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	Enumeration of aerobic mesophilic bacteria, yeast and mould	2	04/10/2013	ACM 006

1. SCOPE AND FIELD OF APPLICATION

The method specifies the procedure for enumeration of aerobic mesophilic bacteria, yeast, and mould present in cosmetic products.

2. PRINCIPLE

Method for enumeration of microorganisms from cosmetic products is direct colony counts. Products that are not soluble in water are initially treated to render them miscible before enumeration procedures are conducted. The possible inhibition of microbial growth by the sample shall be neutralized to allow the detection of viable microorganism. In all cases and whatever the methodology, the neutralization of the antimicrobial properties of the product shall be checked and validated.

3. DILUENTS, NEUTRALIZERS, MEDIA, REAGENTS, APPARATUS AND STRAINS OF MICROORGANISME

3.1 Media and Reagents

The following culture media and diluents are suitable for enumeration of aerobic mesophilic bacteria, yeast, and mould. Other culture media and diluents may be used if they have been demonstrated to be suitable for use.

3.1.1 Neutralizing diluents, diluents and media for enumeration aerobic mesophilic bacteria

3.1.1.1 Neutralizing diluents (may be used as appropriate Annex A)

- Fluid casein digest - soy lecithin - polysorbate 20 medium (SCDLP 20 broth)
- Eugon LT100 liquid broth
- Modified Lethen broth

3.1.1.2 Diluent

- Fluid A
- Buffered peptone solution pH7
- Diluents for the bacterial suspension: Tryptone sodium chloride solution

3.1.1.3 Agar media for counting

- Soybean-casein digest agar medium (SCDA) or tryptic soy agar (TSA)
- Eugon LT 100 agar medium
- LT 100 Agar
- Agar added soybean casein digest medium (agar added SCD broth)

3.1.1.4 Agar medium for cultivation of reference strains

- Soybean-casein digest agar medium (SCDA) or tryptic soy agar (TSA)

3.1.2 Neutralizing diluents, diluents and media for enumeration yeast and Moulds

3.1.2.1 Neutralizing diluents (may be used as appropriate Annex A)

- Fluid casein digest - soy lecithin - polysorbate 20 medium (SCDLP 20 broth)
- Eugon LT100 liquid broth
- Lecithin polysorbate (LP) diluent

3.1.2.2 Diluent

- Fluid A
- Buffered peptone solution pH7
- Phosphate buffer (pH7.2)
- Diluents for yeast suspension: Tryptone sodium chloride solution

3.1.2.3 Agar media for counting

- Sabouraud dextrose chloramphenicol agar medium (SDCA)
- Potato dextrose agar medium with antibiotics
- Glucose-peptone (GP) agar medium with antibiotics
- Malt extract medium

3.1.2.4 Agar medium for cultivation of reference strain

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- Sabouraud dextrose agar medium (SDA)

3.1.3 Reagent

- Aqueous solution of 70% ethanol

3.2 Apparatus

- 3.2.1 Pipets, sterile, 1, 5, and 10 ml, graduated
- 3.2.2 Sterile instruments: forceps, scissors, scalpel and blades, spatulas, and microspatulas
- 3.2.3 Test tubes, screw-cap, 16 x 125, and 20 x 150 mm
- 3.2.4 Dilution bottles, screw-cap
- 3.2.5 Balance, sensitivity of 0.01 g
- 3.2.6 Petri dishes, sterile, plastic, 15 x 100 mm
- 3.2.7 Incubators, 25 ± 2.5°C and 32.5 ± 2.5°C
- 3.2.8 Laminar flow hood with HEPA filter (if available)

3.3 Strains of microorganisms

- 3.3.1 For testing the efficacy of neutralizers for bacterial enumeration, two strains representative of both Gram negative and Gram positive microorganisms, respectively are used :

- *Pseudomonas aeruginosa* ATCC 9027 (equivalent strain : CIP 82.118 or NCIMB 8626 or NBRC 13275 or KCTC 2513 or other equivalent national collection strain)
- *Staphylococcus aureus* ATCC 6538 (equivalent strain : CIP 4.83 or NCIMB 9518 or NBRC 13276 or KCTC 1916 or other equivalent national collection strain)

An alternative to the Gram negative strain may be: *Escherichia coli* ATCC 8739 (equivalent strain : CIP 53.126 or NCIMB 8545 or NBRC 3972 or KCTC 2571 or other equivalent national collection strain).

- 3.3.2 For testing the efficacy of neutralizers for yeast and mould enumeration, one yeast reference strain is used.

Candida albicans ATCC 10231 or equivalent strain (IP 48.72 or NCPF 3179 or NBRC 1594 or KCTC 17025 or TISTR 5779) or other equivalent national collection strain.

The selected yeast strain being considered more susceptible to antifungal activity than moulds may be accepted as representative of fungi (yeast and mould) for the validation of the methodology. However, in case of specific needs, the test for the efficacy of neutralizers may be performed with an additional mould reference strain, using a suitable protocol for the preparation of a calibrated inoculum.

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

4. SAMPLE HANDLING

- 4.1 Analyze samples as soon as possible after receiving them. If necessary, store samples to be tested at room temperature. Do not incubate, refrigerate, or freeze samples before or after analysis.
- 4.2 Inspect samples carefully before opening and note any irregularities of sample container.

5. PROCEDURE

5.1 General Recommendation

- 5.1.1 Use sterile material, equipment and aseptic techniques to prepare the sample, initial suspension and dilutions. In the case of the preparation of an initial suspension, the time which elapses between the end of the preparation and the moment the

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inoculum comes into contact with the culture medium shall not exceed 45 min, unless specifically mentioned in the established protocols or document.

- 5.1.2 Before opening and removing sample contents, disinfect surface of sample container with aqueous solution of 70% ethanol. Use laminar flow hood if possible. Leave the cleaned surface to dry before opening.
- 5.1.3 Weigh 10 g or mL of sample from representative portion of contents for microbial analysis.
- 5.1.4 For products weighing less than 1 g or mL, analyze entire contents. If only one sample unit is available and multiple analyses are requested (i.e., microbial, toxicological, and chemical), take sub-sample for microbiological examination before those for other analyses. In this situation, amount of sub-sample used for microbial analysis will depend on other analyses to be performed. For example, if total sample content is 5 mL, use 1 or 2 mL portion for microbial analyses.
- 5.1.5 The amount of sample and diluent given here can be adjusted according to amount of sample available. If sample has many sub-samples, amount of test material can be increased and workload streamlined by compositing. Analysts should use their best judgment as to when and how much material to composite.

5.2 Preparation of the initial suspension

5.2.1 General

The initial suspension is prepared from a sample of at least 1 g or 1 ml of the well – mixed product under test. The initial suspension is usually 1:10 dilution. Larger volumes of diluent 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.

5.2.2 Water - miscible products

Transfer the sample *S* of product to an appropriate volume (e.g. 9 ml) of neutralizing diluent or diluent.

5.2.3 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 (e.g. 9 ml) of neutralizing diluent or diluent.

5.3 Counting methods

Usually, the initial suspension is the first counted dilution. If needed, additional serial dilutions (e.g. 1:10 dilution) may be performed from the initial suspension using the same diluent (according to the expected level of contamination of the product).

Generally counting is performed using at least two Petri dishes. But it is possible to use only one Petri dish in case of routine testing, or if counts are performed on successive dilution of the same sample or according to previous results.

5.3.1 Pour-plate method

In Petri dishes 85 mm to 100 mm in diameter, add 1 ml of the initial suspension and/or sample dilution prepared as validated (see clause 5.4) and pour 15 ml to 20 ml of the melted agar medium (see 3.1.1.3, 3.1.2.3) kept in a water bath at no more than 48°C. If larger Petri dishes are used, the amount of agar medium is 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.

5.3.2 Surface spread method

In Petri dishes 85 mm to 100 mm in diameter, put 15 ml to 20 ml of the melted agar medium (see 3.1.1.3, 3.1.2.3) kept in a water bath at no more than

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48°C. If larger Petri dishes are used, the volume of the agar is increased accordingly.

Allow plates to cool and solidify, for example in a microbiological cabinet or in an incubator. Spread over the surface of the medium a measured volume of not less than 0.1 ml of the initial suspension and/or sample dilution prepared as validated (see clause 5.4).

5.3.3 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 prepared as validated (preferably representing at least 1 g or 1 ml of the product) on to the membrane. Filter immediately and wash the membrane (follow the procedure developed during the validation, (see clause 5.4). Transfer the membrane onto the surface of the agar medium (see 3.1.1.3, 3.1.2.3).

5.3.4 Incubation

For enumeration of bacteria : Unless otherwise stated, invert the inoculated dishes and place them in the incubator set at 32.5°C ± 2.5°C for 72 h ± 6 h. After incubation, the dishes shall, if possible, be examined immediately. Otherwise, they may be stored, unless otherwise specified, for up to a maximum of 24 h in the refrigerator.

For enumeration of yeast and mould : Unless otherwise stated invert the inoculated dishes and place them in the incubator set at 25°C ± 2.5°C for 3 d to 5 d or use the alternative condition (An alternative condition for incubation is 22.5°C ± 2.5°C for 5 d to 7 d using the culture medium without antibiotic). After incubation, the dishes shall, if possible, be examined immediately. Alternatively, they may be stored, unless otherwise specified, for up to a maximum of 24 h in the refrigerator at 5°C ± 3°C.

Note 1 In certain cases where there is a potential for confusing particles from the product with counted colonies, it can be useful to prepare duplicate dishes containing the same sample dilutions and agar medium which are stored in the refrigerator for comparison with incubated dishes.

Note 2 An intermediate check can be performed where both yeast and mould are suspected.

5.4 Counting of colonies (plate counts and membrane filtration methods)

After incubation, count the colonies :

For enumeration of bacteria :

- in petri dishes containing 30 colonies to 300 colonies
- on membranes containing 15 colonies to 150 colonies

For enumeration of yeast and mould :

- In Petri dishes containing 15 colonies to 150 colonies
- On membranes containing 15 colonies to 150 colonies

5.5 Expression of results

Method of calculation for plate count (see Annex B)

5.6 Neutralization of the antimicrobial properties of the Cosmetic product

5.6.1 General

The different tests described below demonstrate that the microorganisms can grow under the conditions of analysis.

The three strains (see clause 3.3) used to demonstrate the validity of these properties are generally sensitive to antimicrobial agents.

5.6.2 Preparation of inoculum

Prior to the test, and for each strain, inoculate the surface of soybean casein digest agar (SCDA) for bacteria and sabouraud dextrose agar (SDA) with *Candida*

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albicans or other suitable (non-selective, non-neutralizing) medium. Incubate at $32.5^{\circ}\text{C} \pm 2.5^{\circ}\text{C}$ for 18 h to 24 h. To harvest the culture, use a sterile loop, streak the surface of the culture and re-suspend into the diluent to obtain a calibrated suspension of about 1×10^8 CFU/ml (bacteria) or about 1×10^6 CFU/ml (*C. albicans*) (e.g. using a spectrophotometer). Use this suspension and its dilutions within 2 h.

5.6.3 Validation of counting methods

5.6.3.1 Principle

For each strain, mix the neutralized sample (initial suspension or sample dilution according to the antimicrobial activity or the low solubility of the product) with a dilution of the reference strain. Plate on a Petri dish or filter on membrane. After incubation, check the nature of the colonies and compare the count with a control (without the sample).

If the count is less than 50% (0.3 log) of the control, modify the procedure (diluent, neutralization agents or combination of both, see Annex A). 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 in log the difference exceeds 0.3. Failure of the inoculum to grow invalidates the test unless it is possible to consider that contamination of the product with this microorganism is unlikely.

5.6.3.2 Validation of the pour-plate method

Mix 9 ml of the initial suspension and / or the sample dilution(s) in neutralizing diluent (see 3.1.1.1, 3.1.2.1) with 1 ml of a suspension of microorganisms containing 1,000 CFU/ml to 3,000 CFU/ml. Transfer 1 ml in a Petri dish (preferably in duplicate) and pour 15 ml to 20 ml of the melted agar medium (see 3.1.1.3, 3.1.2.3) kept in a water bath at no more than 48°C . In parallel, prepare and plate a control using the same diluent and the same suspension of microorganisms but without the sample.

After incubation for 24 h to 72 h at $32.5^{\circ}\text{C} \pm 2.5^{\circ}\text{C}$ (bacteria) or after incubation for 3 d to 5 d at $25^{\circ}\text{C} \pm 2.5^{\circ}\text{C}$ (*C. albicans*), count the colonies on the plates and compare the counts obtained for the test and of the control. The diluent and the counting method are validated at the 1:10 dilution (when 1 ml of the initial suspension is used) if the validation count is at least 50% (0.3 log) of the control count.

5.6.3.3 Validation of the surface spread method

Mix 9 ml of the initial suspension in neutralizing diluent (see 3.1.1.1, 3.1.2.1) with 1 ml of a suspension of microorganisms containing 10,000 CFU/ml to 30,000 CFU/ml. (or less if 0.5 ml or 1 ml are spread). Spread at least 0.1 ml on a solidified agar plate (preferably in duplicate). In parallel, prepare and plate a control using the same diluent and the same suspension of microorganisms but without the sample.

After incubation for 24 h to 72 h at $32.5^{\circ}\text{C} \pm 2.5^{\circ}\text{C}$ (bacteria) or after incubation for 3 d to 5 d at $25^{\circ}\text{C} \pm 2.5^{\circ}\text{C}$ (*C. albicans*), count the colonies on the plates and compare the counts obtained for the test and of the control. The diluent and the counting method are validated at the 1:10 dilution (when 1 ml of the initial suspension is used) if the validation count is at least 50% (0.3 log) of the control count.

5.6.3.4 Validation of the membrane filtration method

Mix to the volume of initial suspension or of the sample dilution used in the test a suitable amount of a calibrated suspension of microorganisms corresponding to approximately 100 CFU.

Filter immediately the entire volume and wash the membrane using defined volumes of water, diluent or neutralizing diluent. Transfer the membrane on to the surface of a suitable agar medium.

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In parallel, prepare a control under the same conditions as above, but without the product. Filter and wash the control under the same conditions.

After incubation for 24 h to 72 h at $32.5^{\circ}\text{C} \pm 2.5^{\circ}\text{C}$ (bacteria) or after incubation for 3 d to 5 d at $25^{\circ}\text{C} \pm 2.5^{\circ}\text{C}$ (*C. albicans*), count the colonies on the membranes and compare the counts obtained for the test and of the control. The membrane filtration method and the diluent are validated if the validation count is at least 50% (0.3 log) of the control count.

6. REMARKS

Cosmetic products are not expected to be aseptic. However, they must be completely free of high virulence microbial pathogens, and the total number of aerobic microorganisms per gram must be below. The microorganisms which may be specified as pathogenic differ from country to country according to the national practices or regulations. Pathogens or opportunistic pathogens whose incidence would be of particular concern, especially in eye-area cosmetic products, include *S. aureus*, *Streptococcus pyogenes*, *P. aeruginosa* and other species, and *Klebsiella pneumoniae*. Some microbes normally regarded as nonpathogenic may be opportunistically pathogenic.

7. REFERENCES

- 7.1 ISO 21149. 2006. Cosmetics – Microbiology – Enumeration and detection of aerobic mesophilic bacteria.
- 7.2 ISO 16212. 2008. Cosmetics – Microbiology – Enumeration of yeast and mould.
- 7.3 Singer, S. 1987. The use of preservative neutralizers in diluents and plating media, Cosmetics and Toiletries, 102, 55.

Harmonised method :

- Issued by the microbial analysis group at the harmonization workshop in Kuala-Lumpur, on September 13th to 17th, 2004
- Approved by the harmonization workshop delegates workshop in Kuala-Lumpur, on September 13th to 17th, 2004, Modified after the Bangkok training, Nov 29th to Dec 3rd, 2004
- Modified (format only) in Brunei workshop Aug 30th to 31st, 2005
- Modified and approved after the final review in Singapore, Nov 30th to Dec 2nd, 2005
- Modified and approved after the Malaysia workshop, Jul 10th to 12th, 2006
- Modified and approved after The 2nd Meeting of the ASEAN Cosmetic Testing Laboratory Committee (ACTLC). 22 May 2013. Vientiane, Lao PDR

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ANNEX A
(Informative)

Table A1. Neutralizers of antimicrobial activity of preservatives and rinsing liquids (ref. 7.1)

Preservative	Chemical compounds able to neutralize preservative's antimicrobial activity	Examples of suitable neutralizers and rinsing 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 <i>Rinsing liquid</i> : 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 <i>Rinsing liquid</i> : 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 <i>Rinsing liquid</i> : polysorbate 80, 3 g/l + L- histidine, 0.5 g/l.
Oxidizing compounds	Sodium thiosulfate	Sodium thiosulphate, 5 g/l. <i>Rinsing liquid</i> : 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. <i>Rinsing liquid</i> : 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. <i>Rinsing liquid</i> : tryptone, 1 g/l +NaCl, 9 g/l; polysorbate 80, 5 g/l.
Metallic salts (Cu, Zn, Hg) Organo-mercuric compounds	Sodium bisulfate, 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.

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	D/E neutralizing broth ^a <i>Rinsing liquid: sodium thioglycollate, 0.5 g/l.</i>
^a D/E neutralizing broth (Dey/Engley neutralizing broth)	

ANNEX B Method of calculation for plate count

Calculate the number N of microorganisms present in the sample S , using:

m , the arithmetic mean of the counts obtained from the duplicates in Equation (1)

c , the number of colonies counted on a single plate in Equation (2), or

\bar{x}_c , the weighted mean of the counts obtained from two successive dilutions in Equation (3),

according to the following equations:

$$N = m / (V \times d) \quad (1)$$

$$N = c / (V \times d) \quad (2)$$

$$N = \bar{x}_c / (V \times d) \quad (3)$$

where

m is the arithmetic mean of the counts obtained from the duplicates;

V is the volume of inoculum applied to each dish, in millilitre;

d is the dilution factor corresponding to the dilution made for the preparation of the initial suspension or for the first counted dilution;

c is the number of colonies counted on a single plate;

\bar{x}_c is the weighted mean of the colonies counted from two successive dilutions and is calculated as follows:

$$\bar{x}_c = \frac{\sum C}{(n_1 + 0,1 n_2)}$$

where

$\sum C$ is the sum of colonies counted on all the dishes retained from two successive dilutions;

n_1 is the number of dishes counted for the initial suspension (or for the first counted dilution);

n_2 is the number of dishes counted for the 1/10 dilution of the initial suspension (or for the second counted dilution).

Round off the result calculated to two significant figures. For this, if the last figure is below 5, the preceding figure is not modified; if the last figure is 5 or more, the preceding figure is increased by one unit. Proceed stepwise until two significant figures are obtained. Note the number N obtained.

1. Interpretation

1.1 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 in log, the difference exceeds 0,3.

For a count to be precise, only plates with more than 30 colonies and less than 300 colonies and membranes with more than 15 colonies and less than 150 colonies should be taken into account. Check that the counts are obtained from dilutions validated according to the chosen method (see Clause 5.6).

1.2 Where the number of CFU is more than 30 and less than 300 on plates or more than 15 and less than 150 on membranes, where S is the mass or the volume of the sample, 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 .

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Express the result as a number between 1,0 and 9,9 multiplied by the appropriate power of 10 (see Example 1, Example 2, Example 3 and Example 7).

1.3 Where the number of CFU is less than 30 on plates or 15 on membranes, express the result as follows:

- if S is at least 1 g or 1 ml, and V is at least 1 ml:
the estimated number of aerobic mesophilic bacteria per millilitre or per gram of the sample is = N/S ;
- if S is less than 1 g or 1 ml, and/or V is less than 1 ml:
the estimated number of aerobic mesophilic bacteria in the sample is = N .

Where S is the mass or the volume of the sample.

Express the result as a number between 1,0 and 9,9 multiplied by the appropriate power of 10 (see Example 4, Example 5 and Example 6).

1.4 Where no colony is observed, the result is reported as follows:

- less than $1/d \times V \times S$ of aerobic mesophilic bacteria per gram or millilitre of the product (S is at least 1 g or 1 ml);
- less than $1/d \times V$ of aerobic mesophilic bacteria in the sample S (note the tested quantity of sample taking into account S and V) (S is less than 1 g or 1 ml);

where d is the dilution factor of the initial suspension and V is 1 (for counting with the pour-plate method and for membrane filtration) or 0.1 (for the spread plate method) (see Example 8).

2. Examples

EXAMPLE 1 Two dishes for one dilution

$S = 1$ g or 1 ml; $V = 1$; counts obtained: for the dilution 10^{-1} , 38 and 42.

For Equation (1):

$N = m/(V \times d) = 40/(1 \times 10^{-1}) = 40/0,1 = 400$ or 4×10^2 aerobic mesophilic bacteria per millilitre or per gram of the sample.

EXAMPLE 2 One dish for one dilution

$S = 1$ g or 1 ml; $V = 1$; count obtained: for the dilution 10^{-1} : 60.

For Equation (2):

$N = c/(V \times d) = 60/(1 \times 10^{-1}) = 60/0,1 = 600$ or 6×10^2 aerobic mesophilic bacteria per millilitre or per gram of the sample.

EXAMPLE 3 Two dishes for two dilutions

$S = 1$ g or 1 ml; $V = 1$; counts obtained: for the dilution 10^{-2} , 235 and 282; for the dilution 10^{-3} , 31 and 39.

For Equation (3):

$N = \frac{x_c}{d} / (V \times d) = 235 + 282 + 31 + 39 / (1 \times 2 + 0,1 \times 2) \times 10^{-2} = 587 / 0,022 = 26\ 682$.

Rounding the result as specified above gives 27 000 or $2,7 \times 10^4$ aerobic mesophilic bacteria per millilitre or per gram of the sample.

EXAMPLE 4 Two membrane filters for one dilution

$S = 1$ g or 1 ml; $V = 1$; counts obtained: for the dilution 10^{-1} , 18 and 22.

For Equation (1):

$N = m/(V \times d) = 20/(1 \times 10^0) = 20/0,1 = 200$ or 2×10^2 aerobic mesophilic bacteria per millilitre or per gram of the sample.

EXAMPLE 5 One membrane filter for one dilution

$S = 1$ g or 1 ml; $V = 1$; count obtained: for the dilution 10^{-1} , 65.

For Equation (2):

$N = c/(V \times d) = 65/(1 \times 10^{-1}) = 65/0,1 = 650$ or $6,5 \times 10^2$ aerobic mesophilic bacteria per millilitre or per gram of the sample.

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EXAMPLE 6 Two membrane filters for two dilutions

S = 1 g or 1 ml; V = 1; counts obtained: for the dilution 10^{-1} , 121 and 105; for the dilution 10^{-2} , 15 and 25.

For Equation (3):

$$N = \frac{xc}{(V \times d)} = \frac{121 + 105 + 15 + 25/1}{(2 + 0,1 \times 2)} \times 10^{-1} = \frac{266}{0,22} = 1\ 209.$$

Rounding the result as specified above gives 1 200 or $1,2 \times 10^3$ aerobic mesophilic bacteria per millilitre or per gram of the sample.

EXAMPLE 7 Two dishes for one dilution

S = 1 g or 1 ml; V = 1; counts obtained for the dilution 10^{-1} , 28 and 22.

For Equation (1):

$$N = \frac{m}{(V \times d)} = \frac{25}{(1 \times 10^{-1})} = 25/0,1 = 250.$$

The estimated number is 250 or $2,5 \times 10^2$ aerobic mesophilic bacteria per millilitre or per gram of the sample.

Example 8

S = 1 g or 1 ml; V = 1; counts obtained for the dilution 10^{-1} , 0 and 0.

For Equation (1):

$$\begin{aligned} N &\leq \frac{1}{(V \times d)}, \\ &\leq \frac{1}{(1 \times 10^{-1})}, \\ &\leq 1/0,1, \\ &\leq 10. \end{aligned}$$

The estimated number is less than 10 aerobic mesophilic bacteria per millilitre or per gram of the sample.

Example 9

S = 1 g or 1 ml; V = 1; counts obtained for dilution 10^{-1} , 0 and 3.

For Equation (1):

$$\begin{aligned} N &\leq \frac{m}{(V \times d)}, \\ &\leq \frac{1,5}{(1 \times 10^{-1})}, \\ &\leq 1,5/0,1, \\ &\leq 15. \end{aligned}$$

The estimated number is less than 15 mesophilic bacteria per millilitre or per gram of the sample.

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ANNEX C

Diluents, Neutralizer and culture media

1. Neutralizing diluents

Fluid casein digest – soy lecithin – polysorbate 20 medium (SCDLP 20 broth)

pancreatic digest of casein	20	g
soy lecithin	5	g
polysorbate 20	40	g
water	960	ml

Dissolve the polysorbate 20 in 960 ml of water by mixing while heating in a water bath at $49^{\circ}\text{C} \pm 2^{\circ}\text{C}$. 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 pH shall be equivalent to 7.3 ± 0.2 when measured at room temperature.

Lethen Broth (Modified)

letheen broth	25.7	g
trypticase peptone	5	g
thiotone peptone	10	g
yeast extract	2	g
sodium bisulfite	0.1	g
distilled water	1	liter

Dispense 90 mL into screw-cap bottle. Autoclave 15 min at 121°C . Final pH, 7.2 ± 0.2 .

2. Diluent

Fluid A

peptic digest of animal tissue	10	g
water	1000	ml

Dissolve 1 g of peptone in water to make 1l. Heat with frequent agitation. Dispense into suitable containers. Sterilize in the autoclave at 121°C for 15 min. After sterilization the pH shall be equivalent to 7.1 ± 0.2 when measured at room temperature.

Tryptone sodium chloride solution

tryptone, pancreatic digest of casein	1	g
sodium chloride	8.5	g
water	1000	ml

Dissolve the components in the water by mixing while heating. Dispense into suitable containers. Sterilize in the autoclave at 121°C for 15 min. After sterilization the pH shall be equivalent to 7.0 ± 0.2 when measured at room temperature.

3. Culture media for counting

Soybean - casein digest agar medium (SCDA) or tryptic soy agar (TSA)

Pancreatic digest of casein	15	g
Papaic digest of soybean meal	5	g
Sodium chloride	5	g
Agar	15	g
water	1000	ml

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.

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Sabouraud dextrose chloramphenicol agar medium (SDCA)

dextrose	40	g
peptic digest of animal tissue	5	g
pancreatic digest of casein	5	g
chloramphenicol	0.05	g
agar	15	g
water	1000	ml

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 pH shall be equivalent to 5.6 ± 0.2 when measured at room temperature.

Potato dextrose agar medium with antibiotics

potato extract	4	g
glucose	20	g
agar	15	g
chloramphenicol	0.05	g
water	1000	ml

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, the pH 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 sterile solution just prior to use.

Glucose – peptone (GP) agar medium with antibiotics

glucose	20	g
yeast extract	2	g
magnesium sulfate	0.5	g
peptone	5	g
monobasic potassium phosphate	1	g
agar	15	g
chloramphenicol	0.05	g
water	1000	ml

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, the pH shall be equivalent to 5.7 ± 0.1 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 sterile solution just prior to use.

Malt extract medium

malt extract	30	g
soya peptone, papaic digest of soybean meal	3	g
agar	15	g
chloramphenicol	0.05	g
water	1000	ml

Dissolve the components (including the chloramphenicol) or the dehydrated complete medium in the water by heating. Dispense the medium into suitable containers. Sterilize in an autoclave at 115°C for 10 min. After sterilization the pH shall be equivalent to 5.6 ± 0.2 when measured at room temperature.