INTERNATIONAL STANDARD

ISO 8607

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Artificial insemination of animals — Frozen semen of breeding bulls — Enumeration of living aerobic microorganisms

Insémination artificielle des animaux — Semences congelées de taureaux reproducteurs — Dénombrement des micro-organismes aérobies vivants

Reference number
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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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.

ISO 8607 was prepared by Technical Committee ISO/TC 34, *Food products*.

This first edition of ISO 8607 cancels and replaces ISO/TR 8607:1991, which has been technically revised.

Introduction

The quantitative microbiological control of the hygienic collection and handling of bovine semen is of great importance in order to predict the efficiency of artificial insemination and to fulfil the requirements of biosecurity (see reference [1]). For the same reason, the investigation of bacterial contamination and the possible presence of facultative pathogenic microorganisms in the preserved bovine semen is also very important.

There is a need for an international method suitable for the determination of the microbial count in frozen semen, which indicates the hygienic status during collection, handling and storage. The aim of the colony-count method specified in this International Standard is to enumerate the saprophytic microorganisms that are originally present in and/or are transmitted to the bovine semen from the environment. With this method only the total count of bacteria is detected, mainly the aerophilic and mesophilic saprophytic ones, as well as a few facultative pathogenic microorganisms that are not very sensitive to environmental conditions.

Since samples of frozen bovine semen contain additional antibiotics, the determination of microbiological contamination of this type of sample is slightly different from the commonly used microbiological methods. When examining preserved semen samples in low dilutions, the number of colonies may be lower than expected and do not follow the usual proportions. Therefore relatively high decimal dilutions should be used to compensate for the inhibition effect of the antibiotics. As a result of the necessary high dilutions, 15 or less colonies can be observed in each Petri dish and this result should be accepted. This differs from the usual microbiological examinations of food where the sample dilution can be chosen in such a way that the number of colonies is more than 15 in a Petri dish so more precise examination is possible.

Microbial cells often occur as clumps or groups in the samples. Whereas shaking samples and dilutions may uniformly distribute the clumps of bacteria, this may not completely disrupt the clumps themselves into single cells. Consequently, each colony that appears on the medium can arise from a clump of cells or from a single cell and therefore it is more precise to express the result as the number of colony-forming units (CFU) of microorganisms than to give the number of microorganisms (see reference [2]).

This International Standard does not specify a tolerable limit value for the total CFU of bacteria, which may be a consumer requirement in trade. This should be given in commercial contracts.

A list of publications related to this International Standard is given in the Bibliography.

Artificial insemination of animals — Frozen semen of breeding bulls — Enumeration of living aerobic microorganisms

1 Scope

This International Standard specifies a method for the enumeration of living aerobic microorganisms present in the frozen semen of breeding bulls. The colonies growing in a solid medium after aerobic incubation at 37 °C are counted. The microbiological contamination of the sample is expressed as a number of colony-forming units of microorganisms per millilitre of the test sample.

2 Normative references

The following referenced documents are indispensable for the application 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 6887-1, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*

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

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 6887-1 and the following apply.

3.1
semen
product of the genital organs of a male, intended for the fertilization of a female

3.2
ejaculate
quantity of semen obtained as a result of mating the male

3.3
dose
quantity of semen which is packaged individually and carries a unique identification, intended for a single artificial insemination

3.4
series of doses
group of doses of semen obtained from one bull and prepared from one or more ejaculates, obtained on the same day and subjected to the same treatment

3.5
living aerobic microorganisms
bacteria, yeasts and moulds which grow aerobically at 37 °C under the conditions specified in this International Standard

3.6
colony-forming unit
CFU
single microbial cell, or clumps or a group of cells, forming one colony on the medium under the conditions specified in this International Standard

4 Principle

Two poured plates are prepared using a specified culture medium. These are deep inoculated with a specified quantity of test sample, followed by aerobic incubation at 37 °C.

The number of CFU of microorganisms per millilitre of the test sample is calculated from the number of colonies obtained.

5 Diluent and culture medium

For general guidance, see ISO 7218.

Chemical products shall be of recognized analytical quality and suitable for microbiological analysis.

The water used shall be distilled water or of equivalent quality (see ISO 7218).

5.1 Diluent

The diluent is a peptone salt solution as specified in ISO 6887-1. Its composition, preparation and use are given only for the convenience of the users of this International Standard.

In order to improve the reproducibility of the results, it is recommended that, for the preparation of the diluent, dehydrated basic components or a dehydrated complete preparation should be used. The manufacturer's instructions shall be rigorously followed.

5.1.1 Composition

Enzymatic digest of casein	1,0 g
Sodium chloride (NaCl)	8,5 g
Water	1 000 ml

5.1.2 Preparation

Dissolve the components in the water, by heating if necessary.

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

5.1.3 Distribution and sterilization

Dispense the diluent in volumes as necessary for the preparation of the initial suspensions into test tubes or flasks (6.3) of appropriate capacity.

Dispense the diluent in volumes as necessary for the preparation of the decimal dilutions into test tubes or flasks (6.3) in quantities such that, after sterilization, each tube or flask contains 9,0 ml. The uncertainty of measurement of this final volume, after sterilization, shall not exceed ± 2 %.

5.2 Agar medium

5.2.1 Composition

Meat extract	10,0 g
Anhydrous D-glucose ($C_6H_{12}O_6$)	1,0 g
Dehydrated yeast extract	2,5 g
Peptone	3,0 g
Sodium chloride (NaCl)	2,0 g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)	2,0 g
Gelatine	10,0 g
Agar in powder or flake form	12,0 g to 18,0 g[1)]
Water	1 000 ml

5.2.2 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 7,2 ± 0,2 at 25 °C.

Dispense the medium into tubes or flasks (6.3), in quantities such that the container is half-full.

Sterilize in an autoclave (6.1) at 121 °C ± 1 °C for 15 min.

If the medium is to be used immediately, cool it to 44 °C to 47 °C in the water bath (6.8) and then add 10 % (by volume) inactivated and sterilized[2)] bovine or sheep serum. Otherwise, before beginning the microbiological examination, completely melt the medium in the boiling water bath (6.9), cool to 44 °C to 47 °C in another water bath (6.8), and then add 10 % (by volume) inactivated and sterilized[2)] bovine or sheep serum.

6 Apparatus

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

Usual microbiological laboratory apparatus and, in particular, the following.

6.1 Sterilizing oven (for dry sterilization) or **autoclave** (for wet sterilization), see ISO 7218.

6.2 Incubator, capable of being maintained at 37 °C ± 1 °C.

6.3 Test tubes, of 16 mm diameter and 160 mm length, or **flasks**, of capacity not greater than 500 ml.

6.4 Petri dishes, made of glass or plastic, of 90 mm to 100 mm diameter.

6.5 Pipettes, having a nominal capacity of 1 ml, graduated in 0,1 ml divisions.

Blow-out pipettes shall not be used.

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1) According to the gel strength of the agar.

2) By ultrafiltration (0,2 nm filter).

6.6 pH-meter, electric, accurate to ± 0,1 pH unit at 25 °C.

6.7 Water bath, capable of being maintained at 37 °C ± 1 °C.

6.8 Water bath, capable of being maintained at 45 °C ± 1 °C.

6.9 Boiling water bath.

6.10 Colony-counting equipment, consisting of an illuminated base with a dark background, fitted with a magnifying lens suitable for use at a magnification of ×1,5, and a mechanical or electronic digital counter.

7 Sampling

Choose at random from a series of doses the necessary number of doses of deep-frozen semen of any type (pellets or minitubes of 0,25 ml or 0,5 ml) so that the volume of sample is 1,0 ml per series of doses.

Store the test samples in liquid nitrogen.

When required for examination, the test samples may be transferred from the large liquid-nitrogen storage container to a small liquid-nitrogen laboratory container.

8 Preparation of test sample

Before use, thaw the test sample in the water bath (6.7) set at 37 °C for 3 min.

Thawed test samples may be kept in a refrigerator at 4 °C but for no longer than 1 h.

9 Procedure

9.1 Test portion, initial suspension and dilutions

Prepare the initial suspension in accordance with ISO 6887-1 in a safety cabinet. The number of further dilutions to be carried out depends on the antibiotics content of the initial suspension as specified below.

a) If the initial suspension contains the usual content of antibiotics [i.e. 10^3 International Units (IU)[3)] of penicillin and 1 mg of streptomycin or another broad-spectrum antibiotic per millilitre], use a 10^{-5} final dilution.

b) If the quantity of antibiotics differs from that mentioned in a), use a final dilution such that the content of penicillin is not more than 0,1 IU/ml and that of the broad-spectrum antibiotic not more than 0,1 µg/ml.

NOTE A higher antibiotics concentration can inhibit growth of the microorganisms and so produce false results.

9.2 Inoculation and incubation

9.2.1 Take two sterile Petri dishes (6.4). Transfer, by means of a sterile pipette (6.5), 1 ml of the final dilution of the initial suspension (see 9.1) to each dish.

Take two other sterile Petri dishes. Using a new sterile pipette, transfer to each dish 1 ml of the dilution which is diluted by one order of magnitude less than the final one.

3) The IU is the determined quantity of internationally accepted reference material. In the case of penicillin, more than one reference material is accepted. For example, for benzyl penicillin, potassium salt, 1 mg = 1 670 IU.

9.2.2 Pour about 15 ml of the agar medium (5.2), at 45 °C ± 1 °C, into each Petri dish. The time elapsing between the end of the preparation of the initial suspension and the moment when the medium is poured into the dishes shall not exceed 15 min.

Carefully mix the inoculum with the medium and allow the mixture to solidify by leaving the dishes to stand on a cool horizontal surface.

9.2.3 Invert the prepared plates and place them in the incubator (6.2) set at 37 °C for 72 h ± 3 h.

9.3 Control plates

Inoculate and incubate two control plates in parallel with the operations specified in 9.2 but using 1 ml of diluent (5.1) in place of the final dilution (see 9.1).

9.4 Interpretation of results

9.4.1 Examination of control plates

In all cases, carry out an initial examination of the control plates (9.3) to determine whether colonies are present within the medium. If colonies are present, discard the control plates and the plates containing the test sample and repeat the procedure.

If colonies are not present, proceed to an examination of the plates containing the test sample according to 9.4.2.

9.4.2 Colony count

Count the colonies within each of the plates containing the test sample by means of the colony-counting equipment (6.10) or using the naked eye.

Count only well-distinguishable colonies, which have grown within the medium and on the surface of the medium. Reject any plate in which more than half of the surface is overgrown.

10 Expression of results

NOTE This clause is based on ISO 4833:1991 (see reference [3]).

10.1 Method of calculation

10.1.1 General

If the counts obtained from the plates containing the final dilution are greater than the counts obtained from the plates containing the previous dilution (one order of magnitude lower than the final one), include only the counts obtained from the final dilution.

10.1.2 Dishes containing between 15 and 300 colonies

Retain dishes containing not more than 300 colonies at two consecutive dilutions. It is necessary that one of these dishes contain at least 15 colonies.

Calculate the number N of colony-forming units (CFU) per millilitre of test sample using the following equation:

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

where

$\sum C$ is the sum of colonies counted on all the dishes retained;

n_1 is the number of dishes retained at the first of two consecutive dilutions;

n_2 is the number of dishes retained at the second dilution;

d is the dilution factor corresponding to the first dilution.

Round the result calculated to two significant figures.

Take as the result the number of colony-forming units per millilitre of test sample, expressed as a number between 1,0 and 9,9 multiplied by 10^x where x is the appropriate power of 10.

EXAMPLE

The colony count gave the following results:

— at the first dilution (10^{-4}): 168 and 215 colonies respectively on the two dishes retained;

— at the second dilution (10^{-5}): 14 and 25 colonies respectively on the two dishes retained:

$$N = \frac{\sum C}{(n_1 + 0{,}1\, n_2)\, d} = \frac{168 + 215 + 14 + 25}{\left[2 + (0{,}1 \times 2)\right] \times 10^{-4}} = \frac{422}{0{,}000\ 22} = 1\ 918\ 181$$

Rounding the result as specified above gives 1 900 000 or $1{,}9 \times 10^6$ colony-forming units per millilitre of test sample.

10.1.3 Dishes containing 15 or less colonies

If the two dishes, corresponding to the first dilution of two consecutive ones, contain less than 15 colonies, calculate the estimated number, N_E, of colony-forming units per millilitre of test sample as an arithmetic mean of the colonies counted on the two dishes using the following equation:

$$N_E = \frac{m}{d}$$

where

N_E is the estimated number of colony-forming units per millilitre;

m is the arithmetic mean of the colonies counted on the two dishes;

d is the dilution factor for the first of the two consecutive dilutions.

10.1.4 No colonies

If no colony growth is observed for the final dilution, express the result as less than $1 \times d^{-1}$ CFU per millilitre of test sample, where d is the dilution factor for the final dilution.

10.2 Precision

10.2.1 Dishes containing between 15 and 300 colonies (see 10.1.2)

For statistical reasons alone, in 95 % of cases the confidence limits of this method vary from ± 12 % to ± 37 % (see reference [4]). In practice, even greater variation may be found especially among results obtained by different microbiologists.

10.2.2 Dishes containing 15 or less colonies (see 10.1.3)

The confidence limits for the estimation of small numbers of colony-forming units are given in Table A.1.

11 Test report

The test report shall specify:

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

— the sampling method used, if known;

— the test method used, with reference to this International Standard;

— all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result;

— the test result obtained, and if small numbers of colonies have been detected, state the confidence limits at the 95 % level.

Annex A
(normative)

Confidence limits for the estimation of small numbers of colony-forming units of microorganisms

Table A.1 — Confidence limits when the number of colonies on dishes retained is 15 or less

Number of colony-forming units (CFU)	Confidence limits at the 95 % level	
	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

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[1] RADKE H., CORBOZ L. and FLÜKIGER A. *9th European A. I. Vets Meeting*, 1997, Neuchâtel, Switzerland

[2] VANDERZANT C. and SPLITTSTOESSER D. F. *Compendium of methods for the microbiological examination of foods*. American Public Health Association, 1992, pp. 78-79

[3] ISO 4833:1991[4], *Microbiology — General guidance for the enumeration of micro-organisms — Colony count technique at 30 °C*

[4] COWELL N. D. and MORISETTI M. D. *J. Sci. Food. Agric.*, **20**, 1969, pp. 573-579

4) Under revision.

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