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Surface active agents — Microbiology — Microbiological test methods for liquid hand dishwashing

Agents de surface — Microbiologie — Méthodes d'essai microbiologique pour les détergents liquides de lavage de vaisselle à la main

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Foreword

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This document was prepared by Technical Committee ISO/TC 91, *Surface active agents*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Surface active agents — Microbiology — Microbiological test methods for liquid hand dishwashing

1 Scope

This document provides microbiological test methods for enumeration and detection of aerobic mesophilic bacteria, detection of *Escherichia coli* and *Pseudomonas aeruginosa* in liquid hand dishwashing.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies

ISO 21148, *Cosmetics — Microbiology — General instructions for microbiological examination*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
liquid hand dishwashing
liquid detergent which is used for dishwashing by hand

3.2
product
portion of an identified liquid hand dishwashing product received in the laboratory for testing

3.3
sample
portion of the product (at least 1 g or 1 ml) which is used in the test to prepare the initial suspension

3.4
initial suspension
suspension (or solution) of the sample in a defined volume of an appropriate enrichment broth

3.5
sample dilution
dilution of the initial suspension

3.6
aerobic mesophilic bacteria
mesophilic bacteria growing aerobically under the conditions specified in this document

3.7
Pseudomonas aeruginosa
gram-negative rod (bacilli), motile; smooth colonies pigmented (light brown or greenish)

Note 1 to entry: 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.

3.8
Escherichia coli
gram-negative rod (bacilli), motile, smooth colonies

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

3.9
enrichment broth
non-selective liquid medium containing suitable neutralizers and/or dispersing agents and validated for the product under test

4 Principle

This document provides enumeration and detection of aerobic mesophilic bacteria on a non-selective agar medium and enrichment medium, detection of *Escherichia coli* and *Pseudomonas aeruginosa* by presence or absence of bacterial growth after enrichment.

Microorganisms to be tested might be different from country to country according to the practices or regulations. Users can choose enumeration and/or detection methods for those microorganisms which are mentioned in this document based on their needs.

In order to ensure product quality and safety for consumers, it is advisable to perform appropriate microbiological risk analysis so as to determine the type of the product to which this document is applicable. For example, products considered to present a low microbiological risk include those with low water activity, those with extreme pH values, etc. (see ISO 29621).

Alternative microbiological methods may be substituted for the tests presented here provided that their equivalence has been demonstrated or the method has been otherwise validated.

The possible inhibition of microbial growth by the sample shall be neutralized to allow the detection of viable microorganism. In all cases the neutralization of the antimicrobial properties of the product shall be checked.

5 Diluents and culture media

5.1 General

General recommendations for microbiological examinations are specified in ISO 21148.

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

5.2 Neutralizing diluent and diluents

5.2.1 General

The diluent is used to disperse the sample. It may contain neutralizers if the specimen to be tested has antimicrobial properties. The efficacy of the neutralization shall be demonstrated before the

determination or detection of the count. Information related to the suitable neutralizers is given in Annex A.

5.2.2 Neutralizing diluent: Fluid casein digest — soy lecithin — polysorbate 20 medium (SCDLP 20 broth)

5.2.2.1 Composition

Pancreatic digest of casein,	20,0 g
Soy lecithin,	5,0 g
Polysorbate 20,	40,0 ml
Water,	1 000 ml

5.2.3 Diluent for the bacterial suspension (Tryptone sodium chloride solution)

5.2.3.1 Composition

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

5.2.3.2 Preparation

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.

5.3 Culture medium for counting

5.3.1 General

Culture media may be prepared as follows, or from dehydrated culture media according to the instructions of the manufacturer. Ready-to-use media may be used when their composition and/or growth yields are comparable to those of the formulae given herein.

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

5.3.2.1 Composition

Pancreatic digest of casein,	15,0 g
Papaic digest of soybean meal,	5,0 g
Sodium chloride,	5,0 g
Agar,	15,0 g
Water,	1 000 ml

5.3.2.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by mixing while heating. Dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization and cooling down, the pH shall be equivalent to 7,3 ± 0,2 when measured at room temperature.

5.3.3 Other medium for counting

5.3.3.1 Eugon LT 100 agar medium

5.3.3.2 Composition

Pancreatic digest of casein,	15,0 g
Papaic digest of soybean meal,	5,0 g
L-cystine,	0,7 g
Sodium chloride,	4,0 g
Sodium sulphite,	0,2 g
Glucose,	5,5 g
Egg lecithin,	1,0 g
Polysorbate 80,	5,0 g
Octoxynol 9,	1,0 g
Agar,	15,0 g
Water,	1 000 ml

5.3.3.3 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. Mix gently to avoid foam. Dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization and cooling down, the pH shall be equivalent to 7,0 ± 0,2 when measured at room temperature.

5.4 Culture medium for detection

5.4.1 General

An enrichment broth shall be used for bacterial detection.

The enrichment broth is used to disperse the sample and to increase the initial microbial population. It may contain neutralizers if the specimen to be tested has antimicrobial properties.

5.4.2 Enrichment broth: Eugon LT 100 broth

5.4.2.1 Eugon LT 100 broth

5.4.2.2 General

This medium contains ingredients which neutralize inhibitory substances present in the sample: lecithin and polysorbate 80, dispersing agent: octoxynol 9.

5.4.2.3 Composition

Pancreatic digest of casein,	15,0 g
Papaic digest of soybean meal,	5,0 g
L-cystine,	0,7 g
Sodium chloride,	4,0 g
Sodium sulfite,	0,2 g
Glucose,	5,5 g
Egg lecithin,	1,0 g
Polysorbate 80,	5,0 g
Octoxynol 9,	1,0 g
Water,	1 000 ml

5.4.2.4 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 pH shall be equivalent to 7,0 ± 0,2 when measured at room temperature.

5.5 Culture media for isolation and identification

5.5.1 MacConkey agar medium

5.5.1.1 Composition

Pancreatic digest of gelatin,	17,0 g
Pancreatic digest of casein,	1,5 g
Peptic digest of animal tissue,	1,5 g
Lactose,	10,0 g
Bile salts mixture,	1,5 g
Sodium chloride,	5,0 g
Agar,	13,5 g

Neutral red,	30,0 mg
Crystal violet,	1,0 mg
Water,	1 000 ml

5.5.1.2 Preparation

Dissolve all solid components in the water and boil for 1 min to effect solution. Dispense in suitable containers and sterilize at 121 °C for 15 min. The pH, after sterilization and cooling down, shall be equivalent to 7,1 ± 0,2 when measured at room temperature.

5.5.2 Levine eosin-methylene blue agar medium (EMB agar medium)

5.5.2.1 Composition

Pancreatic digest of gelatin,	10,0 g
Potassium dihydrogen phosphate (KH_2PO_4),	2,0 g
Agar,	15,0 g
Lactose,	10,0 g
Eosine Y,	400 mg
Methylene blue,	65 mg
Water,	1 000 ml

5.5.2.2 Preparation

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

— 5 ml of 20 % lactose solution,

— 2 ml of 2 % eosin Y solution, and

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

The finished medium may not be clear. Dispense in suitable containers and sterilize at 121 °C for 15 min.

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

5.5.3 Cetrimide agar medium

5.5.3.1 Composition

Pancreatic digest of gelatin,	20,0 g
Magnesium chloride,	1,4 g
Potassium sulfate,	10,0 g

Cetrimide (cetyltrimethylammonium bromide),	0,3 g
Agar,	13,6 g
Glycerin,	10,0 ml
Water,	1 000 ml

5.5.3.2 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 pH shall be equivalent to 7,2 ± 0,2 when measured at room temperature.

5.5.4 Pseudomonas agar medium for detection of pyocyanin (Pseudomonas agar P)

5.5.4.1 Composition

Pancreatic digest of gelatin,	20,0 g
Anhydrous magnesium chloride,	1,4 g
Anhydrous potassium sulfate,	10,0 g
Agar,	15,0 g
Glycerin,	10,0 ml
Water,	1 000 ml

5.5.4.2 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 pH shall be equivalent to 7,2 ± 0,2 when measured at room temperature.

6 Apparatus and glassware

The laboratory equipment, apparatus and glassware specified in ISO 21148.

7 Strains of microorganisms

For testing the efficacy of neutralizers, the representative of Gram negative is used. *Pseudomonas aeruginosa* ATCC (American Type Culture Collection) 9027 or *Escherichia coli* ATCC 8739[1)] or equivalent strain may be used.

1) ATCC®9027 and ATCC®8739 are a trademark of American Type Culture Collection. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

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

8 Handling of the product and laboratory samples

If necessary, store products to be tested at room temperature. Do not incubate, refrigerate or freeze products and samples before or after analysis. Sampling of products should be carried out, as described in ISO 21148. Analyse samples as specified in ISO 21148 and in accordance with the following procedure.

9 Procedure

9.1 General recommendation

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

In the case of the preparation of an initial suspension, the time which elapses between the end of the preparation and the moment the inoculum comes into contact with the culture medium shall not exceed 45 min, unless specifically mentioned in the established protocols or documents.

9.2 Preparation of the initial suspension

The initial suspension is prepared by adding at least 1 g or 1 ml of the well-mixed product to at least 9 ml of neutralizing diluents (5.2.2) or diluent (5.2.3).

The initial suspension is usually 1:10 dilution. More dilutions may be required if high levels of contamination are expected and/or if anti-microbial properties are still present in 1:10 dilution.

Avoid the development of foam as it might impact the final results.

9.3 Enumeration methods

9.3.1 Dilutions for enumeration

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 dilutions of the same sample or according to previous results.

9.3.2 Plate-count methods

9.3.2.1 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 and pour 15 ml to 20 ml of the melted agar medium (5.3.2) kept in a water bath at no more than 48 °C. 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 the plates sufficiently to disperse them. Allow the mixture in the Petri dishes to solidify on a horizontal surface at room temperature.

9.3.2.2 Surface spread method

In Petri dishes 85 mm to 100 mm in diameter, put 15 ml to 20 ml of the melted agar medium (5.3.2) kept in a water bath at no more than 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 described in Clause 11.

9.3.2.3 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 described in Clause 11 (preferably representing at least 1 g or 1 ml of the product) onto the membrane. Filter immediately and wash the membrane (follow the suitability test procedure, see Clause 11). Transfer the membrane onto the surface of the agar medium (5.3.2).

9.3.2.4 Incubation

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 be examined immediately, if possible. Otherwise, they may be stored, unless otherwise specified, for up to a maximum of 24 h in the refrigerator.

NOTE 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.

9.3.2.5 Expression of results

Calculate the number N of microorganisms present in the sample according to the following formulae:

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

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

$$N = wm/(V \times d) \quad (3)$$

where

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

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

d is the dilution factor corresponding to the dilution made for the preparation of the initial suspension (9.2) or for the first counted dilution;

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

wm is the weighted mean of the colonies counted from two successive dilutions with $wm = \Sigma c/(n_1 + 0{,}1\ n_2)$;

Σc 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.

9.3.2.6 Interpretation

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 log.

For a count to be precise, only plates with more than 30 colonies and less than 300 colonies should be taken into account. Check that the counts are obtained from dilutions validated according to the chosen method (see Clause 11).

Where the number of CFU is more than 30 and less than 300 on plates or more than 15, 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 is = 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 is = N.

Where S is the mass or the volume of the sample (9.2).

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

Where the number of CFU is less than 30 on plates, 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 (9.2).

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

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 ml);

— less than $1/d \times V$ of aerobic mesophilic bacteria in the sample (S is less than 1 g or ml).

Where d is the dilution factor of the initial suspension (9.2) and V is 1 (for counting with the pour-plate method).

EXAMPLE 1 Two dishes for one dilution

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

(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; V = 1; count obtained: for the dilution 10^{-1}: 60

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

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

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.

10 Method for detection and identification

10.1 Enrichment

Add at least 1 g or 1 ml of the well-mixed product to at least 9 ml of enrichment culture media (5.3.3) and incubate it at 32,5 °C ± 2,5 °C for at least 20 h.

10.2 Isolation

Using a sterile pipette, transfer 0,1 ml to 0,5 ml of the incubated suspension on the surface of a Petri dish (diameter 85 mm to 100 mm) containing approximately 15 ml to 20 ml of Soybean-casein digest agar medium (SCDA) or tryptic soy agar (TSA) for aerobic mesophilic bacteria, MacConkey agar medium for *Eschericha coli* and Cetrimide agar medium for *Pseudomonas aeruginosa* in order to obtain isolated colonies

Do not invert the inoculated plates (or wait for the absorption of the incubated suspension by the agar before inverting) and incubate at 32,5 °C ± 2,5 °C for 48 h to 72 h.

10.3 Detection of aerobic mesophilic bacteria

After incubation, check the agar surface and record the presence or absence of growth and ay aerobic mesophilic bacteria after 72 h. Express the results “presence/absence of aerobic mesophilic bacteria per sample S of product".

10.4 Detection and Identification of *Escherichia coli*

10.4.1 General

Check for characteristic colonies on the incubated plate of MacConkey agar (10.2), see Table 1.

Table 1 — Morphologic characteristics of *Escherichia coli* on MacConkey agar medium

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

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

10.4.2 Gram’s stain

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

10.4.3 Culture on Levine eosin-methylene blue agar medium (EMB agar medium)

Inoculate the surface of the Levine eosin-methylene blue agar medium with suspect isolated colonies grown on MacConkey agar medium, so that isolated colonies develop. Invert the Petri dish and then incubate at 32,5 °C ± 2,5 °C for at least 24 h (maximum 48 h).

Check for characteristic colonies as specified in Table 2.

Table 2 — Morphologic characteristics of *Escherichia coli* on Levine eosin-methylene blue agar medium

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

10.4.4 Expression of results

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

Presence of *Escherichia coli* in the sample, *S*.

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

10.5 Detection and identification of *Pseudomonas aeruginosa*

10.5.1 General

Check the characteristic colonies on the incubated plate of Cetrimide agar medium 10.2 (see Table 3)

Table 3 — Morphological characteristics of *Pseudomonas aeruginosa* on selective medium

Selective medium	Characteristic colonial morphology of *Pseudomonas aeruginosa*
Cetrimide agar medium	Yellow-green pigment (pyocyanin), which fluoresces under UV light.

Proceed the following tests for the suspected isolated colonies on Cetrimide agar medium.

10.5.2 Gram's stain

This test is described in ISO 21148. Check for Gram negative rod.

10.5.3 Oxidase test

This test is described in ISO 21148. Check for oxidase positive test.

10.5.4 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 °C ± 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.

10.5.5 Expression of results

If the identification of the colonies confirms the presence of this species, express the result as: presence of *Pseudomonas aeruginosa* in the sample, *S*.

If no growth after enrichment is observed and/or if the identification of the colonies does not confirm the presence of this species, express the result as: absence of *Pseudomonas aeruginosa* in the sample, *S*.

11 Neutralization of the antimicrobial properties of the product

11.1 General

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

11.2 Preparation of inoculum

Prior to the test, and for each strain, inoculate the surface of soybean casein digest agar (SCDA) or other suitable (non-selective, non-neutralizing) medium with *Escherichia coli or Pseudomonas aeruginosa*. Incubate the plate at 32,5 °C ± 2,5 °C for 18 h to 24 h.

To harvest the bacterial culture, use a sterile loop, streak the surface of the culture and re-suspend it into the diluent to obtain a calibrated suspension of about 1×10^8 CFU/ml (e.g. using a spectrophotometer; see Annex C of ISO 21148). Use this suspension and its dilutions within 2 h.

11.3 Suitability of counting methods

11.3.1 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 microorganism. Plate on a Petri dish. 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 (diluents, 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 possible contamination of the product with this microorganism is unlikely.

11.3.2 Suitability test of the pour-plate method

Mix 9 ml of the initial suspension and/or the sample dilution(s) in neutralizing diluent (or other; see 5.2) 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 (5.3.2) 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 °C ± 2,5 °C, count the colonies on the plates and compare the counts obtained for the test and for the control. The diluent and the counting method are satisfactory at the 1:10 dilution (when 1 ml of the initial suspension is used) if the suitability test count is at least 50 % of the control.

11.3.3 Suitability of the surface spread method

Mix 9 ml of the initial suspension in neutralizing diluent (or other, see 5.2) 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 (5.3.2) (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 °C ± 2,5 °C, 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 satisfactory at the 1:10 dilution (when 1 ml of the initial suspension is used) if the count is at least 50 % of the control.

11.3.4 Suitability of the membrane filtration method

Mix to the volume of initial suspension or of the sample dilution used in the test (see Clause 9) with 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 neutralizing diluent (5.2.2) or diluent (5.2.3). Transfer the membrane onto the surface of a suitable agar medium (5.3.2).

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

After incubation for 24 h to 72 h at 32,5 °C ± 2,5 °C, count the colonies on the membranes and compare the counts obtained for the test and for the control. The membrane filtration method and the diluent are satisfactory if the count is at least 50 % of the control

11.4 Suitability of the detection method

11.4.1 Procedure

11.4.1.1 In tubes of 9 ml of diluent (5.2.3), prepare a dilution of the calibrated suspension in order to obtain a final count between 100 CFU/ml and 500 CFU/ml. To count the final concentration of viable microorganisms in the diluted calibrated suspension, transfer 1 ml of the suspension into a Petri dish and pour 15 ml to 20 ml of the melted agar medium (5.3.2) kept in a water bath at no more than 48 °C. Let solidify and then incubate at 32,5 °C ± 2,5 °C for 20 h to 24 h.

11.4.1.2 Prepare in duplicate the initial suspension (9.2) in the conditions chosen for the test (at least 1 g or 1 ml of product under test, defined volume of enrichment broth) in a tube or flask.

11.4.1.3 Introduce aseptically 0,1 ml of the diluted calibrated suspension of microorganisms into one tube or flask (suitability test). Mix then incubate both tubes or flasks (suitability test and non-inoculated control) at 32,5 °C ± 2,5 °C for 20 h to 24 h.

11.4.1.4 For each tube or flask (suitability test and non-inoculated control), Using a sterile loop, streak an aliquot (same conditions as in the test) of the incubated mixture onto the surface of the MacConkey agar medium or Cetrimide agar medium (approximately 15 ml to 20 ml) in the Petri dish (diameter 85 mm to 100 mm). Incubate the plates at 32,5 °C ± 2,5 °C for 24 h to 48 h.

11.4.2 Interpretation

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

The neutralization and the detection method is satisfactory if a growth characteristic of *Escherichia coli* or *Pseudomonas aeruginosa* occur on the suitability test plate and no growth occurs on the control plate.

When growth is detected on the control plate (contaminated products), the neutralization and the detection method are satisfactory if *Escherichia coli* or *Pseudomonas aeruginosa* is recovered on the suitability test plates.

Failure of growth on the suitability test plates indicates that antimicrobial activity is still present and necessitates a modification of the conditions of the method by an increase in the volume of nutrient broth, the quantity of product remaining the same, or by incorporation of a sufficient quantity of inactivating agent in the enrichment broth, or by an appropriate combination of modifications so as to permit the growth of *Escherichia coli or Pseudomonas aeruginosa*.