anses
French agency for food, environmental and occupational health safety

Laboratory for Food Safety
Maisons-Alfort location

EURL CPS
European Union Reference Laboratory for Coagulase Positive Staphylococci
http://eurl-staphylococci.anses.fr

Guide for implementation of Standard methods EN ISO 6888-1 and EN ISO 6888-2 in accordance with EN ISO 7218

Version 1.3 – [18-07-2019]

Alexandra CAUQUIL, Unit Staphylococci, Bacillus, Clostridium

French Agency for Food, Environmental and Occupational Health Safety – Food Safety Laboratory
Maisons-Alfort location and Boulogne-sur-Mer location
14, rue Pierre et Marie Curie - F94706 Maisons-Alfort Cedex - Telephone: + 33 149 771 350 - Fax: + 33 149 771 380
Boulevard du Bassin Napoléon - F62200 Boulogne-sur-Mer - Téléphone : +33 321 992 500 – Télécopie : + 33 321 991 725
www.anses.fr - @Anses_fr

TABLE OF CONTENT

1 INTRODUCTION

Several methods are used by EURL and NRLs for Coagulase Positive Staphylococci (CPS) to implement the CPS microbiological criteria of the Regulation 2073/2005 on microbiological criteria for foodstuffs. One the EURL CPS activities is to draft guides and standard operating procedures (SOPs), which aim at facilitating the implementation of these methods.

This document provides a guide for the implementation of the Standard methods EN ISO 6888-1 [1] and EN ISO 6888-2 [2], cited as reference methods in Regulation 2073/2005, in accordance with the Standard EN ISO 7218 [3] giving general guidance and requirements for food microbiological analysis.

There are different steps of analysis: sampling, transport, receipt, storage, test portion, dilution, incubation and test report. This document gives guidance for each of these different steps.

2 SAMPLING

It is important that the laboratory receive a sample which is representative of the batch of product and has not been damaged or changed during transport and storage.

3 TRANSPORT

The mode of transportation of the samples to the laboratory shall ensure that they are kept under conditions which will minimize any alteration in the number of microorganisms present.

Deliver samples to the laboratory promptly with the original storage conditions maintained as nearly as possible.

4 RECEIPT

The laboratory must check the condition of the samples on receipt.

If their condition is unsatisfactory or if the weight of samples are insufficient, the laboratory can refuse the samples.

Record different information: date of receipt, details on sampling, client's name and address.

The sample should be examined as soon as possible and preferably within 24h.

5 STORAGE

Store the samples under conditions, which will minimize alteration in the number of microorganisms present:

- Stable products : 18°C to 27°C
- Frozen products: below -15°C and preferably below -18°C
- Products not stable at ambient temperature: 3°C±2°C (see series EN ISO 6887 [4])
- Swab samples (see EN ISO 18593 [5] and EN ISO 17604 [6])

6 TEST PORTION, INITIAL SUSPENSION AND DILUTION

Refer to the relevant part of EN ISO 6887, for specific rules for taking the test portion and preparing the initial suspension.

The test portion should be representative of the laboratory sample and is used for the preparation of the initial suspension. Test portion size is in general 10g for enumeration of coagulase positive staphylococci (CPS).

The initial suspension, also named primary dilution may be: a suspension, a solution or an emulsion obtained after weighing or measuring a quantity of the product under examination mixed with, normally, a nine-fold quantity of diluent, allowing large particles, if present, to settle (see Figure 1).

The time lapse between preparing the initial suspension of the sample and inoculating the last tube should be less than 30 min, according to EN ISO 7218/1 (see Figure 1).

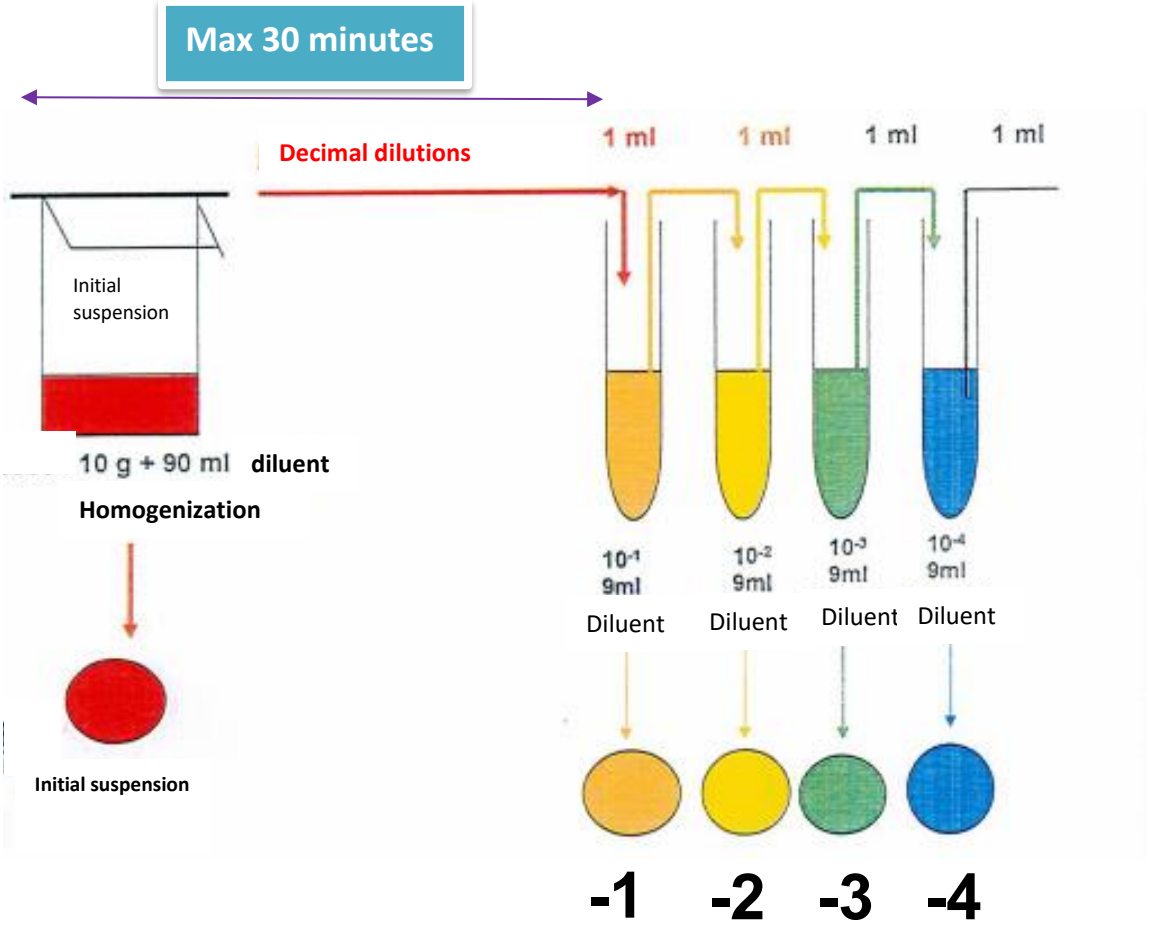

Figure 1. Preparation of initial suspension and decimal dilutions

7 INCUBATION / ENUMERATION

7.1 EN ISO 6888-1 [1]

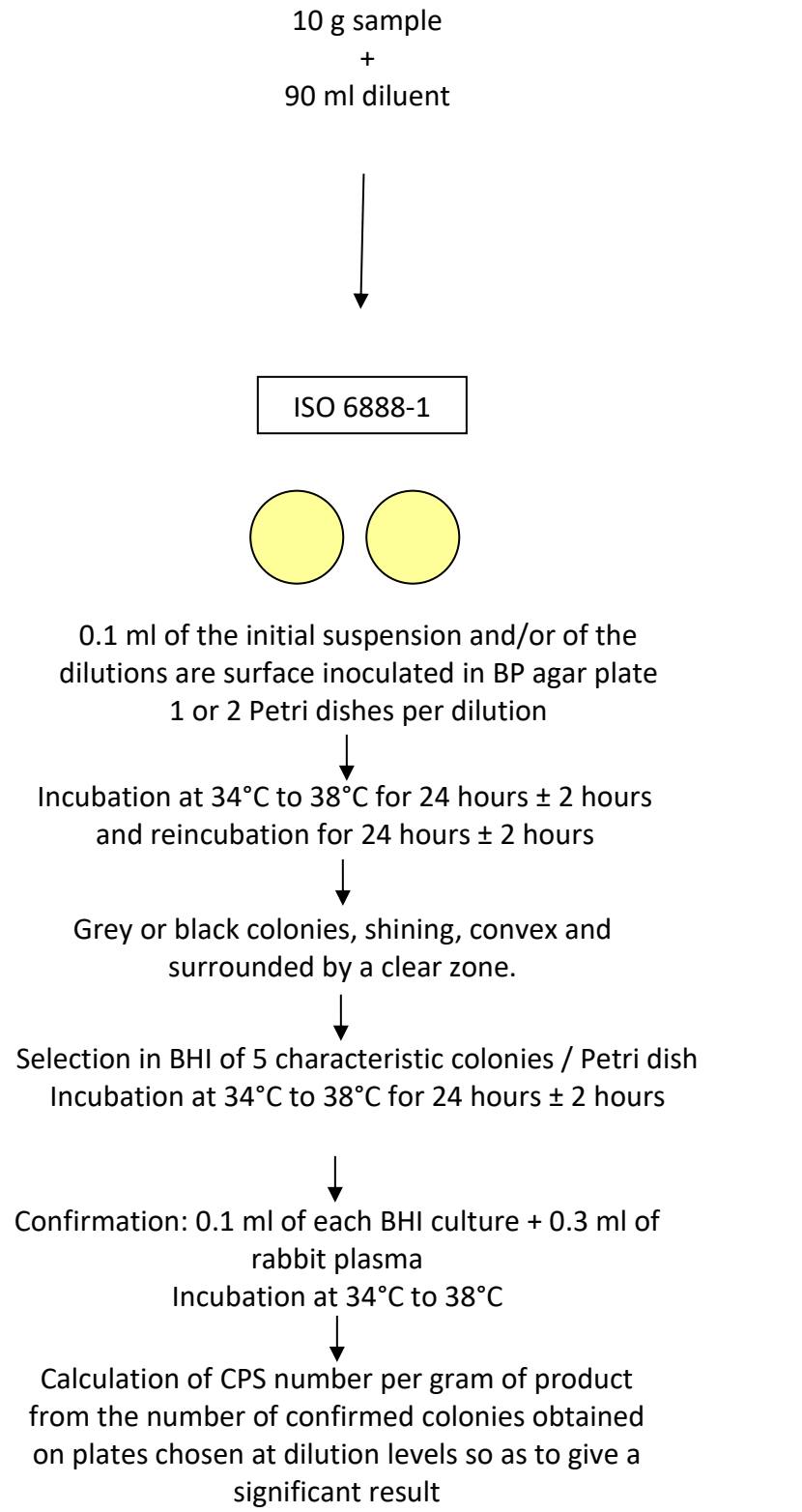

Figure 2. Scheme of the method EN ISO 6888-1

It is important to note: 1 ml of initial suspension may be spread on 3 Petri dishes of 90 mm or 1 ml of initial suspension on 1 Petri dish of 140 mm to increase the sensitivity of enumeration results. It is advised for CPS enumeration because of low numbers of coagulase positive staphylococci often found in food.

For colony-count techniques in food microbiology and according to EN ISO 7218, use one plate per dilution with at least two successive dilutions, for laboratory under quality assurance (EN ISO 17025 accredited). If only one dilution is performed or if laboratory is not under quality assurance then use two plates per dilution. Two plates per dilution may also be systematically used to improve reliability of results.

7.2 EN ISO 6888-2 [2]

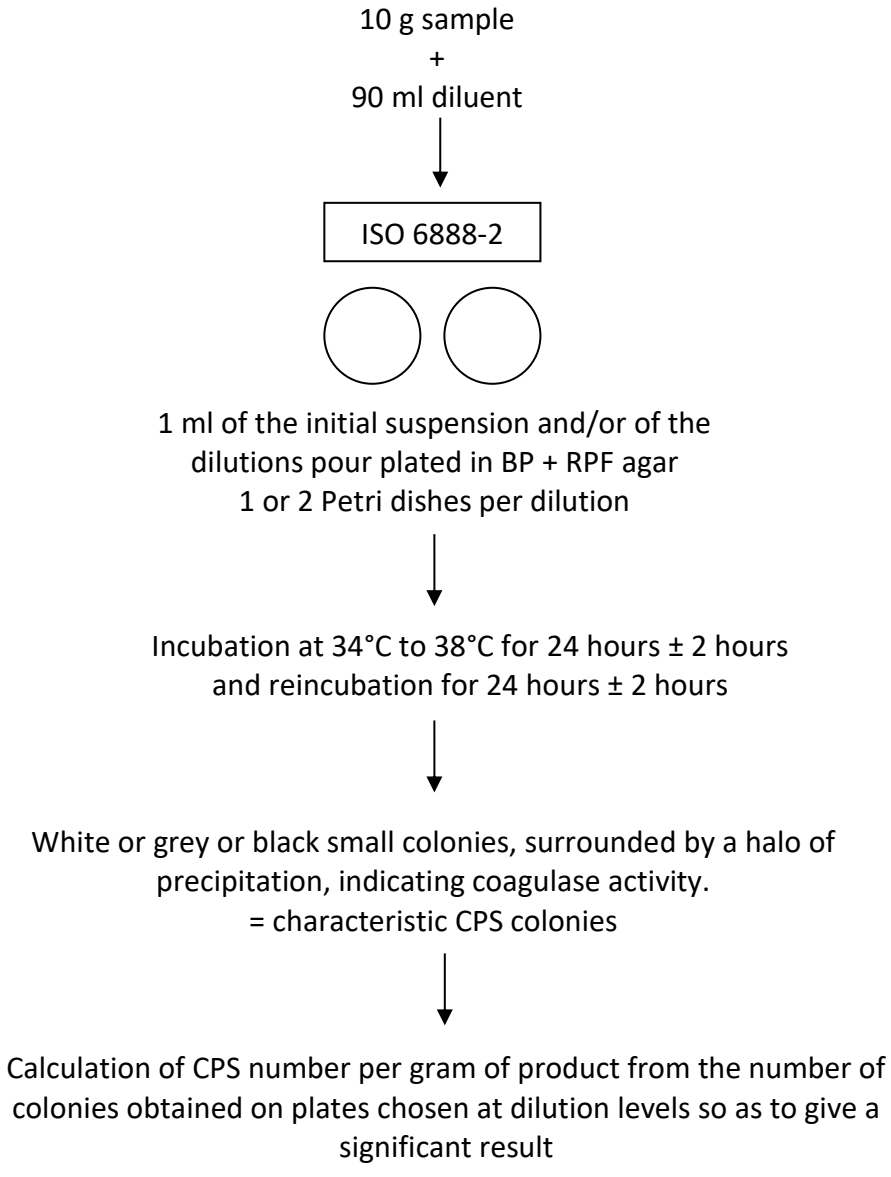

Figure 3. Scheme of the method EN ISO 6888-2

For colony-count techniques in food microbiology and according to EN ISO 7218, use one plate per dilution with at least two successive dilutions, for laboratory under quality assurance (EN ISO 17025 accredited). If only one dilution is performed or if laboratory is not under quality assurance then use two plates per dilution. Two plates per dilution may also be systematically used to improve reliability of results.

8 TEST REPORT

The test report shall specify the following:

- all information necessary for the complete identification of the sample;
- the sampling method used, if known;
- the test method used, with reference to this part of EN ISO 6888 (part 1 or part 2);
- the incubation temperature used;
- all operating details not specified in this part of EN ISO 6888 (part 1 or part 2), or regarded as optional, together with details of any incidents which may have influenced the test results;
- the results obtained.

8.1 ENUMERATION LIMIT

In accordance with EN ISO 6888-1, the first dilution (initial suspension) partially determines the value of the lower enumeration limit, which also depends on the technique used (surface plating with a 1 ml inoculum in a 1/10 suspension, for which this limit is 10 cfu per gram). The expression of results for the negative sample should be expressed as “less than … cfu/g”.

Example for EN ISO 6888-1:

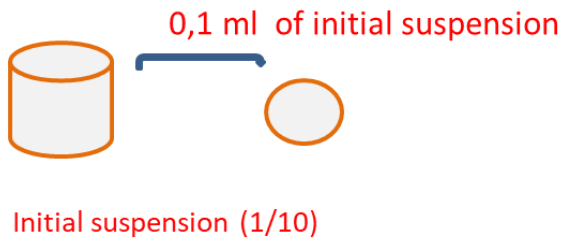

Figure 4. Example of expression of results for the negative sample with EN ISO 6888-1

The result for a negative sample is <10 cfu/ml (initial suspension) or <100 cfu/g.

Example for EN ISO 6888-2:

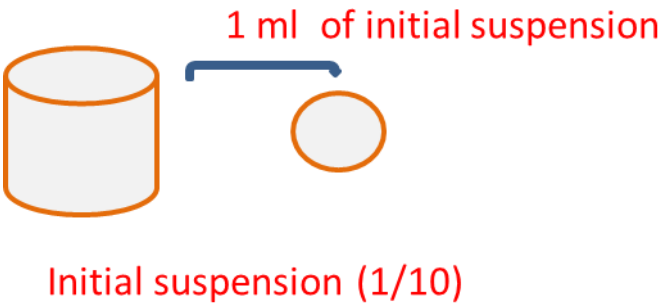

Figure 5. Example of expression of results for the negative sample with EN ISO 6888-2

The result for the negative sample is <1 cfu/ml (initial suspension) or <10 cfu/g.

8.2 CALCULATION ACCORDING TO EN ISO 7218/A1 [7]

8.2.1 GENERAL CASE: 1 PLATE PER DILUTION WITH AT LEAST TWO SUCCESSIVE DILUTIONS

Conditions:

- one plate per dilution at two successive dilutions,
- at least one plate with at least 10 colonies,
- maximum countable number of colonies:
  - 300 colonies with 150 typical and/or atypical colonies at two successive dilutions for EN ISO 6888-1
  - 300 colonies with 100 typical colonies[1] at two successive dilutions for EN ISO 6888-2

[1] A limit of 100 typical colonies per plate for RPFA in EN ISO 6888-2 (instead of 150 in EN ISO 7218), has been set to be able to count well separated colonies and to see their coagulase halo.

The confidence intervals are given in the following table (see Annex B1 of EN ISO 7218/A1).

Table B.1 — Weighted means and confidence intervals δ for relevant numbers of colonies

Weighted mean of number of colonies counted on two successive dilutions	System "1 plate per dilution" Confidence interval δ	System "2 plates per dilution" Confidence interval δ
300	268 to 332	277 to 323
150	127 to 173	134 to 166
100	**81 to 119**	**87 to 113**
30	**20 to 40**	**23 to 37**
15	7 to 22	10 to 20
10	4 to 16	6 to 14
7	Not applicable	3 to 10

In accordance with EN ISO 7218 [3], the result is calculated as follows:

$$N = \frac{\sum C}{V \times 1{,}1 \times d}$$

where:

$\sum C$ is the sum of the coagulase-positive staphylococcal colonies on the two dishes retained from two successive dilutions, at least one of which contains a minimum of 10 colonies

V is the volume of inoculum placed in on each dish, in millilitres

d is the dilution rate corresponding to the first dilution retained [d = 1 when the undiluted liquid product (test sample) is retained]

8.2.1.1 EN ISO 6888-1

With EN ISO 6888-1, after confirmation, calculate the number a of identified CPS using the following equation:

$$a = \frac{b}{A} \times C$$

where:

b is the number of colonies complying with identification criteria among the identified colonies A

C is the total number of presumptive colonies counted on the dish

Then, calculate the number *N* of identified CPS present in the test sample, using the following equation:

$$N = \frac{\sum a}{V \times 1{,}1 \times d}$$

where:

$\sum a$ is the sum of the coagulase-positive staphylococcal colonies identified on the two dishes retained from two successive dilutions, at least one of which contains a minimum of 10 colonies

V is the volume of inoculum placed in on each dish, in millilitres

d is the dilution rate corresponding to the first dilution retained [d = 1 when the undiluted liquid product (test sample) is retained]

Example for EN ISO 6888-1:

Number of typical colonies obtained after confirmation at the first countable dilution: 128

Number of typical colonies obtained after confirmation at the second dilution: 13

So, $\sum a$ = 128+13 = 141

V = 0,1 ml

d = 10^{-2}

$$N = \frac{\sum a}{\text{V X 1,1 X d}} = \frac{141}{0.1 \text{ X } 1{,}1 \text{ X } 10^{-2}} = 1{,}3 \text{ x } 10^5$$

8.2.1.2 EN ISO 6888-2

In accordance with EN ISO 7218, calculate the result as follows:

$$N = \frac{\sum C}{V \times 1{,}1 \times d}$$

where:

$\sum C$ is the sum of CPS colonies on the two dishes retained from two successive dilutions, at least one of which contains a minimum of 10 colonies

V is the volume of inoculum placed in on each dish, in millilitres

d is the dilution rate corresponding to the first dilution retained [d = 1 when the undiluted liquid product (test sample) is retained]

Example for EN ISO 6888-2:

Number of colonies obtained at the first countable dilution: 78

Number of colonies obtained at the second dilution: 8

So, $\sum C$ = 78+8 = 86

V = 1 ml

d = 10^{-3}

$$N = \frac{\sum C}{\text{V X 1,1 X d}} = \frac{86}{1 \text{ X } 1{,}1 \text{ X } 10^{-3}} = 7{,}8 \text{ x } 10^4$$

8.2.2 2 PLATES PER DILUTION ON 2 SUCCESSIVE DILUTIONS (EN ISO 7218/A1 ANNEX D3)

Calculate the result with the following formula:

$$N = \frac{\sum C}{V \times \left[n_1 + (0{,}1 \times n_2) \right] \times d}$$

where:

$\sum C$ is the sum of CPS colonies on all the dishes retained from two successive dilutions, and where at least one contains a minimum of 10 colonies

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

n_1 is the number of dishes retained at the first dilution

n_2 is the number of dishes retained at the second dilution

d is the dilution factor corresponding to the first dilution retained [d = 1 when the undiluted liquid product (test sample) is used]

Example for EN ISO 6888-1:

Number of typical colonies obtained after confirmation at the first countable dilution on the plate A: 128

Number of typical colonies obtained after confirmation at the first countable dilution on the plate B: 126

Number of typical colonies obtained after confirmation at the second dilution on the plate A: 13

Number of typical colonies obtained after confirmation at the second dilution on the plate B: 12

So, $\sum a$ = 128+126+13+12 = 279

V = 0,1 ml

n_1 = 2

n_2 = 2

d = 10^{-2}

$$N = \frac{\sum a}{\text{V X } [n_1 + (0{,}1 \text{ X } n_2)] \text{ X d}} = \frac{279}{0{,}1 \text{ X } [2 + (0{,}1 \text{ X } 2)] \text{ X } 10^{-2}} = 1{,}3 \text{ x } 10^5$$

Example for EN ISO 6888-2:

Number of colonies obtained at the first countable dilution on the plate A: 78

Number of colonies obtained at the first countable dilution on the plate B: 76

Number of colonies obtained at the second dilution on the plate A: 8

Number of colonies obtained at the second dilution on the plate B: 7

So, $\sum C$ = 78+76+8+7= 169

V = 1 ml

d = 10^{-3}

$$N = \frac{\sum C}{\text{V X } [n_1 + (0{,}1 \text{ X } n_2)] \text{ X d}} = \frac{169}{1 \text{ X } [2 + (0{,}1 \text{ X } 2)] \text{ X } 10^{-3}} = 7{,}7 \text{ x } 10^4$$

8.2.3 SPECIAL CASES

8.2.3.1 CASE 1

If the number of colonies is from 3 to 1, the precision of the result is too low and report the result as:
« Microorganisms are present but less than 4/cfu per gram or per ml »

8.2.3.2 CASE 2

If the plate contains less than 10 colonies, but at least 4, report the result as:
Number Estimated NE : $\frac{C}{V \times d \times n}$ in cfu/ml of initial suspension

where:
c is the number of colonies
d is the first dilution retained
V is the volume of the inoculum used in each dish
n is the number of Petri dish

8.2.3.3 CASE 3

Where the counting of colonies (total colonies, typical colonies or presumptive colonies) for each one of the dishes for all inoculated dilutions produces a number greater than 150:
« more than: (150/Vd) X (b/A) »

where:
d is the dilution of the last inoculated dilution
V is the volume of the inoculum used in each dish
b is the number of colonies complying with identification among the inoculated colonies
A

8.3 EXPRESSION OF RESULTS ON THE TEST REPORT

Express the calculated result as:

- rounded to two significant figures
  If third figure is less than 5 => no modification of the preceding figure
  If third figure is greater or equal to 5 => increase preceding figure by one unit
- with a number between 1,0 and 9,9 x 10^N
- whole number with two significant figures
- N of microorganisms
  per millilitre (liquid products)
  or per gram (other products)

Take as the result the CPS number per millilitre (liquid products) or per gram (other products), expressed as a number between 1,0 and 9,9 inclusive, multiplied by 10^x where x is the appropriate power of 10.

9 BIBLIOGRAPHY

[1] Standard EN ISO 6888-1: “Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species) -- Part 1: Technique using Baird-Parker agar medium”.

[2] Standard EN ISO 6888-2: “Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species) -- Part 2: Technique using rabbit plasma fibrinogen agar medium”.

[3] Standard EN ISO 7218: “General requirements and guidance for microbiological examinations”

[4] Standard EN ISO 6887 series: “Preparation of test samples, initial suspension and decimal dilutions for microbiological examination”

[5] Standard EN ISO 18593: “Horizontal methods for sampling techniques from surfaces using contact plates and swabs”

[6] Standard EN ISO 17604: “Carcass sampling for microbiological analysis”

[7] Standard EN ISO 7218/A1: “General requirements and guidance for microbiological examinations- Amendment1”