for example, Polysorbate 80). Disperse the sample within the solubilizing agent and add an appropriate volume of broth. In case if a common/universal neutralizing broth is used, having solubilizing agents, then both water miscible and immiscible products shall be treated in the same manner as broth will contain emulsifying agents.

6.4 *Pseudomonas aeruginosa*

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

NOTE

1 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.

2 *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.

6.4.1 *Diluents and culture media*

a) *Enrichment broth* — Eugon LT 100 broth or any other validated neutralizing enrichment medium,

b) *Selective agar for isolation* — Cetrimide agar medium, and

c) *Selective agar for confirmation* — Pseudomonas agar medium for detection of pyocyanin and pyorubin.

6.4.2 *Strains of Micro-Organisms*

For validation of the test conditions, *Pseudomonas aeruginosa* ATCC 9027 standard indicator test strain shall be used.

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

NOTE — ATCC — American Type Culture Collection.

6.4.3 *Procedure — Incubation of the Inoculated Enrichment Broth* Incubate the initial suspension prepared in broth at 32.5 ± 2.5°C for at least 20 h (maximum 72 h).

6.4.4 *Detection and Identification of Pseudomonas aeruginosa — 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 ± 2.5°C for at least 24 h (maximum 48 h).

6.4.4.1 *Identification of Pseudomonas aeruginosa*

Selective medium	: Characteristic colony morphology of Pseudomonas aeruginosa
Cetrimide agar medium	: Yellow — green pigment (pyocyanin), which fluoresces under UV light

6.4.5 *Confirmation of Pseudomonas aeruginosa*

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.

6.4.6 *Gram's Stain*

This test is described in Annex E. Check for gram negative rods.

6.4.7 *Oxidase Test*

This test is described in Annex E. Check for oxidase positive culture.

6.4.8 *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 cetrimide agar medium, so that individual colonies develop. Incubate at 32.5 ± 2.5°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.

6.4.9 *Expression of Results*

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

'*Pseudomonas aeruginosa* present in the sample'.

b) 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:

'*Pseudomonas aeruginosa* absent in the sample'.

6.5 ***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.

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

6.5.1 *Diluents and Culture Media*

a) *Enrichment broth* — Eugon LT 100 broth/ Fluid lactose medium with neutralizing and dispersing agents/Fluid lactose medium/ Modified letheen broth/D/E neutralizing broth/Soyabean casein digest lecithin polysorbate 80 medium.

b) *Selective agar for isolation* — MacConkey agar medium.

c) *Selective agar for confirmation* — Levine Eosin — Methylene Blue agar medium.

6.5.2 *Strains of Micro-Organisms*

For validation of the test, use *Escherichia coli* ATCC 8739 standard indicator test strain. The culture should be reconstituted according to the procedures provided by the supplier of reference strain.

6.5.3 *Incubation of the Inoculated Enrichment Broth*

Incubate the initial suspension prepared in broth (*see* **6.1**) at 32.5 ± 2.5°C for at least 20 h (maximum 72 h).

6.5.4 *Detection and Identification of Escherichia coli*

6.5.4.1 *Isolation*

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

Invert the petri dish and then incubate at 32.5 ± 2.5°C for at least 24 h (maximum 48 h).

Morphological characteristics of *Escherichia coli* on MacConkey's agar medium:

Selective medium	: Characteristic colony morphology of *Escherichia coli*
MacConkey's medium	: Brick-red, may have surrounding zone of precipitated bile

6.5.4.2 *Identification of Escherichia coli*

6.5.4.2.1 *General*

Proceed with following tests for the suspect colonies isolated on MacConkey agar medium.

The presence of *Escherichia coli* may be confirmed by other suitable, cultural and bio-chemical tests.

6.5.4.2.2 *Gram's stain*

Perform the test specified in Annex E. Check for Gram negative rods (bacilli).

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

Inoculate the surface of 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 ± 2.5°C for at least 24 h (maximum 48 h). Morphological characteristics of *Escherichia coli* on levine eosin methylene blue agar medium:

Selective medium	: Characteristic colony morphology of *Escherichia coli*
Levine eosin ethylene blue agar medium	: Metallic sheen under reflected light and a blueblack appearance under transmitted light

6.5.5 *Expression of the Results*

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

'*Escherichia coli* present in the sample'.

b) If no growth after enrichment is observed and/ or if the identification of the colonies does not confirm presence of this species, express the result as: '*Escherichia coli* absent in the sample'.

6.6 ***Staphylococcus aureus***

Gram-positive cocci, mainly joined in grape-like clusters, smooth colonies generally pigmented in yellow.

NOTE

1 The main characteristic for identification are: growth on specific selective medium, catalase positive and coagulase positive.

2 *Staphylococcus aureus* is an opportunistic pathogenic bacterium for humans that can also be present normal flora on the skin of healthy people without causing infection in them. But it is undesirable in cosmetic products due to its potential pathogenicity.

6.6.1 *Diluents and Culture Media*

a) *Enrichment broth* — Eugon LT 100 broth/ Fluid soyabean casein digest medium/D/E neutralizing broth/Modified latheen broth.

b) *Selective agar medium for isolation of Staphylococcus aureus* — Baird Parker Agar/ Mannitol salt agar medium/Vogel Johnson agar medium.

6.6.2 *Strains of Micro-Organisms*

For validation of the test conditions, *Staphylococcus aureus* ATCC 6538/6538P standard indicator test strain shall be used.

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

6.6.3 *Procedure*

6.6.3.1 *Incubation of the inoculated enrichment broth* Incubate the initial suspension prepared in broth at 32.5 ± 2.5°C for at least 20 h (maximum 72 h).

6.6.4 *Detection and Identification of Staphylococcus aureus*

6.6.4.1 *Isolation*

Using a sterile loop, streak an aliquot of the incubated enrichment broth on surface of Baird Parker Agar medium in order to obtain isolated colonies. Invert the petri dish and then incubate at 32.5 ± 2.5°C for at least 24 h (maximum 48 h).

Check for characteristic colonies

Morphological characteristics of *Staphylococcus aureus* on selective media:

Selective Medium	Aspect of the Colonies of S. Aureus
Baird parker agar medium	Black shiny colonies surrounded by clear zones (2 mm to 5 mm)
V-J Agar medium	Black Colonies surrounded by yellow zones
MSA	Yellow colonies with yellow zones

6.6.4.2 *Identification of Staphylococcus aureus*

6.6.4.2.1 *General*

Proceed with following tests, for the suspect colonies isolated on Baird Parker agar/Mannitol salt agar/Vogel Johnson agar medium. Presence of *Staphylococcus aureus* may be confirmed by other suitable, cultural and bio-chemical tests.

6.6.4.2.2 *Gram's stain*

This test is described in Annex E. Check for gram-positive cocci in clusters.

6.6.4.2.3 *Catalase test*

This test is described in Annex E. Check for a catalase positive test.

6.6.4.2.4 *Coagulase test*

With an inoculating loop, transfer representative suspected well isolated colonies from the agar surface of Baird Parker/Mannitol salt/Vogel Johnson agar medium to individual sterile tubes, each containing 0.5 ml of rabbit or horse, plasma with or without suitable additives.

Incubate at 37 ± 2°C and examine the tubes at 3 h, 4 h and 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. Any other suitable validated test method may be used as an alternate to coagulase test.

6.6.5 *Expression of the Results (Detection of Staphylococcus aureus)*

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

'*Staphylococcus aureus* present in the sample'.

If no growth after enrichment is observed and/or if the identification of the colonies does not confirm presence of this species, express the result as:

'*Staphylococcus aureus* absent in the sample'.

6.7 ***Candida albicans***

This strain of yeasts forms white to beige, creamy and convex colonies on surface of a selective medium.

NOTE — Its main characteristic for identification is production of germ tube and/or pseudomycelium and chlamydospore.

6.7.1 *Diluents and Culture Media*

a) *Enrichment broth* — Eugon LT 100 broth/ Fluid soyabean casein digest medium/ Modified latheen broth/Glucose and peptone added lecithin polysorbate medium/D/E neutralizing broth/Soyabean casein digest lecithin polysorbate 80 medium.

b) *Selective agar medium for isolation of C. albicans* — Sabouraud dextrose chloramphenicol agar/Potato dextrose agar with antibiotics/BIGGY Agar

c) *Selective agar medium for identification of C. albicans* — Corn meal agar with 1 percent polysorbate 80.

6.7.2 *Strains of Micro-Organisms*
For validation of the test conditions, *Candida albicans* ATCC 10231 standard indicator test strain shall be used. The culture should be reconstituted according to procedures provided by the supplier of the reference strain.

6.7.3 *Procedure*

6.7.3.1 *Incubation of the inoculated enrichment broth* Incubate initial suspension prepared in broth at 32.5 ± 2.5°C for at least 20 h but not more than 72 h.

6.7.4 *Detection and Identification of Candida albicans*

6.7.4.1 *Isolation*

Using a sterile loop, streak an aliquot of incubated enrichment broth on to surface of Sabouraud dextrose chloramphenicol agar in order to obtain isolated colonies. Invert the petri dish and then incubate at 32.5 ± 2.5°C for 24 h to 48 h. Bismuth sulphite glucose glycine yeast agar is an alternative to the above mentioned selective medium.

Table 1 Morphological Characteristics of *Candida albicans* on Selective Medium

Selective Medium	Aspect of Colony of Candida albicans
Sabouruads dextrose chloramphenicol agar	White to beige, creamy and convex
Bismuth sulfite glucose glycine yeast agar	Brown black, smooth and circular

6.7.4.2 *Identification*

6.7.4.2.1 *General*

Candida albicans can appear to be dimorphic and is capable of producing pseudohyphae, some true hyphae, and clusters of round blastoconidia as well as large thick-walled chlamydospores. At low ambient temperature the culture might express this pseudomycelial form; however, it can change to the unicellular form at higher temperatures.

Proceed with following tests for the suspect colonies isolated on Sabouraud dextrose chloramphenicol agar medium. Presence of *Candida albicans* may be confirmed by other suitable cultural and biochemical tests.

6.7.4.2.2 *Gram's stain*

Follow the procedure specified in Annex E. The microscopic observation shall reveal a violet colour, short ovoid or elongated cells, sometimes with budding cells.

Further identification for confirmation may be carried out if required using following two methods.

6.7.4.2.3 *Germ tube production*

Place 0.5 ml to 1 ml of serum (foetal calf or horse serum) in a small test tube. Emulsify a small portion of yeast colony to be tested in the serum. Incubate in a water bath, at 37 ± 1°C, for 1.5 h to 2 h, or in an incubator at 37 ± 2°C for 3 h. Place a drop of serum on a slide, put on a cover glass, and examine microscopically for germ tube production. Germ tubes appear as cylindrical filaments originating from the blastospore, without any constriction at the point of origin and without obvious swelling along the length of the filament. Formation of germ tubes characterizes presence of *Candida albicans*. If germ tubes were not formed, the colonies shall be examined for production of hyphae, pseudohyphae and chlamydospores.

6.7.4.2.4 *Culture on corn meal agar with 1 percent polysorbate 80*
Remove a small portion of the yeast colony with an inoculating wire and streak-inoculate the surface of medium across the centre of the plate. Place a sterile cover glass over the inoculum

streak. Incubate at 32.5 ± 2.5°C for up to 3 days. After 24 h, remove the dish lid and examine growth through the cover glass under the microscope with magnification of 100 × to 400 ×.

Candida albicans produces large, highly refractile, thick-walled chlamydospore which may be seen terminally or on short lateral branches.

6.7.5 *Expression of Results* (*Detection of* Candida albicans)

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

'*Candida albicans* present in the sample'

If no growth after enrichment is observed and/or if identification of the colonies does not confirm presence of this species, express the result as:

'*Candida albicans* absent in the sample'.

7.0 Alternate Rapid test method: This method is included as a Rapid alternate test method to the conventional test method for finished products.

Principle for Adenosine Triphosphate Bioluminescence: This is Celsis Rapid Sterility Method which is a growth-based sterility test & it detects microbial contamination based on the presence of microbial Adenosine triphosphate (ATP) in a sample. It a well-established technology with luminometers and reagents available from multiple instrument manufacturers. The energy from living cells is stored as ATP and can be measured as light when exposed to luciferase from the American firefly. Each ATP molecule consumed by luciferase produces 1 photon of light. The result detected by a luminometer is typically expressed in relative light units (RLU) and is instrument, reagent, and organism dependent. The ATP content of different microorganisms ranges from 2 to 4×10^{-18} mole/cfu for gram-negative bacteria, 5 to 8×10^{-18} mole/cfu for gram-positive bacteria, and 300 to 800×10^{-18} mole/cfu for fungi . Given the high signal-to-noise ratio of the measurement and the background ATP in microbiological culture media, the microbiologically relevant instrument detection limit in broth is in the order of 5000 RLU equivalent to approximately 103 cfu. This LOD will detect the presence of microorganisms at levels which are 3–4 logs less within an aliquot of the media than that is required for visual detection of growth in the media. For a rapid microbial test for the release of sterile short-life products, an enrichment culture either in liquid media to reach a threshold ATP level or on a membrane filter on solid media for the formation of microbial colonies could be used with an incubation time of 2–7 days.

NOTE**:** Refer USP -1071& 1223 for details of method and validation, respectively.

ANNEX A
(*Clause* 1)
MICROBIAL LIMITS FOR COSMETIC FINISHED PRODUCTS

Product Categories	**Total Viable count** (cfu/g)**	**Yeast & Mould count (cfu/g)**	**Gram negative pathogens *(E. coli, Pseudomonas aeruginosa)***	***S. aureus***	***C. albicans***
Oral care and related products					
Toothpowders, gels, Toothpastes, mouthwash and other similar preparations	500 max	100 max	Absent per gram	Absent per gram	Absent per gram
Eye Care					
Eyebrow pencil, eyeliner, eye-definer pencil, Highlighter pencil, eye-shadow and other similar preparations	100 max	100 max	Absent per gram	Absent per gram	Absent per gram
Lip Care					
Lipsticks, Lipsalves, Lipgloss, Lip-definer pencils and other similar preparations	100 max	100 max	Absent per gram	Absent per gram	Absent per gram
Baby Care (Products for children under 3 yrs of age)					
Baby cream, lotion, shampoo, head to toe wash, baby oils and other similar preparations for babies	100 max	100max	Absent per gram	Absent per gram	Absent per gram
Hair care					
Rinse off: Shampoos, conditioners, gels, and other similar hair preparations	1000 max	100 max	Absent per gram	Absent per gram	Absent per gram
Leave on: Hair creams, gel, conditioners, masks, wax and other similar hair preparations	1000 max	100 max	Absent per gram	Absent per gram	Absent per gram
Skin care					
Skin care : Skin creams, lotions, skin powders, gels, facewashes, shave products (shaving lotions, creams, after shave lotions, gels etc.) and other similar preparations	1000 max	100 max	Absent per gram	Absent per gram	Absent per gram

Toiletries					
Body washes, body scrubs, soaps, bathing bars and other similar preparations	1000 max	100 max	Absent per gram	Absent per gram	Absent per gram
Miscellaneous					
Henna Powder	10^7 max	10^4 max	Absent per gram	Absent per gram	Absent per gram

Note 1: Specifications for products that need microbial assessment after conducting Risk Analysis as per Clause 4
Note 2: Due to inherent variability of the plate count method, result considered out of limit if
a $\geq 2 \times 10^2$ CFU/g or ml,
b $\geq 2 \times 10^3$ CFU/g or ml
When colonies of bacteria are detected on Sabouraud Dextrose agar, Sabouraud Dextrose agar containing antibiotics may be used.
**Total Aerobic Mesophilic Microorganisms : (Bacteria plus yeast and mould)

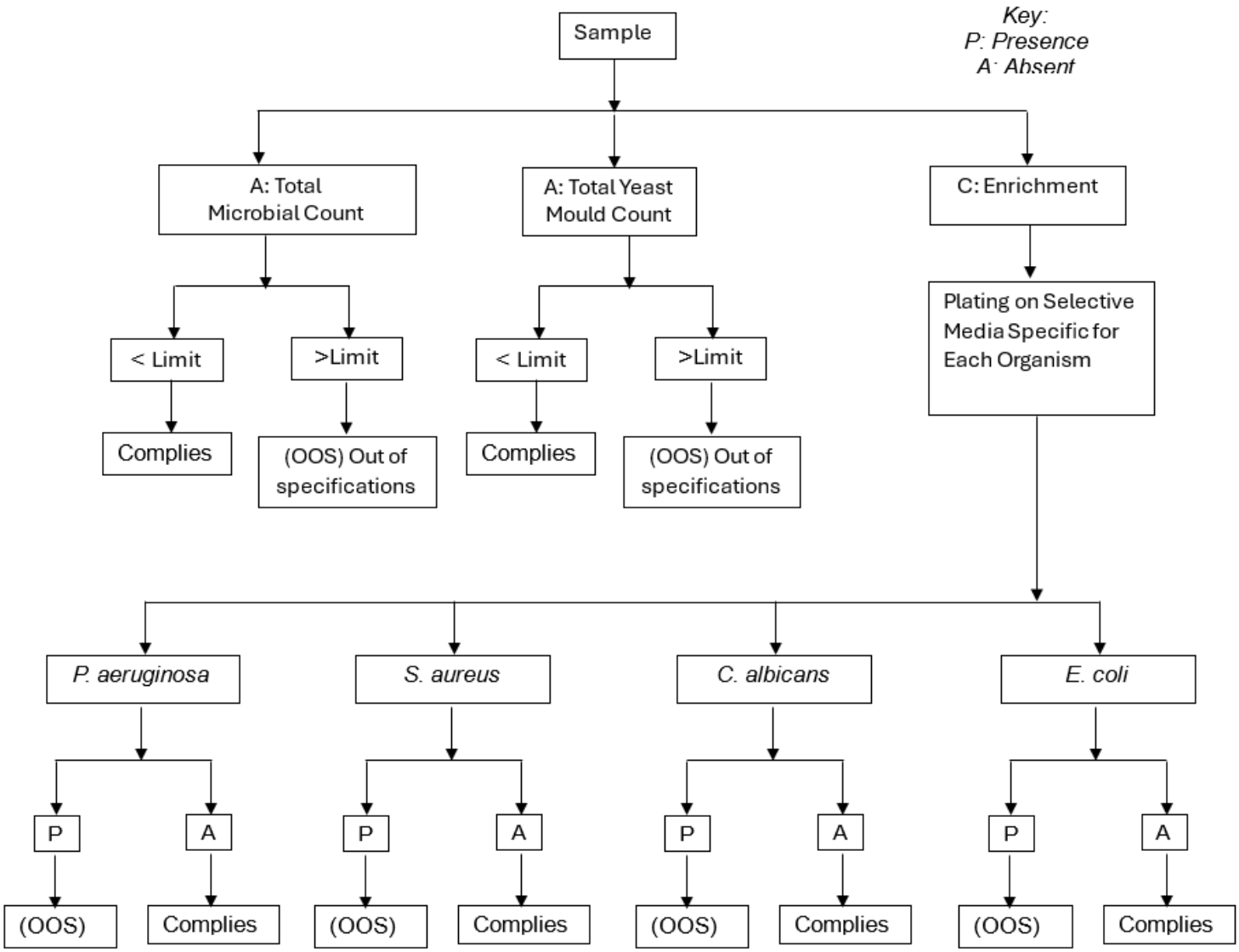
ANNEX B
(*Clause* 1)
LIST OF RECOMMENDED NEUTRALIZERS FOR DIFFERENT PRESERVATIVES

Preservative	Chemical compound able to Neutralize Preservative's Antimicrobial Activity	Example of Suitable Neutralizers
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[1)] TAT Broth base
Quarternary ammonium compunds Cationic surfactants	Lecithin, saponin, polysorbate 80, Sodium dodecyl sulphate Ethylene oxide condensate of fatty alcohol.	Polysorbate 80, 30 g/l + sodium Dodecyl sulphate, 4g/l +lecithin, 3 g/l. Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l D/E neutralizing broth[1)] TAT Broth base
Aldehydes Formaldehyde-release Agents	Glycine, histidine	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[1)]
Oxidizing compounds	Sodium thiosulphate	Sodium thiosulphate, 5 g/l. D/E neutralizing broth
Isothiazolinones, Imidazoles	Lecithin, saponin, Amines, sulfates, mercaptans, sodium bisulfite, sodium thioglycollate	Polysorbate 80, 30 g/l + saponine, 30 g/l. D/E neutralizing broth[1)] TAT Broth base
Biguanides	Lecithin, saponin, polysorbate 80	Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. D/E neutralizing broth[1)] TAT Broth base
Metallic salts (Cu, Zn, Hg) Organo-mercuric Compounds	Sodium bisulphate, L-cysteine, Sulphydryl compounds, thioglycollic acid	Sodium thioglycollate, 0,5 g/l or 5 g/l. L-cysteine, 0,8 or 1,5 g/l. D/E neutralizing broth[1)]
1) D/E is universal diluent or neutralizing and hence can be used for all product types (see Annex D). However it needs to be validated by appropriate methods wherever applicable.		

ANNEX C

(*Clause* 3.1)

SCHEMATIC FLOW CHART FOR MICROBIOLOGICAL ANALYSIS



ANNEX D
(*Clause* 3.2.9)
COMPOSITION OF DILUENTS AND CULTURE MEDIA

D-1 DILUENTS

D-1.1 Modified Letheen Broth

D-1.1.1 *Composition*

a) Peptic digest of meat	: 20.0 g
b) Pancreatic digest of casein	: 5.0 g
c) Beef extract	: 5.0 g
d) Yeast extract	: 2.0 g
e) Lecithin	: 0.7 g
f) Polysorbate 80	: 5.0 g
g) Sodium chloride	: 5.0 g
h) Sodium bisulfite	: 0.1 g
j) Water	: 1 000 ml

D-1.1.2 *Preparation*

Dissolve successively in boiling water; polysorbate 80 and lecithin until their complete dissolution. Dissolve the other components by mixing whilst 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 *p*H shall be equivalent to 7.2 ± 0.2 when measured at room temperature.

D-1.2 D/E Neutralizing Broth (Dey/Engley Neutralizing Broth)

D-1.2.1 *Composition*

a) Glucose	: 10.0 g
b) Soybean lecithin	: 7.0 g
c) Sodium thiosulfate pentahydrate	: 6.0 g
d) Polysorbate 80	: 5.0 g
e) Pancreatic digest of casein	: 5.0 g
f) Sodium bisulfite	: 2.5 g
g) Yeast extract	: 2.5 g
h) Sodium thioglycollate	: 1.0 g
j) Bromcresol purple	: 0.02 g
k) Water	: 1 000 ml

D-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 *p*H shall be equivalent to 7.6 ± 0.2 when measured at room temperature.

D-1.3 Soybean-Casein-Digest-Lecithin-Polysorbate 80 Medium (SCDLP 80 Broth)

D-1.3.1 *Composition*

a) Casein peptone	: 17.0 g
b) Soybean peptone	: 3.0 g
c) Sodium chloride	: 5.0 g
d) Dipotassium hydrogen phosphate	: 2.5 g
e) Glucose	: 2.5 g
f) Lecithin	: 1.0 g
g) Polysorbate 80	: 7.0 g
h) Water	: 1 000 ml

D-1.3.2 *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 *p*H shall be equivalent to 7.2 ± 0.2 when measured at room temperature.

D-1.4 Fluid Casein Digest — Soy Lecithin — Polysorbate 20 Medium (SCDLP 20 Broth)

D-1.4.1 *Composition*

a) Pancreatic digest of casein	: 20.0 g
b) Soy lecithin	: 5.0 g
c) Polysorbate 20	: 40.0 ml
d) Water	: 960.0 ml

D-1.4.2 *Preparation*

Dissolve the polysorbate 20 in 960 ml of water by mixing while heating in a water bath at 49 ± 2°C. Add pancreatic digest of casein and soy lecithin. Heat for about 30 min to obtain solution. Mix and dispense the medium into suitable containers. Sterilize in the autoclave at 121°C for 15 min. After sterilization, the *p*H shall be equivalent to 7.3 ± 0.2 when measured at room temperature.

D-1.5 Eugon LT 100 Broth

D-1.5.1 *Composition*

a) Pancreatic digest of casein	: 15.0 g
b) Papaic digest of soybean meal	: 5.0 g
c) L-cystine	: 0.7 g
d) Sodium chloride	: 4.0 g
e) Sodium sulfite	: 0.2 g
f) Glucose	: 5.5 g
g) Egg lecithin	: 1.0 g
h) Polysorbate 80	: 5.0 g
j) Octoxynol	: 91.0 g
k) Water	: 1 000 ml

D-1.5.2 *Preparation*

Dissolve successively polysorbate 80, octoxynol 9 and egg lecithin 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, the *p*H shall be equivalent to 7.0 ± 0.2 when measured at room temperature.

D-1.6 Fluid Lactose Medium

D-1.6.1 *Composition*

a) Beef extract	: 3.0 g
b) Pancreatic digest of gelatin	: 5.0 g
c) Lactose	: 5.0 g
d) Water	: 1 000 ml

D-1.6.2 *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 *p*H shall be equivalent to 6.9 ± 0.2 when measured at room temperature.

D-1.7 Fluid Lactose Medium with Neutralizing and Dispersing Agents

D-1.7.1 *Composition*

a) Beef extract	: 3.0 g
b) Pancreatic digest of gelatin	: 5.0 g
c) Lactose	: 5.0 g
d) Egg lecithin	: 1.0 g

e) Polysorbate 80	: 5.0 g
f) Octoxynol	: 91.0 g
g) Water	: 1 000 ml

D-1.7.2 *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 *p*H shall be equivalent to 6.9 ± 0.2 when measured at room temperature.

D-1.8 Glucose and Peptone Added Lecithin- Polysorbate 80 Medium (GPLP 80 Broth)

D-1.8.1 *Composition*

a) Glucose	: 20.0 g
b) Yeast extract	: 2.0 g
c) Magnesium sulfate	: 0.5 g
d) Peptone	: 5.0 g
e) Potassium dihydrogen phosphate	: 1.0 g
f) Lecithin	: 1.0 g
g) Polysorbote 80	: 7.0 g
h) Water	: 1 000 ml

D-1.8.2 *Preparation*

Dissolve the components or the dehydrated complete medium successively in boiling water to complete dissolution. Dispense the medium into suitable containers. Sterilize in an autoclave at 121°C for 15 min. After sterilization and cooling of the solution, the *p*H shall be equivalent to 5.7 ± 0.2 when measured at room temperature.

D-1.9 Peptone Broth

D-1.9.1 *Composition*

a) Peptone	: 10.0 g
b) Sodium chloride	: 5.0 g
c) Distilled or deionized water	: 1 000 ml
d) Final *p*H (at 25°C)	: 7.2 ± 0.2

D-1.9.2 *Preparation*

Dissolve the components successively in water to complete dissolution. Dispense the medium into suitable containers. Sterilize in an autoclave at 121°C for 15 min. After sterilization and cooling of the solution, the *p*H shall be equivalent to 7.2 ± 0.2 when measured at room temperature.

D-1.10 Inactivating Diluent

D-1.10.1 *Composition*

a) Peptone	: 10.0 g
b) Tween 80	: 20.0 g
c) Distilled or deionized water	: 1 000 ml
d) *p*H	: 7.2 ± 0.2

For creams and lotions, add 20.0 g Isopropyl myristate for proper emulsification.

D-1.10.2 *Preparation*

Dissolve the components successively in water to complete dissolution. Dispense the medium into suitable containers. Sterilize in an autoclave at 121°C for 15 min. After sterilization and cooling of the solution, the *p*H shall be equivalent to 7.2 ± 0.2 when measured at room temperature.

D-1.11 TAT Broth Base

D-1.11.1 *Composition*

a) Casein enzymic hydrolysate	: 20.0 g
b) Azolectin	: 5.0 g
c) Distilled or deionized water	: 1 000 ml
d) *p*H	: 7.2 ± 0.2

D-1.11.2 *Preparation*

Dissolve the components successively in water to complete dissolution. Add 40ml of polysorbate 20 and heat if necessary. Dispense the medium into suitable containers. Sterilize in an autoclave at 121°C for 15 min. After sterilization and cooling of the solution, the *p*H shall be equivalent to 7.2 ± 0.2 when measured at room temperature.

D-2 CULTURE MEDIA

D-2.1 Soybean — Casein Digest Agar Medium (SCDA) or Tryptic Soy Agar (TSA)

D-2.1.1 *Composition*

a) Pancreatic digest of casein	: 15.0 g
b) Papaic digest of soybean meal	: 5.0 g

c) Sodium chloride	: 5.0 g
d) Agar	: 15.0 g
e) Water	: 1 000 ml

D-2.1.2 *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 *p*H shall be equivalent to 7.3 ± 0.2 when measured at room temperature.

D-2.2 Sabouraud Dextrose Agar (SDA)

D-2.2.1 *Composition*

a) Dextrose	: 40.0 g
b) Peptic digest of animal tissue	: 5.0 g
c) Pancreatic digest of casein	: 5.0 g
d) Agar	: 15.0 g
e) Water	: 1 000 ml

D-2.2.2 *Preparation*

Dissolve the components or the dehydrated complete medium in the water by heating. Dispense the medium into suitable containers. Sterilize in an autoclave at 121°C for 15 min. After sterilization the *p*H shall be equivalent to 5.6 ± 0.2 when measured at room temperature.

D-2.3 Sabouraud Dextrose Chloramphenicol Agar

D-2.3.1 *Composition*

a) Dextrose	: 40.0 g
b) Peptic digest of animal tissue	: 5.0 g
c) Pancreatic digest of casein	: 5.0 g
d) Chloramphenicol	: 0.050 g
e) Agar	: 15.0 g
f) Water	: 1 000 ml

D-2.3.2 *Preparation*

Dissolve the components (including the chloramphenicol) or the dehydrated complete medium in the water by mixing while heating. Dispense the medium into suitable containers. Sterilize in an autoclave at 121°C for 15 min. After sterilization the *p*H shall be equivalent to 5.6 ± 0.2 when measured at room temperature.

D-2.4 Corn Meal Agar with 1 Percent Polysorbate 80

D-2.4.1 *Composition*

a) Infusion from corn meal	: 50.0 g
b) Agar	: 15.0 g
c) Polysorbate 80	: 10.0 g
d) Water	: 1 000 ml

D-2.4.2 *Preparation*

Dissolve the components or the dehydrated complete medium in the water by mixing while heating. Dispense the medium into suitable containers. Sterilize in an autoclave at 121°C for 15 min. After sterilization the *p*H shall be equivalent to 6.0 ± 0.2 when measured at room temperature.

D-2.5 Potato Dextrose Agar Medium (PDA)

D-2.5.1 *Composition*

a) Potato extract	: 4.0 g
b) Dextrose	: 20.0 g
c) Agar	: 15.0 g
d) Water	: 1 000 ml

D-2.5.2 *Preparation*

Dissolve the components or the dehydrated complete medium in the water by mixing while heating. Dispense the medium into suitable containers. Sterilize in an autoclave at 121°C for 15 min. After sterilization and cooling of the solution, the *p*H shall be equivalent to 5.6 ± 0.2 when measured at room temperature.

D-2.6 Potato Dextrose Agar Medium with Antibiotics

D-2.6.1 *Composition*

a) Potato extract	: 4.0 g
b) Dextrose	: 20.0 g
c) Agar	: 15.0 g
d) Chloramphenicol	: 0.05 g
e) Water	: 1 000 ml

D-2.6.2 *Preparation*

Mix all the components and dispense the medium into suitable containers. Sterilize in the autoclave at 121°C for 15 min. After sterilization and cooling of the solution, the *p*H shall be equivalent to 5.6 ± 0.2 when measured at room temperature. Alternatively, chloramphenicol may be replaced

by use of 0.10 g of benzylpenicillin potassium and 0.10 g of tetracycline per litre of medium, added as a sterile solution just prior to use.

D-2.7 Levine Eosin-Methylene Blue Agar Medium

D – 2.7.1 Composition

a) Pancreatic digest of gelatin	: 10.0 g
b) Potassium dihydrogen phosphate (KH2PO4)	: 2.0 g
c) Agar	: 15.0 g
d) Lactose	: 10.0 g
e) Eosine Y	: 400 mg
f) Methylene blue	: 65 mg
g) Water	: 1 000 ml

D-2.7.2 *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 :

a) 5 ml of 20 percent lactose solution,
b) 2 ml of 2 percent eosin Y solution, and
c) 2 ml of 0.033 percent methylene blue solution.

The finished medium may not be clear. Dispense in suitable containers and sterilize at 121°C for 15 min. The *p*H, after sterilization and cooling down, shall be equivalent to 7.1 ± 0.2 when measured at room temperature.

D-2.8 MacConkey Agar Medium

D-2.8.1 *Composition*

a) Pancreatic digest of gelatin	: 17.0 g
b) Pancreatic digest of casein	: 1.5 g
c) Peptic digest of animal tissue	: 1.5 g
d) Lactose	: 10.0 g
e) Bile salts mixture	: 1.5 g
f) Sodium chloride	: 5.0 g
g) Agar	: 13.5 g
h) Neutral red	: 30.0 mg
j) Crystal violet	: 1.0 mg

k) Water	: 1 000 ml

D-2.8.2 *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 *p*H after sterilization and cooling down, shall be equivalent to 7.1 ± 0.2 when measured at room temperature.

D-2.9 Cetrimide Agar Medium

D-2.9.1 *Composition*

a) Pancreatic digest of gelatin	: 20.0 g
b) Magnesium chloride	: 1.4 g
c) Potassium sulfate	: 10.0 g
d) Cetrimide (cetyltrimethyl ammonium bromide)	: 0.3 g
e) Agar	: 13.6 g
f) Glycerin	: 10.0 ml
g) Water	: 1000 ml

D-2.9.2 *Preparation*

Dissolve all solid components in the water, and add the glycerin. 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 *p*H shall be equivalent to 7.2 ± 0.2 when measured at room temperature.

D-2.10 Pseudomonas Agar Medium for Detection of Pyocyanin (Pseudomonas Agar P)

D-2.10.1 *Composition*

a) Pancreatic digest of gelatin	: 20.0 g
b) Anhydrous magnesium chloride	: 1.4 g
c) Anhydrous potassium sulfate	: 10.0 g
d) Agar	: 15.0 g
e) Glycerin	: 10.0 ml
f) Water	: 1 000 ml

D-2.10.2 *Preparation*

Dissolve all solid components in the water, and add the glycerin. 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 *p*H shall be equivalent to 7.2 ± 0.2 when measured at room temperature.

D-2.11 Baird Parker Agar Medium

D-2.11.1 *Base Medium*

D-2.11.1.1 *Composition*

a) Pancreatic digest of casein : 10.0 g
b) Yeast extract : 1.0 g
c) Meat extract : 5.0 g
d) Sodium pyruvate : 10.0 g
e) L-glycine : 12.0 g
f) Lithium chloride : 5.0 g
g) Agar : 12 g to 22 g
h) Water to a final volume of : 950 ml

D-2.11.1.2 *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 *p*H shall be equivalent to 7.2 ± 0.2 when measured at room temperature.

D-2.11.2 *Potassium Tellurite Solution*

D-2.11.2.1 *Composition*

a) Potassium tellurite (K_2TeO_3) : 1.0 g
b) Water : 100 ml

D-2.11.2.2 *Preparation*

Dissolve the potassium tellurite completely in the water with minimal heating. Sterilize by filtration using 0.22 m pore size membranes. The solution may be stored at the maximum for one month at 3 ± 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.

D-2.11.3 *Egg-Yolk Emulsion (Concentration Approximately 20 percent 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 percent (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 ± 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 ± 2°C for a maximum of 72 h.

D-2.11.3.1 *Complete medium*

D-2.11.3.1.1 *Composition*

a) Base medium (*A*) : 100 ml
b) Potassium tellurite solution (*B*) : 1.0 ml
c) Egg-yolk emulsion (*C*) : 5.0 ml

D-2.11.3.1.2 *Preparation*

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

D-2.12 Mannitol-Salt Agar Medium (Chapman Agar)

D-2.12.1 *Composition*

a) Beef extract : 1.0 g
b) Pancreatic digest of casein : 5.0 g
c) Pancreatic digest of beef : 5.0 g
d) Sodium chloride : 75.0 g
e) D-mannitol : 10.0 g
f) Agar : 15.0 g
g) Phenol red : 0.025 g
h) Water : 1 000 ml

D-2.12.2 *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 *p*H shall be equivalent to 7.4 ± 0.2 when measured at room temperature.

D-2.13 Vogel-Johnson Agar Medium

D-2.13.1 *Composition*

a) Pancreatic digest of casein : 10.0 g
b) Yeast extract : 5.0 g
c) Mannitol : 10.0 g
d) Dibasic potassium phosphate : 5.0 g
e) Lithium chloride : 5.0 g
f) Glycine : 10.0 g

g) Agar : 16.0 g
h) Phenol red : 0.025 g
j) Water : 1 000 ml

D-2.13.2 *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 *p*H shall be equivalent to 7.2 ± 0.2 when measured at room temperature.

D-2.14 BiGGY Agar

D-2.14.1 *Composition*

a) Bismuth ammonium citrate : 5.0 g
b) Sodium sulphite : 3.0 g
c) Dextrose : 10.0 g
d) Glycine : 10.0 g
e) Yeast extract : 1.0 g
f) Agar : 16.0 g
g) *p*H : 6.8 ± 0.2

D-2.14.2 *Preparation*

Suspend 45 g of the powder in 1 litre of purified water. Mix thoroughly. Heat with frequent agitation and boil for not more than 1 min to completely dissolve the powder. DO NOT AUTOCLAVE. Cool to approximately 45-50°C. Swirl to disperse the insoluble material and pour into plates. Test samples of the finished product for performance using stable, typical control cultures.

NOTE — Composition of media described above are indicative; minor changes in media composition may occur depending on the manufacturer this is generally acceptable.

ANNEX E
(*Clauses* 6.4.6, 6.4.7, 6.5.4.2.2 *and* 6.7.4.2.2)
BASIC IDENTIFICATION TECHNIQUES

E-1 GRAM'S STAINING

E-1.1 General

This staining of bacterial cells allows description for the morphology of the bacteria and classification of them into two groups as a function of whether or not they are capable of retaining the violet stain of crystal violet under the test conditions. This division results mainly from differences in the structure of the cell walls of the two groups and it is correlated with other major

differences between the two groups. There are a number of ways to conduct a Gram's stain, but all follow the sequences given below.

E-1.2 Solutions

E-1.2.1 *General*

Commercially available solutions may be used. In this case, follow the manufacturer's recommendations.

E-1.3 Crystal Violet Solution (Composition)

a) Crystal violet : 2.0 g
b) Ethanol (95 percent) : 20 ml
c) Ammonium oxalate : 0.8 g
d) Water : 80 ml

E-1.3.1 *Preparation*

Dissolve the crystal violet in the ethanol and the ammonium oxalate in the distilled water. Mix the two solutions and allow the mixture to stand for 24 h prior to use.

E-1.4 Iodine Solution (Composition)

a) Iodine : 1.0 g
b) Potassium iodide (KI) : 2.0 g
c) Water : 100 ml

E-1.4.1 *Preparation*

Dissolve the potassium iodide in 10 ml of distilled water, add the iodine in fractions.

After dissolution, make up to 100 ml in a volumetric flask.

E-1.5 Safranin Solution (Composition)

a) Safranin O : 0.25 g
b) Ethanol (95 percent) : 10 ml
c) Water : 100 ml

E-1.5.1 *Preparation*

Dissolve the safranin in the ethanol then mix with the distilled water. Make up volumetrically to a final volume of 100 ml. When crystal violet is used, the stability of the solution should be verified. For the verification, mix one drop of the crystal violet solution with one drop of iodine solution on a glass slide to see a chemical reaction. If the crystallization is seen on the glass slide, do not use the crystal violet solution. Commercially available Gram stain kits can be used.

E-1.6 Staining Technique

After fixing (for example with a flame) the bacterial film on the microscope slide prepared from a culture 18 h to 24 h, or when the broth is turbid, cover the film with the crystal violet solution. Allow it to react for 1 min. Gently rinse the inclined slide with water for a few seconds. Cover the slide with the iodine solution. Allow it to react for 1 min. Gently rinse the inclined slide with water for a few seconds. Pour gently and continuously a film of ethanol (95 percent) onto the inclined slide over a period of no more than 30 s and until no more of the violet colour is emitted. Gently rinse the inclined slide with water in order to eliminate the ethanol. Cover the slide with the solution of safranin for 1min. Gently rinse the inclined slide with water. Dry the slide.

E-1.7 Interpretation

Examine the slide under the high-power objective (100X) of the microscope after application of oil on the smear. Those bacterial cells which appear blue or violet are termed Gram-positive; those which are coloured dark pink to red are termed Gram-negative.

E-1.7.1 *Test for Catalase*

E-1.7.1.1 *General*

The detection of this enzyme, which decomposes hydrogen peroxide (H_2O_2) into water and oxygen, can be carried out using a broth culture, an agar culture or one single colony on an agar medium.

E-1.7.1.2 *From a broth culture*

Add to 1 ml of the culture, 0.5 ml of a 10-volume [3 percent (mass fraction)] hydrogen peroxide solution. Observe the occurrence of oxygen bubbles (catalase positive) or absence (catalase negative).

E-1.7.1.3 *From an agar medium culture*

Cover the culture with 1 ml to 2 ml of a 10-volume [3 percent (mass fraction)] hydrogen peroxide solution. Observe immediately and after 5 min whether or not oxygen bubbles have formed.

E-1.7.1.4 *From a colony*

Place separately two drops of a 10-volume hydrogen peroxide solution on a microscope slide. Pick off a colony with a sterile glass or plastic rod (especially not a metallic wire) and gently emulsify it in one of the two drops. Observe immediately and over several minutes (at least 1 min) whether or not oxygen bubbles have formed. In the event of doubt, cover each of the drops with a cover slide and compare the occurrence of bubbles under both cover slides. The observation can be conducted macroscopically or using a low magnification microscope.

E-1.7.2 *Test for Oxidase*

E-1.7.2.1 *General*

The detection of oxidase is carried out by the change in colour of a compound at the time of oxidation under the action of this enzyme.

E-1.7.2.2 *Reagent (Composition)*

N,*N*,*N'*,*N'*-Tetramethyl-3-*p*- phenylenedia-amine dihydrochloride ($C_{10}H_{16}N_2.2HCl$)	: 1.0 g
Water	: 100 ml

E-1.7.2.3 *Preparation*

Dissolve the reagent in cold water. Prepare the reagent immediately prior to use. Commercially available disks or sticks may be used. In this case, follow the manufacturer's recommendations.

E-1.7.2.4 *Technique*

Moisten a piece of filter paper with the reagent. Take a sample of the bacterial culture obtained from an agar medium using a platinum wire or a glass or plastic rod (a nickel/chrome wire gives false positive) and deposit it on the moistened filter paper.

E-1.7.2.5 *Interpretation of the result*

In the case of the presence of oxidase, a violet to purple colour appears within a period of between 5 s and 10 s. If the colour has not changed within 10 s, the test is considered as being negative.

A number of new technologies that enable rapid testing and staining are available. Standard prevalidated products from reputed manufacturers may be used as alternative test method for some of the above test.

ANNEX F
(*Foreword*)
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