INTERNATIONAL STANDARD

ISO 6888-1

Second edition
2021-08

Microbiology of the food chain — Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) —

Part 1: Method using Baird-Parker agar medium

Microbiologie de la chaîne alimentaire — Méthode horizontale pour le dénombrement des staphylocoques à coagulase positive (Staphylococcus aureus *et autres espèces) —*

Partie 1: Méthode utilisant le milieu gélosé de Baird-Parker

Reference number
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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology* in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 463, *Microbiology of the food chain,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces the first edition (ISO 6888-1:1999), which has been technically revised. It also incorporates the amendments ISO 6888-1:1999/Amd 1:2003 and ISO 6888-1:1999/Amd 2:2018. The main changes compared with the previous edition are as follows:

— the title has been changed to relate to the “Food chain”;

— the status of this document and ISO 6888-2 has been clarified;

— the document has been aligned with ISO 7218:2007, i.e. pour molten agar medium at 44 °C to 47 °C;

— all occurrences, when appropriate, have been changed from “35 °C or 37 °C” to “34 °C to 38 °C”;

— all occurrences of incubation time, when appropriate, have been changed from “18 h to 24 h” to “24 h ± 2 h”;

— requirements have been added to use ISO 11133;

— all available standards related to sampling techniques have been updated;

— a description of typical and atypical colonies on Baird-Parker agar (BPA) medium has been updated;

— the rabbit plasma fibrinogen agar (RPFA) medium has been added as an alternative to the coagulase test for confirmation;

— the flow diagram procedure in Annex A has been updated;

— culture media and reagents with performance testing in Annex B have been added;

— results of the interlaboratory study (from ISO 6888-1:1999/Amd 1:2003, Precision data) has been updated;

— the Bibliography has been updated.

A list of all parts in the ISO 6888 series can be found on the ISO website.

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.

Introduction

This document, ISO 6888-2 and ISO 6888-3 describe three horizontal methods for the detection and enumeration of coagulase-positive staphylococci among which enterotoxinogenic strains are encountered. It is mainly concerned with *Staphylococcus aureus*, but also with *S. intermedius* and certain strains of *S. hyicus*.

For the purposes of this document, the confirmation of typical and atypical colonies is based on a positive coagulase reaction, but it is recognized that some strains of *Staphylococcus aureus* give weakly positive coagulase reactions. These latter strains can be confused with other bacteria but they can be distinguished by the use of additional tests not included in this document, such as tests for sensitivity to lysostaphin, and production of haemolysin, thermostable nuclease and acid from mannitol (see ISO 7218 and Reference [15]).

The main technical changes listed in the Foreword, introduced in this document compared with the previous edition are considered as minor (see ISO 17468). They have a minor impact on the performance characteristics of this method.

Results of the interlaboratory study and samples tested are described in Annex C.

Microbiology of the food chain — Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) —

Part 1: Method using Baird-Parker agar medium

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting staphylococci are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials. Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety aspects, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

1 Scope

This document specifies a horizontal method for the enumeration of coagulase-positive staphylococci by counting the colonies obtained on a solid medium (Baird-Parker medium)[10] after aerobic incubation at 34 °C to 38 °C and coagulase confirmation.

This document is applicable to:

— products intended for human consumption;

— products intended for animal feeding;

— environmental samples in the area of food and feed production, handling, and

— samples from the primary production stage.

This horizontal method was originally developed for the examination of all samples belonging to the food chain.

Because of the large variety of products in the food chain, it is possible that this horizontal method is not appropriate in every detail for all products. Nevertheless, it is expected that the required modifications are minimized so that they do not result in a significant deviation from this horizontal method.

Based on the information available at the time of publication of this document, this method is not considered to be (fully) suited to the examination of fermented products or other products containing technological flora based on *Staphylococcus* spp (e.g. *S. xylosus*) (such as cheeses made from raw milk and certain raw meat products) likely to be contaminated by:

— staphylococci forming atypical colonies on a Baird-Parker agar medium;

— background flora that can obscure the colonies being sought.

Nevertheless, both this document and ISO 6888-2 are given equivalent status.

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 6887 (all parts), *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

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 http://www.electropedia.org/

3.1
coagulase-positive staphylococci
bacteria that form either typical or atypical colonies, or both, on the surface of a selective culture medium (Baird-Parker agar medium) and that show a positive coagulase reaction in a tube test or on rabbit plasma fibrinogen agar

Note 1 to entry: The typical and atypical colonies are described in 9.3.1.

3.2
enumeration of coagulase-positive staphylococci
determination of the number of *coagulase-positive staphylococci* (3.1) per gram, per millilitre, per square centimetre or per sampling device/sampled area

Note 1 to entry: A sampled area is an area not defined by a numerical size, for example, a hot tap, a door handle.

4 Principle

4.1 General

Inoculation of the surface of a solid selective culture medium, with a specified quantity of the test sample if the product is liquid, or with a specified quantity of the initial suspension in the case of other products.

Inoculation, under the same conditions, using decimal dilutions of the test sample.

4.2 Incubation

Aerobic incubation of the plates at 34 °C to 38 °C and examination after both 24 h and 48 h.

4.3 Enumeration and confirmation

Calculation of the number of coagulase-positive staphylococci per gram, per millilitre, per square centimetre or per sampling device of sample from the number of either typical or atypical colonies, or both, obtained on plates at dilution levels chosen to give a significant result, and confirmed by a positive coagulase test result, within the counting limits of the method and in accordance with ISO 7218.

NOTE See Annex A for a flow diagram.

5 Culture media and reagents

Follow current laboratory practices in accordance with ISO 7218.

The composition of culture media and reagents and their preparation are specified in Annex B.

For performance testing of culture media and reagents, follow the procedures in accordance with either Annex B or ISO 11133, or both.

For the diluent(s), see the relevant part of the ISO 6887 series.

6 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

6.1 Apparatus for dry sterilization (oven) and wet sterilization (autoclave).

See ISO 7218.

6.2 Incubator, capable of maintaining the inoculated media within the temperature range 34 °C to 38 °C.

NOTE The range 34 °C to 38 °C for incubation of media includes the use of incubators set at 35 °C ± 1 °C, 36 °C ± 2 °C or 37 °C ± 1 °C.

6.3 Water bath, or similar apparatus, capable of being maintained at 44 °C to 47 °C.

6.4 Sterile tubes, bottles or flasks with caps, of appropriate capacity. Bottles or flasks with non-toxic metallic or plastic screw-caps may be used.

6.5 Sterile Petri dishes, with a diameter of approximately 90 mm or a larger size (optional; diameter of approximately 140 mm), made of glass or plastic.

6.6 Straight wire (see ISO 7218) or **Pasteur pipette**.

Loops (of diameter approximately 3 mm) and wires, made of platinum/iridium or nickel/chromium, or glass rods, or equivalent sterile disposable loops or inoculating needles.

6.7 Sterile graduated pipettes or automatic pipettes of nominal capacities of 1 ml, 2 ml and 10 ml, graduated in 0,1 ml, 0,1 ml and 0,5 ml divisions, respectively. See ISO 7218.

Graduated pipettes and pipettor tips should be fitted with a non-absorbent cotton wool plug to prevent contamination when used to manipulate microbial cultures.

6.8 Spreaders, sterile, made of glass or plastic.

6.9 pH-meter, capable of being read to the nearest 0,01 pH unit, enabling measurements to be made with a tolerance of ±0,1 pH unit. The pH meter shall be equipped with either manual or automatic temperature compensation. See ISO 7218.

6.10 Refrigerator, capable of operating at 5 °C ± 3 °C.

6.11 Membranes, with a 0,2 µm pore size.

7 Sampling

Sampling is not part of the method specified in this document. Follow the specific International Standard dealing with the product concerned. If there is no specific International Standard dealing with the sampling of the product concerned, the parties concerned should come to an agreement on this subject.

Recommended sampling techniques are given in the following documents:

— ISO/TS 17728 for food and animal feed;

— ISO 707 for milk and milk products;

— ISO 6887-3 for fish and fishery products;

— ISO 13307 for the primary production stage;

— ISO 17604 for carcasses;

— ISO 18593 for surfaces.

It is important that the laboratory receives a sample that is representative. The sample should not have been damaged or changed during transport or storage.

8 Preparation of the test sample

Prepare the test sample from the laboratory sample in accordance with the specific International Standard dealing with the product concerned: follow the procedures specified in the ISO 6887 series and, if necessary, ISO 18593. If there is no specific International Standard available, the parties concerned should come to an agreement on this subject.

9 Procedure

9.1 Test portion, initial suspension and dilutions

Refer to the relevant part of the ISO 6887 series.

9.2 Inoculation and incubation

Transfer, by means of a sterile pipette (6.7), 0,1 ml of the test sample if liquid, or 0,1 ml of the initial suspension (10^{-1} dilution) in the case of other products, to a Baird-Parker agar (BPA) plate (see B.2). For enumeration techniques in microbiology of the food chain, the number of Petri dishes to be used according to the tested dilutions is stated into ISO 7218. Repeat the procedure for further decimal dilutions if necessary.

If, for certain products, it is desirable to count low numbers of coagulase-positive staphylococci, the level of detection can be raised by a factor of 10 by inoculating 1,0 ml of the test sample liquid, or 1,0 ml of the initial suspension for other products, either on the surface of one plate (d = 140 mm) or on the surface of three small agar plates (d = 90 mm). The number of Petri dishes to be used according to the dilutions tested is stated in ISO 7218.

Carefully spread the inoculum as quickly as possible over the surface of the agar plate, trying not to touch the sides of the Petri dish, using the spreader (6.8). Allow the plates to dry with their lids on for about 15 min at laboratory temperature.

NOTE 1 For inoculation using a spiral plater, see ISO 7218.

Invert the dishes prepared above and place them for 24 h ± 2 h in the incubator (6.2) set at 34 °C to 38 °C. Then re-incubate for a total of 48 h ± 4 h.

NOTE 2 Colonies with typical appearance after 24 h ± 2 h incubation can lose their typical appearance after 48 h ± 4 h incubation, due to overgrowth with enlargement of the clear zone during the second phase of incubation. Counting only at 48h ± 4h can lead to low counts or no counts.

9.3 Counting of colonies

9.3.1 General description of colonies growing on BPA medium

9.3.1.1 Colonies presumed to be coagulase-positive staphylococci

Typical colonies are black or grey, shining and convex (1 mm to 1,5 mm in diameter after incubation for 24 h ± 2 h, and 1,5 mm to 2,5 mm in diameter after incubation for 48 h ± 4 h) and are surrounded by a clear zone, which can be partially opaque. After incubation for at least 24 h, an opalescent ring immediately in contact with the colonies can appear in this clear zone.

Atypical colonies have the same size as typical colonies and can present one of the following morphologies:

— shining black colonies with or without a narrow white edge; the clear zone is absent or barely visible and the opalescent ring is absent or hardly visible;

— grey colonies free of clear zone.

Atypical colonies are formed mainly by strains of coagulase-positive staphylococci contaminating, for example, dairy products, shrimps and giblets. They are less often formed by strains of coagulase-positive staphylococci contaminating other products.

9.3.1.2 Colonies not presumed to be coagulase-positive staphylococci

Other colonies are all the remaining colonies possibly present on the plates that do not show the typical or atypical appearance described in 9.3.1.1 and are considered as the background flora.

NOTE Bacteria belonging to genera other than staphylococci can give colonies with an appearance similar to staphylococci. Microscopic examination of Gram stain, before confirmation, will enable the distinction of other genera from staphylococci.

9.3.2 Colony counting procedure

After incubation for 24 h ± 2 h, mark on the bottom of the plates the positions of any typical colonies present.

Re-incubate all plates at 34 °C to 38 °C for a further 24 h ± 2 h and mark any new typical colonies. Also mark any atypical colonies present.

For enumeration, only retain plates containing a maximum of 300 colonies in total (typical, atypical, background flora), and including a maximum of either 150 typical or atypical colonies, or both, at two successive dilutions.

EXAMPLE 1

0 typical colonies, 150 atypical colonies and 150 background flora.

EXAMPLE 2

150 typical colonies, 0 atypical colonies and 150 background flora.

EXAMPLE 3

150 typical colonies, 150 atypical colonies and 0 background flora.

One of the plates shall contain at least 10 colonies (either typical or atypical colonies, or both). Select for confirmation (see 9.4) a given number (in general five typical colonies if there are only typical colonies, or five atypical colonies if there are only atypical colonies, or five typical and five atypical colonies if both types are present, from each plate).

If there are less than 10, either typical or atypical colonies, or both, present on plates inoculated with undiluted liquid product or the lowest dilution of other products, it is possible to make an estimated count as described in ISO 7218.

If a 1,0 ml inoculum was spread over three plates (see 9.2), treat these plates as one in all subsequent counting and confirmation procedures.

To make an estimated count of lower numbers of coagulase-positive staphylococci, retain all plates that contain any typical and atypical colonies. Select all such colonies for confirmation within the limits set out above.

9.4 Confirmation

9.4.1 General

The confirmation of coagulase-positive staphylococci is undertaken by a tube test (see 9.4.2). Alternatively, it can be undertaken by a plate test using RPFA medium (see 9.4.3 and References [11], [13] and [14]).

NOTE An alternative procedure can be used to confirm isolates as coagulase-positive staphylococci, providing the suitability of the relevant procedure is verified (see ISO 7218).

9.4.2 Tube test

From the surface of each selected colony (see 9.3.1), remove an inoculum with a sterile wire (6.6) and transfer it to a tube or bottle of brain-heart infusion (BHI) broth (see B.3). With the same wire, spread the suspension on a non-selective medium (blood agar or nutrient agar) and incubate at 34 °C to 38 °C for 24 h ± 2 h to check the purity of the selected colony (homogeneous morphology).

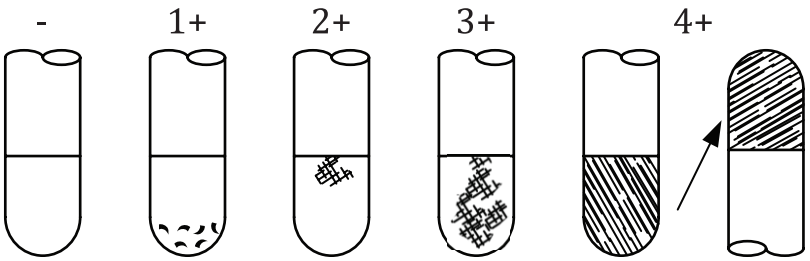
Incubate the brain-heart infusion broth, preferably in a water bath, at 34 °C to 38 °C for 24 h ± 2 h.

Aseptically add 0,1 ml of each culture to 0,3 ml of the rabbit plasma (see B.4) (unless other amounts are specified by the manufacturer) in sterile haemolysis tubes or bottles (6.4), and incubate at 34 °C to 38 °C.

By tilting the tube, examine for clotting of the plasma after 5 h ± 1 h of incubation and, if the test is negative, re-examine after 24 h ± 2 h of incubation, or examine at the incubation times specified by the manufacturer.

Consider the coagulase test to be positive if the cultures yield at least 3 + coagulase reactions according to the scoring guidance in Figure 1. Reactions from 1 + to 2 + are considered as intermediate.

As a negative control, for each batch of plasma, add 0,1 ml of sterile brain-heart infusion broth (see B.3) to the recommended quantity of rabbit plasma (see B.4) and incubate. For the test to be valid, the control plasma shall show no signs of clotting.



Score		Coagulase interpretation at 24 h
–	no clot	negative
1 +	small unorganized clot(s)	intermediate shall be confirmed by another method (see Introduction)
2 +	intermediate small organized clot	intermediate shall be confirmed by another method (see Introduction)
3 +	large organized clot	positive
4 +	entire contents of the tube coagulate and is not displaced when the tube is inverted	positive

Figure 1 — Scoring of coagulase test reactions

9.4.3 Plate test using RPFA medium

From the surface of each selected colony (see 9.3.1), take an inoculum with a sterile wire (6.6) and stab inoculate on RPFA plates (see B.5).

Several colonies, in addition to one positive control strain and one negative control strain, can be inoculated onto one plate such that the individual colonies have well separated precipitation zones.

NOTE For guidance, up to 10 colonies can be inoculated onto one plate (d = 90 mm).

Incubate the plates at 34 °C to 38 °C for 24 h ± 2 h to 48 h ± 4 h.

After incubation, the staphylococci form black or grey or even white, small colonies surrounded by a halo of precipitation, indicating coagulase activity.

Before reading, the plates can be stored in the refrigerator at 5 °C ± 3 °C for 24 h ± 2 h to 48 h ± 4 h.

10 Expression of results

For calculation of the results, follow the procedure(s) in accordance with ISO 7218. Calculate and report the results as the number of coagulase-positive staphylococci in cfu (colony forming unit) per gram, millilitre, per square centimetre or per sampling device.

In cases where no colonies of the target organism have been detected, follow ISO 7218 for the expression of results for special cases.

11 Performance characteristics of the method

11.1 Interlaboratory study

Results of the interlaboratory study to determine the precision of the method are summarized in Annex C. Repeatability and reproducibility limits were determined using four sample types (cheese, meat, egg powder and reference materials) contaminated at various levels.

The values derived from the interlaboratory study may not be applicable to concentration ranges and sample types other than those given in Annex C.

11.2 Repeatability limit

The absolute difference between two single ($\log_{10}$-transformed) test results (number of coagulase-positive staphylococci per gram or per millilitre) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within the shortest feasible time interval, will in no more than 5 % of cases exceed the repeatability limit *r*.

As a general indication of the repeatability limit *r*, the following values (medians of values per matrix and contamination level, see Annex C) may be used when testing food samples in general:

— r = 0,28, range [0,17; 0,50] (expressed as an absolute difference between log10-transformed test results); or

— r = 1,90, range [1,48; 3,16] (expressed as a ratio of the higher to the lower of the two test results on the normal scale).

EXAMPLE A first test result of 10 000 or $1,0 \times 10^4$ or $\log_{10}$ 4,00 of coagulase-positive staphylococci per gram of food was obtained in a given laboratory. Under repeatability conditions, the difference between $\log_{10}$-transformed results is not intended to be greater than ± 0,28 $\log_{10}$ units. So the result from a second test result of the same sample is expected to be between 3,72 (4,00 – 0,28) $\log_{10}$ units and 4,28 (4,00 + 0,28) $\log_{10}$ units. For non-log-transformed results, the ratio between the first test result and the second test result from the same sample is not intended to be greater than 1,90. So the second test result is expected to be between 5 263 (= 10 000/1,90) cfu per gram and 19 000 (=100 000 × 1,90) cfu per gram.

For reference materials (capsules containing approximately 5 000 cfu, see Annex C), the following values may be used:

— r = 0,19 (expressed as an absolute difference between $\log_{10}$-transformed test results); or

— r = 1,55 (expressed as a ratio of the higher to the lower of the two test results on the normal scale).

11.3 Reproducibility limit

The absolute difference between two single ($\log_{10}$-transformed) test results (number of coagulase-positive staphylococci per gram or per millilitre) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in no more than 5 % of cases exceed the reproducibility limit *R*.

As a general indication of the reproducibility limit *R*, the following values (medians of values per matrix and contamination level, see Annex C) can be used when testing food samples in general:

— R = 0,43, range [0,29; 0,66] (expressed as a difference between $\log_{10}$-transformed test results); or

— R = 2,70, range [1,95; 4,57] (expressed as a ratio of the higher to the lower of the two test results on the normal scale).

EXAMPLE 1 A test result of 10 000 or $1,0 \times 10^4$ or $\log_{10}$ 4,00 coagulase-positive staphylococci per gram of food product was obtained in a first laboratory. Under reproducibility conditions, the difference between $\log_{10}$-transformed results is expected to be greater than ±0,43 $\log_{10}$ units. So, the results from a second laboratory are expected to be between 3,57 (4,00 – 0,43) $\log_{10}$ units and 4,43 (4,00 + 0,43) $\log_{10}$ units. For non-log-transformed results, the ratio between the test results from this first laboratory and a second laboratory is not intended to be greater than 2,70. So, the result from the second laboratory is expected to be between 3 704 (= 10 000/2,70) cfu per gram and 27 000 (=10 000 × 2,70) cfu per gram.

EXAMPLE 2 A laboratory wants to know the maximum level it can find, which is still in compliance with a pre-set limit (e.g. a limit of 10^5 or $\log_{10}$ 5). For this, the *R* value (on the log scale) is multiplied by a factor of 0,59[1)]. The maximum value is 0,25 (0,43 × 0,59) as a difference between $\log_{10}$-transformed test results or 1,78 ($10^{0,25}$) as a ratio between test results. So results up to $\log_{10}$ 5,25 ($\log_{10}$ 5 + $\log_{10}$ 0,25) or 17 800 (=10 000 × 1,78) do not indicate non-compliance with the limit.

For reference materials (capsules containing approximately 5 000 cfu, see Annex C) the following values may be used:

— R = 0,39 (expressed as a difference between $\log_{10}$-transformed test results); or

— R = 2,4 (expressed as a ratio of the higher to the lower of the two test results on the normal scale).

12 Test report

The test report shall specify, at least, the following information:

— the test and confirmation method used, with reference to this document, i.e. ISO 6888-1:2021;

— the sampling method used, if known;

— all operating conditions not specified in this document, or regarded as optional, together with details of any incidents that could have influenced the test result(s);

— any deviations from this document;

— all information necessary for the complete identification of the sample;

— the test result(s) obtained;

— measurement uncertainty;

— the date of the test.

13 Quality assurance

The laboratory should have a clearly defined quality control system to ensure that the equipment, reagents and techniques are suitable for the method. The use of positive controls, negative controls and blanks are part of the method. Performance testing of culture media and reagents is specified in Annex B and described in ISO 11133.

1) The factor 0,59 reflects the fact that a test with a one-sided 95 % interval is used to test whether the limit is exceeded; it is obtained from the following formula: 0,59 = 1,64 / (1,96 x √2).

Annex A
(normative)

Flow diagram of the procedure

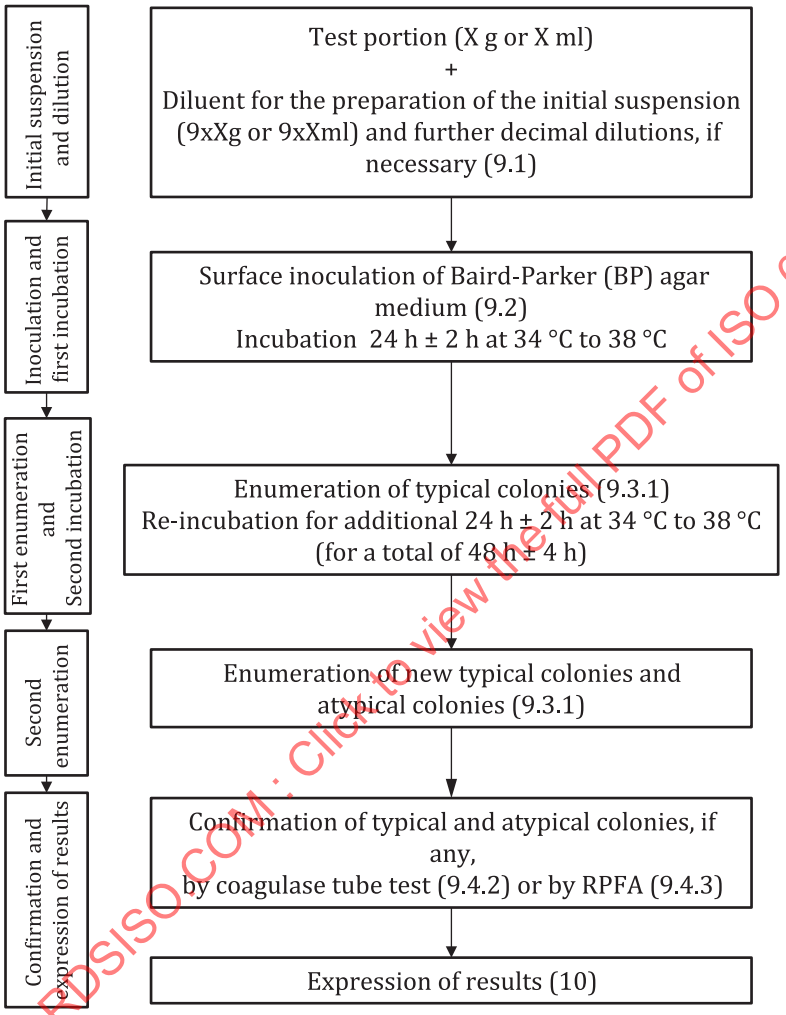


Figure A.1 — Flow diagram of procedure for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) using BPA medium

Annex B
(normative)

Culture media and reagents

B.1 General

The general specifications of ISO 11133 are applicable to the preparation and performance testing of the culture media described in this annex. If culture media or reagents are prepared from dehydrated complete media and reagents or if ready-to-use media and reagents are used, follow the manufacturer's instructions regarding preparation, storage conditions, expiry date and use.

The shelf life of the media and reagents indicated in this annex has been determined in some studies. The user shall verify these under their own storage conditions (in accordance with ISO 11133).

Performance testing of culture media is described in B.6.

B.2 BPA medium[10][16]

B.2.1 Base medium

B.2.1.1 Composition

Enzymatic digest of casein	10,0 g
Yeast extract	1,0 g
Meat extract	5,0 g
Sodium pyruvate (CAS Number 113-24-6)	10,0 g
Glycine (CAS Number 56-40-6)	12,0 g
Lithium chloride (CAS Number 7447-41-8)	5,0 g
Agar	12 g to 22 g[a]
Water, to a final volume of	1 000 ml
[a] Depending on the gel strength of the agar.	

B.2.1.2 Preparation

Dissolve the components or the dehydrated complete base in the water by boiling.

Adjust the pH (6.9), if necessary, so that after sterilization it is 7,2 ± 0,2 at 25 °C.

Dispense the medium in quantities of 100 ml into flasks or bottles (6.4) of appropriate capacity.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

Store the medium in closed containers at 5 °C (6.10) for up to six months.

B.2.2 Solutions for BPA medium

B.2.2.1 Potassium tellurite solution

B.2.2.1.1 Composition

Potassium tellurite[a] (K_2TeO_3) (CAS Number 7790-58-1)	1,0 g
Water	100 ml

[a] It should be ensured beforehand that the potassium tellurite available is suitable for this test (see B.2.2.1.2).

B.2.2.1.2 Preparation

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

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

Sterilize by filtration using 0,2 µm pore size membranes (6.11).

The solution may be stored at 5 °C (6.10) for up to one month.

Discard the solution if a white precipitate forms.

B.2.2.2 Egg yolk emulsion (concentration approximately 20 % (e.g. 200 ml/l) or according to the manufacturer's instructions)

B.2.2.2.1 General

If a commercial preparation is available, it may be used.

B.2.2.2.2 Preparation

Use fresh hen eggs with intact shells. Clean the eggs with a brush using a liquid detergent. Rinse them under running water, then disinfect the shells either by immersing them in ethanol (70 % volume fraction, e.g. 700 ml/l)) for 30 s and allowing 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 (6.4) and add four times their volume of sterile water. Mix thoroughly. Heat the mixture in the water bath (6.3) set at 44 °C to 47 °C for 2 h and leave for 18 h to 24 h at 5 °C (6.10) to allow a precipitate to form. Aseptically collect the supernatant liquid into a fresh sterile flask for use.

The emulsion may be stored at 5 °C (6.10) for up to 72 h.

B.2.2.3 Sulfamezathine (sulfamethazine, sulfadimidine) solution[16]

B.2.2.3.1 Composition

Sulfamezathine ($C_{12}H_{14}N_4O_2S$) (CAS Number 57-68-1)	0,2 g
Sodium hydroxide solution, (NaOH) = 0,1 mol/l	10 ml
Water	90 ml

B.2.2.3.2 Preparation

Dissolve the sulfamezathine in the sodium hydroxide solution.

Dilute to 100 ml with the water.

Sterilize by filtration using 0,2 µm pore size membranes.

The solution may be stored at 5 °C (6.10) for up to one month.

B.2.3 Complete BPA medium

B.2.3.1 Composition

Base medium (see B.2.1)	100 ml
Potassium tellurite solution (see B.2.2.1)	1 ml
Egg yolk emulsion (see B.2.2.2)	5 ml
Sulfamethazine solution (see B.2.2.3) (if necessary)	2,5 ml

B.2.3.2 Preparation

Melt the base medium, then let it cool down to 44 °C to 47 °C in a water bath (6.3).

Under aseptic conditions, add the two solutions (see B.2.2.1 and B.2.2.2) and if necessary (if *Proteus* species are suspected in the test sample) the sulfamethazine solution (see B.2.2.3), previously warmed to 44 °C to 47 °C in a water bath. Mix thoroughly after each addition by rotation to minimize foaming.

B.2.4 Preparation of BPA plates

Pour the appropriate quantity of the complete medium (see B.2.3) into sterile plates in order to obtain an agar thickness of about 3 mm and allow it to solidify.

The prepared BPA plates may be stored, prior to drying, at 5 °C (6.10) for up to 14 d.

For plates prepared commercially, the instructions of the manufacturers should be followed.

If necessary, dry the plates before use according to the instructions given in ISO 11133.

B.3 BHI broth

B.3.1 Composition

Enzymatic digest of animal tissues	10,0 g
Dehydrated calf brain infusion	12,5 g
Dehydrated beef heart infusion	5,0 g
Glucose ($C_6H_{12}O_6$) (CAS Number 50-99-7)	2,0 g
Sodium chloride (CAS Number 7647-14-5)	5,0 g
Disodium hydrogenphosphate, anhydrous (Na_2HPO_4) (CAS Number 7558-79-4)	2,5 g
Water	1 000 ml

B.3.2 Preparation

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

Adjust the pH, if necessary, so that after sterilization it is 7,4 ± 0,2 at 25 °C.

Dispense the medium in quantities of 5 ml to 10 ml into tubes or bottles (6.4) of appropriate capacity.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

Store the medium in closed containers at 5 °C (6.10) for up to three months.

B.4 Rabbit plasma

Use commercially available dehydrated rabbit plasma and rehydrate it according to the manufacturer's instructions.

If dehydrated rabbit plasma is not available, dilute one volume of fresh sterile rabbit plasma with three volumes of sterile water.

Add ethylenediaminetetraacetic acid (EDTA, CAS Number 60-00-4) solution to give 0,1 % EDTA (e.g. 1 g/l EDTA) in the rehydrated or diluted plasma, if potassium citrate or sodium citrate has been used as the plasma anticoagulant. Oxalated or heparinized plasma does not require EDTA, see Reference [10].

Unless stated by the manufacturer, the rehydrated or diluted plasma shall be used immediately.

Before use, test each batch of plasma with coagulase-positive strains of staphylococci and strains of coagulase-negative staphylococci. Performance testing is described in B.6.

B.5 RPFA medium

B.5.1 Base medium

Prepare the base medium as stated in B.2.1, with the exception of the distribution of the base medium, in quantities of 90 ml per flask or bottle (6.4) of appropriate capacity.

B.5.2 Solutions for RPFA medium

B.5.2.1 Potassium tellurite solution

Prepare the potassium tellurite solution as indicated in B.2.2.1.

B.5.2.2 Bovine fibrinogen solution

B.5.2.2.1 Composition

Bovine fibrinogen	5 g to 7 g[a]
Water	100 ml
[a] Depending on the purity of the bovine fibrinogen.	

B.5.2.2.2 Preparation

Under aseptic conditions, dissolve the bovine fibrinogen in the water just prior to use.

B.5.2.3 Rabbit plasma and trypsin inhibitor solution

B.5.2.3.1 Composition

Rabbit plasma with EDTA for coagulase (EDTA coagulase plasma)	30 ml
Trypsin inhibitor	30 mg

B.5.2.3.2 Preparation

Under aseptic conditions, dissolve the trypsin inhibitor in the rabbit plasma, just prior to use.

B.5.3 Complete RPFA medium

B.5.3.1 Composition

Base medium (see B.5.1)	90 ml
Potassium tellurite solution (see B.2.2.1)	0,25 ml
Bovine fibrinogen solution (see B.5.2.2)	7,5 ml
Rabbit plasma and trypsin inhibitor solution (see B.5.2.3)	2,5 ml

B.5.3.2 Preparation

Melt the base medium, then let it cool down to 44 °C to 47 °C in a water bath (6.3).

Under aseptic conditions, add the three solutions (see B.5.2.1, B.5.2.2 and B.5.2.3) previously warmed to 44 °C to 47 °C in a water bath. Mix thoroughly after each addition by rotation to minimize foaming.

Use the complete medium immediately after its preparation, in order to avoid any precipitation of the plasma. The holding time of molten media must be as shortened as possible (see ISO 11133).

WARNING — If a commercially available solution of bovine fibrinogen or rabbit plasma is used, follow with great care the manufacturer's instructions for the preparation of this solution and of the complete medium (in particular the temperature of the base medium). Otherwise, the medium can completely lose its activity.

B.5.4 Preparation of RPFA plates

Pour the appropriate quantity of the complete medium (see B.5.3) into sterile plates in order to obtain an agar thickness of about 3 mm and allow it to solidify.

The prepared RPFA plates may be stored, prior to drying, at 5 °C (6.10) for up to 14 d.

For plates prepared commercially, the instructions of the manufacturers should be followed.

If necessary dry the plates before use according to the instructions given by ISO 11133.

B.6 Performance testing

The definitions of productivity, selectivity and specificity are specified in ISO 11133. In general, follow the procedures for performance testing described in ISO 11133. Performance testing details are given in Table B.1 and Table B.2.

For the productivity test, it is preferable to use a fresh suspension of microorganism culture, otherwise, 2 readings at 24 ± 2 h and 48 ± 4 h are necessary.

Table B.1 — Performance testing for quality assurance of culture media

Medium	Function	Incubation	Control strain	WDCM number[a]	Reference media	Method of control	Criteria[c]	Characteristic reaction
BPA	Productivity	(24 ± 2) h / 34 °C to 38 °C	*Staphylococcus aureus*	00034[b] 00032	Tryptone soya agar (TSA)	Quantitative	$P_R \geq 0,5$	Black or grey colonies with clear halo (egg yolk clearing reaction)
	Selectivity	(48 ± 4) h / 34 °C to 38 °C	*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibition (0)	—
			Proteus mirabilis[e]	00023	—		Total or partial inhibition (0 to 1)	
	Specificity	(48 ± 4) h / 34 °C to 38 °C	*Staphylococcus saprophyticus*	00159[b]	—	Qualitative	—	Black or grey colonies without egg yolk clearing reaction
			Staphylococcus epidermidis	00036	—			

[a] Refer to the reference strain catalogue on at http://www.wfcc.info for information on culture collection strain numbers and contact details.

[b] Strain to be used as a minimum by the user laboratory.

[c] Growth is categorized as: 0: no growth; 1: weak growth (partial inhibition); 2: good growth; P_R is productivity ratio (see ISO 11133).

[d] Strain free of choice, one of the strains has to be used as a minimum.

[e] Strain to be used if sulfamethazine is added to the medium.

Table B.2 — Performance testing for the quality assurance of the confirmation media and reagents

Confirmation medium/ reagent	Function	Incubation	Control strains	WDCM[a] numbers	Characteristic reactions
Rabbit plasma / BHI	Coagulase test	4 h to 6 h / 34 °C to 38 °C	*Staphylococcus aureus*[c]	00032 00034 00035 00090	Positive reaction: volume of clot occupies more than half of the volume of the liquid
		(24 ± 2) h / 34 °C to 38 °C	*Staphylococcus epidermidis* or *Staphylococcus saprophyticus*	00036 00159	Negative reaction: no sign of clotting of the plasma while control plasma shows no clotting with sterile brain heart infusion broth

[a] Refer to the reference strain catalogue on at http://www.wfcc.info for information on culture collection strain numbers and contact details.

[b] If the batch is already tested for a quantitative test (see ISO 6888-2) or a qualitative test (see ISO 6888-3), this performance testing for the coagulase reaction is not necessary.

[c] Strain free of choice, one of the strains has to be used as a minimum.