TECHNICAL REPORT

ISO TR 8607

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Artificial insemination of animals — Frozen semen of breeding bulls — Enumeration of living aerobic micro-organisms

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

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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.

The main task of technical committees is to prepare International Standards, but in exceptional circumstances a technical committee may propose the publication of a Technical Report of one of the following types:

— type 1, when the required support cannot be obtained for the publication of an International Standard, despite repeated efforts;

— type 2, when the subject is still under technical development or where for any other reason there is the future but not immediate possibility of an agreement on an International Standard;

— type 3, when a technical committee has collected data of a different kind from that which is normally published as an International Standard ("state of the art", for example).

Technical Reports of types 1 and 2 are subject to review within three years of publication, to decide whether they can be transformed into International Standards. Technical Reports of type 3 do not necessarily have to be reviewed until the data they provide are considered to be no longer valid or useful.

ISO/TR 8607, which is a Technical Report of type 1, was prepared by Technical Committee ISO/TC 34, *Agricultural food products*.

The decision was taken to publish this document as a Technical Report of type 1 since, at the committee draft stage, no consensus could be reached with regard to technical comments put forward by several member bodies.

International Organization for Standardization
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Artificial insemination of animals — Frozen semen of breeding bulls — Enumeration of living aerobic micro-organisms

1 Scope

This Technical Report specifies a method for the enumeration of living aerobic micro-organisms present in the frozen semen of breeding bulls, by counting the colonies obtained in a solid medium after aerobic incubation at 37 °C.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this Technical Report. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this Technical Report are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 6887:1983, *Microbiology -- General guidance for the preparation of dilutions for microbiological examination*.

ISO 7218:1985, *Microbiology — General guidance for microbiological examinations*.

3 Definitions

For the purposes of this Technical Report, the following definitions 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 micro-organisms: Bacteria, yeasts and moulds which grow aerobically at 37 °C under the operating conditions specified in this Technical Report.

4 Principle

4.1 Deep inoculation of two poured plates containing a specified culture medium with a specified quantity of test sample.

4.2 Aerobic incubation of the two plates at 37 °C ± 1 °C for 72 h.

4.3 Calculation of the number of micro-organisms per millilitre of test sample from the number of colonies obtained.

5 Culture medium and diluent

5.1 General

For general guidance, see ISO 7218.

5.2 Diluent

Use the diluent specified in ISO 6887.

5.3 Agar medium

5.3.1 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 ($Na_2HPO_4.12H_2O$)	2,0 g
Gelatine	10,0 g
Agar in powder or flake form	13,0 g to 15,0 g[1]
Water	1 000 ml

1) According to the gel strength of the agar.

5.3.2 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 ± 0,1 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 45 °C ± 0,5 °C in the water-bath (6.8) and then add 10 % (*V*/*V*) inactivated and sterilized[1] bovine or sheep serum. Otherwise, before beginning the microbiological examination, completely melt the medium in a boiling water-bath, cool to 45 °C ± 0,5 °C in the water-bath (6.8), and then add 10 % (*V*/*V*) inactivated and sterilized[1] bovine or sheep serum.

6 Apparatus and glassware

NOTE 1 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 Apparatus for dry sterilization (oven) or **wet sterilization (autoclave)**, see ISO 7218.

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

1) By ultrafiltration or tyndallization.

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 (not blow-out pipettes), having a nominal capacity of 1 ml, graduated in 0,1 ml divisions.

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 ± 0,5 °C.

6.8 Water-bath, capable of being maintained at 45 °C ± 0,5 °C.

6.9 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 (paillettes of 0,25 ml or 0,5 ml, pellets, minitubes) so that the quantity of semen sample is 1,0 ml per series of doses.

Store the test samples in liquid nitrogen.

NOTE 2 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 the test sample

Before use, thaw the test sample in a water-bath (6.7) at 37 °C ± 0,5 °C for 3 min.

IMPORTANT — Thawed test samples may be kept in a refrigerator at 4 °C 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. 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 I.U. penicillin and 1 mg streptomycin or other broad-spectrum antibiotics per millilitre of diluent, use a 10^{-4} 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 I.U. and that of broad-spectrum antibiotics not more than 0,1 µg per millilitre of diluent.

NOTE 3 A higher antibiotics concentration may inhibit growth of the micro-organisms and produce false results.

9.2 Control plates

Inoculate and incubate two control plates in parallel with the operations specified in 9.3 but using 1 ml of diluent (5.2) in place of the final dilution (9.1).

9.3 Inoculation and incubation

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

9.3.2 Pour about 15 ml of the agar medium (5.3), at 45 °C ± 0,5 °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.

9.3.3 Invert the prepared plates and place them in the incubator (6.2) maintained at 37 °C ± 1 °C for 72 h.

9.4 Interpretation of results

9.4.1 Examination of control plates

In all cases, carry out an initial examination of the control plates (9.2) 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 recommence the procedure.

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

9.4.2 Colony count

If no colonies are observed within the medium in the control plates after the incubation period (see 9.4.1), count the colonies within each of the plates containing the test sample by means of the colony counting equipment (6.9) or using the naked eye.

Count only well-distinguishable colonies which have grown within the medium. Do not count colonies which have grown on the surface of the medium. Reject any plate in which more than half of the surface is overgrown.

10 Expression of results

10.1 Plates containing colonies

Take as the result the arithmetic mean of the number of colonies found in the two plates multiplied by $1/d$, where d is the dilution factor of the final dilution [$d = 10^{-4}$ for final dilutions prepared in accordance with 9.1 a)], and expressed as micro-organisms per millilitre of test sample.

10.2 Plates not containing colonies

If no colony growth is observed from the final dilution, express the result as less than $1/d$ micro-organisms per millilitre of test sample, where d is the dilution factor of the final dilution.

11 Repeatability

The difference between the number of colonies in the two inoculated sample plates shall not exceed 30 %.

12 Test report

The test report shall specify the method used and the result obtained. It shall also mention all operating details not specified in this Technical Report, or regarded as optional, together with details of any incidents which may have influenced the result.

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

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