# International Standard 6888

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# Microbiology — General guidance for enumeration of *Staphylococcus aureus* — Colony count technique

*Microbiologie — Directives générales pour le dénombrement de* Staphylococcus aureus *— Méthode par comptage des colonies*

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## Foreword

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Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council.

International Standard ISO 6888 was developed by Technical Committee ISO/TC 34, *Agricultural food products,* and was circulated to the member bodies in November 1981.

It has been approved by the member bodies of the following countries:

| | | |
|---|---|---|
| Australia | India | Sri Lanka |
| Brazil | Israel | Tanzania |
| Canada | Italy | Thailand |
| Chile | Mexico | Turkey |
| Czechoslovakia | Netherlands | United Kingdom |
| Egypt, Arab Rep. of | New Zealand | USA |
| Ethiopia | Poland | USSR |
| France | Romania | Venezuela |
| Germany, F.R. | South Africa, Rep. of | Yugoslavia |
| Hungary | Spain | |

The member body of the following country expressed disapproval of the document on technical grounds:

Portugal





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# Microbiology — General guidance for enumeration of *Staphylococcus aureus* — Colony count technique

## 0 Introduction

**0.1** This International Standard is intended to provide general guidance for the examination of products not dealt with by existing International Standards and for the consideration of bodies preparing reference microbiological methods of test for application to food products or to animal feeding stuffs. In view of the large variety of products within this field of application, these guidelines may not be appropriate for some products in every detail, and for some other products it may be necessary to use different methods. Nevertheless, it is hoped that, in all cases, every attempt will be made to apply these guidelines as far as possible and that deviations from them will only be made if absolutely necessary for technical reasons.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which the guidelines have been followed and the reasons which necessitated deviation from them in the case of particular products.

The harmonization of test methods cannot be immediate, and, for certain groups of products, International Standards and/or national standards, that do not comply with these guidelines, may already exist. In cases where International Standards already exist for the product to be tested, they should be followed, but it is hoped that, when they are reviewed, they will be aligned with this International Standard so that, eventually, the only remaining departures from these guidelines will be those necessary for well established technical reasons.

**0.2** For the purpose of this International Standard, the confirmation of *Staphylococcus aureus* is based on a strongly positive coagulase reaction, but it is recognized that some strains of *Staphylococcus aureus* give weakly positive coagulase reactions. These latter strains may be confused with other bacteria but they may be distinguished from such other bacteria by the use of additional tests not included in the International Standard, such as the production of thermonuclease, of acid from mannitol, the production of haemolysin and sensitivity to lysostaphin [1].

**0.3** For statistical reasons, the lowest limit for the number of colonies counted per dish has been set at 15, but, for practical purposes, a count of lower numbers of *Staphylococcus aureus* is often required. The confidence limits of such determinations (estimated counts) are given in the annex.

## 1 Scope and field of application

This International Standard gives general guidance for the enumeration of *Staphylococcus aureus* in products intended for human consumption or feeding of animals.

## 2 Reference

ISO 6887, *Microbiology — General guidance for the preparation of dilutions for microbiological examination.*

## 3 Definition

For the purpose of this International Standard, the following definition applies.

*Staphylococcus aureus:* Micro-organisms which form typical and/or atypical colonies on the surface of a selective culture medium and which show a strongly positive coagulase reaction.

## 4 Principle

**4.1** Inoculation of the surface of a solid selective culture medium, using duplicate plates, 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 or of the initial suspension.



1) See Devriese *et al* (1978) *Staphylococcus hyicus* (Sompolinsky 1953) comb. Nov. and *Staphylococcus hyicus* ssp. chromogens ssp. Nov. *International Journal of Systematic Bacteriology* **28**, pp. 482-490.

*Bacteriological Analytical Manual,* 5th ed. 1978.
US Food and Drug Administration p. XI-4 + 5.

**4.2** Incubation of the plates at 35 °C or 37 °C for 24 to 48 h.

**4.3** Calculation of the number of *Staphylococcus aureus* per millilitre, or per gram, of sample from the number of typical and/or atypical colonies obtained on plates at dilution levels chosen so as to give a significant result, and confirmed by the coagulase test.

## 5 Diluent and culture media

### 5.1 Basic materials

In order to improve the reproducibility of the results, it is recommended that, for the preparation of the diluent and culture media, dehydrated basic components or complete dehydrated media and, for the egg yolk emulsion, a commercially available preparation, be used. The manufacturer's instructions shall be rigorously followed.

Chemical products shall be of recognized analytical quality.

The water used shall be distilled or deionized water, and shall be free from substances that might inhibit growth of *Staphylococcus aureus* under the test conditions.

If the diluent and the culture media are not used immediately, they shall be kept in the dark at a temperature between 0 and +5 °C, in conditions that prevent any change in their composition.

### 5.2 Diluent

See ISO 6887 and the specific standard dealing with the product to be examined.

### 5.3 Agar medium [1)]

#### 5.3.1 Base medium

*Composition*

| | |
|---|---|
| tryptone | 10,0 g |
| yeast extract | 1,0 g |
| meat extract | 5,0 g |
| glycine | 12,0 g |
| lithium chloride | 5,0 g |
| agar | 12 to 20,0 g[2)] |
| water | 1 000 ml |
| and, if necessary: | |
| sulphamezathine solution (5.3.2.2) | 27,5 ml |

*Preparation*

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

Add, if necessary, the 27,5 ml of the sulphamezathine (sulphadimidine, INN) solution (5.3.2.2).

Adjust the pH so that after sterilization it is 7,2 at 25 °C.

Transfer the medium in quantities of 90 ml to flasks or bottles of capacity not more than 300 ml.

Sterilize the medium for 15 min at 121 °C.

The medium may be stored for 1 month between 0 and +5 °C.

#### 5.3.2 Solutions

##### 5.3.2.1 Tellurite solution

*Composition*

| | |
|---|---|
| potassium tellurite[3)] (dipotassium trioxotellurate) | 1,0 g |
| water | 100 ml |

*Preparation*

Dissolve the potassium tellurite in the water with minimal heating.

Sterilize by filtration.

The solution may be stored for several months between 0 and +5 °C.

##### 5.3.2.2 Sulphamezathine (sulphadimidine, INN) solution

(only if the presence of Proteus is suspected)

*Composition*

| | |
|---|---|
| sulphamezathine | 0,2 g |
| sodium hydroxide solution, $c$(NaOH) = 0,1 mol/l | 10 ml |
| water, to a final volume of | 100 ml |

*Preparation*

Dissolve the sulphamezathine in the sodium hydroxide solution.

Make up to a final volume of 100 ml with the water.

##### 5.3.2.3 Sodium pyruvate solution

*Composition*

| | |
|---|---|
| sodium pyruvate | 20,0 g |
| water | 100 ml |

*Preparation*

Dissolve the sodium pyruvate in part of the water.

1) The agar medium is that of Baird-Parker, *J. Appl. Bacteriol.* **25** (1962) 12, with the addition of sulphamezathine [see Smith and Baird-Parker, ibid. **27** (1964) 78] if the presence of Proteus is suspected.

2) According to the manufacturer's instructions.

3) It is recommended to ensure beforehand that the potassium tellurite available is suitable for this test.

Make up to the final volume.

Sterilize by filtration.

The solution may be stored for not more than one month between 0 and +5 °C.

**5.3.2.4 Egg-yolk emulsion** (concentration approximately 20 % [1])

*Preparation* (if a commercial preparation is not available)

Using fresh hens' eggs, separate the yolks from the whites.

Mix the yolks thoroughly with four times their volume of water.

Heat the mixture in the water bath (6.9), controlled at 45 ± 0,5 °C, for 2 h and leave for 18 to 24 h at 0 to +5 °C to allow a precipitate to form.

Decant the supernatant liquid and sterilize it by filtration, unless the emulsion has been separated aseptically.

The emulsion may be stored at 0 to +5 °C for not longer than 72 h.

### 5.3.3 Complete medium

*Composition*

| | |
|---|---|
| base medium (5.3.1) | 90 ml |
| potassium tellurite solution (5.3.2.1) | 1,0 ml |
| sodium pyruvate solution (5.3.2.3) | 5,0 ml |
| egg-yolk emulsion (5.3.2.4) | 5,0 ml |

*Preparation*

Melt the base medium, then cool it to approximately 50 °C by means of the water bath (6.10).

Add the other liquids, mixing well after each addition.

### 5.3.4 Preparation of agar plates

Place 15 to 20 ml of the complete medium (5.3.3), cooled to approximately 45 °C, in sterile Petri dishes and allow to solidify.

The plates may be stored, prior to drying, at 0 to +5 °C for up to 24 h.

Dry the plates, preferably with the lids off and the agar surface downwards, in an oven or incubator (6.3), controlled at 50 ± 1 °C, for 30 min.

## 5.4 Brain-heart infusion broth

*Composition*

| | |
|---|---|
| peptone | 10,0 g |
| dehydrated calf brain infusion | 12,5 g |
| dehydrated beef heart infusion | 5,0 g |
| glucose | 2,0 g |
| sodium chloride | 5,0 g |
| disodium hydrogenorthophosphate ($Na_2HPO_4$) | 2,5 g |
| water | 1 000 ml |

*Preparation*

Dissolve the components or the complete medium in the water by boiling.

Adjust the pH so that after sterilization it is 7,4 at 25 °C.

Transfer the culture medium to tubes or bottles in quantities of 10 ml.

Sterilize the medium for 20 min at 121 °C.

The medium may be stored for several months between 0 and +5 °C.

## 5.5 Rabbit plasma [2]

Use commercially available dehydrated rabbit plasma and rehydrate it according to the manufacturer's instructions. Add EDTA [3] solution to give 0,1 % EDTA in the rehydrated plasma.

If dehydrated rabbit plasma is not available, dilute fresh sterile rabbit plasma 1 + 3 with sterile water.

Before use, test each batch of plasma with weakly and strongly coagulase positive strains of *Staphylococcus aureus* and strains of coagulase negative staphylococci.

# 6 Apparatus and glassware

NOTE — Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment and in particular

**6.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave)** (autoclave operating either separately or as part of an apparatus for preparing and distributing media).

Apparatus that will come into contact with the diluent, culture media, and the sample, except for apparatus that is supplied sterile (in particular plastics apparatus), shall be sterilized by one of the following methods

a) by being kept at 170 to 175 °C for not less than 1 h in an oven; or

b) by being kept at 121 ± 1 °C for not less than 20 min in an autoclave.

**6.2 Incubator,** capable of being controlled at 35 ± 1 °C or at 37 ± 1 °C.

**6.3 Drying cabinet, oven** or **incubator,** ventilated (for drying the surface of agar plates), capable of being controlled at 50 ± 1 °C.

1) According to the manufacturer's instructions.

2) Oxalated or heparinized plasma does not require EDTA. (See Baird-Parker, A.C. *The Staphylococci,* 1972, p. 11., ed. Cohen, J.O. Wiley-Interscience and Sons. Inc. New York, London).

3) Ethylenediaminetetraacetic acid.

**6.4 Test tubes,** of dimensions 18 mm × 180 mm, and **flasks,** of capacity 125 to 300 ml, or **bottles,** for the sterilization and storage of culture media.

Test tubes, of dimensions 10 mm × 75 mm, or bottles are also required for the coagulase test.

**6.5 Petri dishes,** made of glass or plastics material, of diameter 90 to 100 mm, or 140 mm if necessary.

**6.6 Total delivery pipettes** (blowout pipettes), of nominal capacity 1 ml.

**6.7 Pipettes,** calibrated especially for bacteriological use, of nominal capacity 1 ml, graduated in 0,1 ml divisions and having an outlet of diameter 2 to 3 mm.

**6.8 Glass spreaders** (hockey sticks), made from glass rods approximately 3,5 mm in diameter and 20 cm long for example, bent at right angles about 3 cm from one end; the cut ends should be made smooth by heating.

**6.9 Water bath,** or similar apparatus, capable of being controlled at 45 ± 0,5 °C.

**6.10 Water bath,** or similar apparatus, capable of being controlled at 50 ± 0,5 °C.

**6.11 pH meter,** accurate to 0,1 pH unit at 25 °C.

## 7 Sampling

Carry out sampling in accordance with the specific standard appropriate to the product concerned. If such a specific standard is not available, it is recommended that agreement be reached on this subject by the parties concerned.

## 8 Preparation of the test sample

See the specific standard appropriate to the product concerned. If such a specific standard is not available, it is recommended that agreement be reached on this subject by the parties concerned.

## 9 Procedure

NOTE — In the case of heavily contamined samples, it is advisable to make a direct microscopic examination beforehand.

### 9.1 Test portion, initial suspension and dilutions

See ISO 6887 and the specific standard appropriate to the product concerned.

### 9.2 Inoculation

**9.2.1** Transfer, by means of a sterile pipette, 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 each of two agar plates (5.3.4). Repeat the procedure for the $10^{-2}$ dilution and for further dilutions if necessary.

NOTE — If, for certain products, it is desirable to count low numbers of *Staphylococcus aureus,* the limits of detection can be raised by a factor of 10 by inoculating 1,0 ml of the test sample if liquid, or 1,0 ml of the initial suspension for other products. Distribute the inoculum either on the surface of a large agar plate (140 mm) or on the surface of three small agar plates (90 mm). In both cases, prepare duplicates by using two large plates or six small ones.

**9.2.2** Carefully spread the inoculum as quickly as possible over the surface of the agar plate, trying not to touch the sides of the dish, using the glass spreader (6.8). Use a sterile spreader for each plate. Allow the plates to dry with their lids on for about 15 min at room temperature.

### 9.3 Incubation

Invert the plates prepared according to 9.2.2 and incubate them for 24 to 48 h in the incubator (6.2) at 35 ± 1 °C or 37 ± 1 °C.

### 9.4 Selection of plates and interpretation

After incubation for 24 to 26 h, mark on the bottom of the plates the positions of any typical colonies present (see note 1).

Re-incubate all plates at 35 ± 1 °C or 37 ± 1 °C for a further 22 to 24 h, and mark any new typical colonies; also mark any atypical colonies present (see note 1).

Take for enumeration only those plates (9.2.1 and 9.4; see note 2) that contain between 15 and 150 typical and/or atypical colonies (see note 3). Select for confirmation (9.5) 5 typical and/or 5 atypical colonies, as the case may be, from each plate.

If there are less than 15 typical and/or atypical colonies 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 note 4 and 10.6.

NOTES

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

Atypical colonies are similar in appearance but without a clear zone. Atypical colonies are formed frequently by strains of *Staphylococcus aureus* contaminating dairy products. They are not frequently formed by strains of *Staphylococcus aureus* contaminating other products.

2 If a 1,0 ml inoculum was spread over three plates (see the note to 9.2.1), treat these plates as one in all subsequent counting and confirmation procedures.

3 Where each colony type (typical and atypical) predominates at a different dilution of the sample, retain plates from both dilutions for selection of colonies of both types.

4 To make an estimated count of lower numbers of *Staphylococcus aureus* (see 0.3), retain all plates that contain any typical and atypical colonies. Select all such colonies for confirmation within the limits set out above.

### 9.5 Confirmation (coagulase test)

From the surface of each selected colony (9.4), remove an inoculum with a sterile wire and transfer it to a tube or bottle of brain-heart infusion broth (5.4). Incubate for 20 to 24 h at 35 °C or 37 °C.

Add 0,1 ml of each culture aseptically to 0,3 ml of the rabbit plasma (5.5) (unless other amounts are specified by the manufacturer) in sterile tubes of dimensions 10 mm × 75 mm or bottles, and incubate at 35 °C or 37 °C.

Examine for clotting of the plasma after 4 to 6 h.

Consider the coagulase test to be positive if the volume of the clot occupies more than three-quarters of the original volume of the liquid.

As a control, add 0,1 ml of sterile brain-heart infusion broth (5.4) to the recommended quantity of rabbit plasma (5.5) and incubate without inoculation. For the test to be valid, the control plasma shall show no signs of clotting.

## 10 Expression of results

### 10.1 Method of calculation

See the notes to 9.4.

**10.1.1** If at least 80 % of the colonies selected are coagulase positive (9.5), take as the number of *Staphylococcus aureus* the number of presumptive *Staphylococcus aureus* obtained by the count made in 9.4.

**10.1.2** In all other cases, calculate the number from the percentage of the number of presumptive *Staphylococcus aureus* (9.4) that are coagulase positive (9.5).

**10.1.3** For dishes containing between 15 and 150 typical and/or atypical colonies at two consecutive dilutions, calculate the number of *Staphylococcus aureus* for each dilution as specified in 10.1.1 and 10.1.2 and take as the result the arithmetic mean of the two values obtained, unless the ratio of the higher value to the lower value is greater than 2; in this case, take the lower value as the result.

**10.1.4** Calculate the average number of *Staphylococcus aureus* from the counts obtained on the duplicate plates (10.1.1 and 10.1.2) or from the two consecutive dilutions in the case of 10.1.3.

If typical and/or atypical colonies were present on the plates taken for enumeration, count these colonies separately.

Add together the calculated average numbers of *Staphylococcus aureus* obtained for both typical and atypical colonies, taking into account the dilution factor of each.

Retain only two significant figures, proceeding as follows:

— if the number is less than 100, round it to the nearest multiple of 5;

— if the number is greater than 100 and ends in 5, round it to the nearest multiple of 20;

— if the number is greater than 100 and does not end in 5, round it to the nearest multiple of 10.

Multiply this value by the reciprocal of the inoculum volume (see note 2 to 9.4) and then by the reciprocal of the corresponding dilution of the test sample to obtain the number of *Staphylococcus aureus* per millilitre or per gram of product, according to the case.

Express the result as a number between 1,0 and 9,9 multiplied by $10^n$, where $n$ is the appropriate power of 10. An example of the calculation is given in 10.2.

**10.1.5** For estimating low numbers, take the average of the counts of confirmed typical and atypical colonies (9.4) and round to the next highest whole number.

**10.1.6** If the average number of confirmed colonies, as calculated in 10.1.4, is less than 15 from the plates inoculated with the test sample (liquid product) or the initial suspension (other products), report the result as:

a) for liquid products:

— fewer than 15 × $n_e$ *Staphylococcus aureus* per millilitre, where $n_e$ is the reciprocal of the volume of inoculum:

$$n_e = \frac{1}{\text{inoculum volume}}$$

b) for other products:

— fewer than 15 × $N_e$ *Staphylococcus aureus* per gram, where

$$N_e = \frac{1}{\text{inoculum volume}} \times \frac{1}{\text{dilution of test sample}}$$

c) for estimating low numbers in liquid products:

$m \times n_e$ *Staphylococcus aureus* per millilitre

where $m$ is the average number of confirmed colonies;

d) for estimating low numbers in other products:

$m \times N_e$ *Staphylococcus aureus* per gram

where $m$ is the average number of confirmed colonies.

Confidence intervals for estimated counts [c) and d)] are given in the annex.

**10.1.7** If there are no confirmed colonies on plates corresponding to the test sample (liquid product) or the initial suspension (other products), report the result as:

a) for liquid products:

— fewer than $1 \times n_e$ *Staphylococcus aureus* per millilitre

b) for other products:

— fewer than $1 \times N_e$ *Staphylococcus aureus* per gram

## 10.2 Example of calculation of *Staphylococcus aureus* count

For the example, the 0,1 ml of inoculum of the $10^{-2}$ dilution of the sample gave 65 and 85 typical colonies on each plate (9.4).

No atypical colonies were identified on any plates.

All 5 colonies selected from the plate containing 65 colonies were coagulase positive (9.5) and, therefore, all 65 colonies were considered to be *Staphylococcus aureus.*

Three of the 5 colonies selected from the plate containing 85 colonies were coagulase positive and therefore 60 % of the 85, i.e. 51 colonies, were considered to be *Staphylococcus aureus.*

The average count (10.1.4) is

$$\frac{65 + 51}{2} = 58 \text{ } \textit{Staphylococcus aureus}$$

The total count (typical + atypical colonies) is

$58 + 0 = 58$ (10.1.5)

The count of 58 is rounded to the nearest multiple of 5, i.e.: 60.

The number of *Staphylococcus aureus* per gram or per millilitre is

$$\text{count} \times \frac{1}{\text{inoculum volume}} \times \frac{1}{\text{dilution of test sample}}$$

$$= 60 \times \frac{1}{0,1} \times \frac{1}{10^{-2}}$$

$$= 60 \times 10 \times 10^2$$

$$= 60 \times 10^3$$

$= 6,0 \times 10^4$ *Staphylococcus aureus* per gram or per millilitre

## 10.3 Precision of the count (10.1.4)

For statistical reasons, the confidence intervals of this method vary, in 95 % of cases, from ± 16 % to ± 52 %.[1] In practice, even greater variation may be found, especially between results obtained by different workers.

# 11 Test report

The test report shall show the method used, the temperature of incubation, and the results obtained. It shall also mention all operating conditions not specified in this International Standard, or regarded as optional, as well as any incidents that may have influenced the result.

The test report shall include all the information necessary for the complete identification of the sample.



1) See Cowell and Morisetti (1969), *J. Sci. Fd. Agric.* **20**, p. 573.

# Annex

## Confidence intervals for estimated counts

The 95 % confidence intervals for estimated counts, where the average number of confirmed colonies from pairs of plates inoculated with the test sample or initial suspension is less than 15 *Staphylococcus aureus,* are given in the table.

| *Staphylococcus aureus* count | 95 % confidence intervals | |
|---|---|---|
| | lower | upper |
| 1 | < 1 | 2 |
| 2 | < 1 | 4 |
| 3 | < 1 | 5 |
| 4 | 1 | 6 |
| 5 | 2 | 9 |
| 6 | 2 | 10 |
| 7 | 2 | 12 |
| 8 | 3 | 13 |
| 9 | 4 | 14 |
| 10 | 4 | 16 |
| 11 | 5 | 18 |
| 12 | 6 | 19 |
| 13 | 7 | 20 |
| 14 | 7 | 21 |
| 15 | 8 | 23 |