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Milk — Enumeration of microorganisms — Plate-loop technique at 30 °C

Lait — Dénombrement des micro-organismes — Méthode de l'anse sur boîtes de Petri à 30 °C

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Foreword

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ISO 8553|IDF 131 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO and AOAC International in the development of standard methods of analysis and sampling for milk and milk products.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of IDF National Committees casting a vote.

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All work was carried out by the Joint ISO/IDF/AOAC Action Team on *Microbiological harmonization*, of the Standing Committee on *Microbiological methods of analysis*, under the aegis of its project leader, Dr J. Floor (ZA).

Milk — Enumeration of microorganisms — Plate-loop technique at 30 °C

1 Scope

This International Standard specifies a method for the enumeration of microorganisms in raw milk by using the plate-loop technique at 30 °C.

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 4833, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of microorganisms — Colony count technique at 30 °C*

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

ISO 8261|IDF 122, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

ISO/TS 11133-1, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory*

ISO/TS 11133-2, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 2: Practical guidelines on performance testing of culture media*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
microorganisms
bacteria, yeasts and moulds forming countable colonies under the conditions specified in this International Standard

4 Principle

4.1 A poured plate is prepared using a specified culture medium and a specified quantity of the test sample taken by means of a calibrated wire loop.

4.2 The plate is aerobically incubated at 30 °C for 72 h.

4.3 The number of microorganisms per millilitre of test sample is calculated from the number of colonies obtained on the plate (see Clause 10).

5 Diluents, culture medium and reagent

5.1 General

See ISO 7218 and ISO/TS 11133-1.

5.2 Diluents

See ISO 8261|IDF 122.

5.3 Culture medium — Plate count milk agar

5.3.1 Composition

Yeast extract	2,5 g
Enzymatic digestion of casein	5,0 g
Skimmed milk powder [a]	1,0 g
Glucose, anhydrous ($C_6H_{12}O_6$)	1,0 g
Agar	9 g to 18 g[b]
Water	1 000 ml

[a] The skimmed milk powder shall be free from inhibitory substances.

[b] Depending on the gel strength of the agar.

5.3.2 Preparation

5.3.2.1 Preparation from commercial dehydrated medium

Follow the manufacturer's instructions but, in all cases, add the skimmed milk powder even if the manufacturer considers such an addition unnecessary. Adjust the pH, if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.

5.3.2.2 Preparation from dehydrated basic components

Dissolve and disperse in the water, in the following order, the yeast extract, the enzymatic digestion of casein, the glucose and, finally, the skimmed milk powder. Heating the water will assist this procedure. Add the agar and heat to boiling while stirring frequently until the agar is completely dissolved.

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

5.3.2.3 Distribution, sterilization and storage

Dispense the medium into test tubes (6.8), in quantities of 12 ml to 15 ml per tube, or into flasks or bottles (6.9) of capacity not greater than 500 ml. Sterilize for 15 min in an autoclave (6.11) set at 121 °C.

If the medium is to be used immediately, cool it in a water bath (6.6) to between 44 °C and 47 °C before use.

If not, store it in the dark at 3 °C ± 2 °C for no longer than three months under conditions which do not allow any change in its composition and properties. Before commencing the microbiological examination and in order to avoid any delay when pouring the medium, completely melt the stored medium, then cool it in a water bath (6.6) to between 44 °C and 47 °C before use (see 9.2.4).

With regard to a temperature check of the medium and other requirements, see ISO 7218.

5.3.3 Performance testing for the quality assurance of the culture medium

Test the performance of the medium according to ISO/TS 11133-2.

5.4 Disinfecting solution

Use a sodium hypochlorite solution containing 50 mg/l to 100 mg/l of active chlorine or a 1:1 mixture of ethanol (96 %) and acetone. Prepare a fresh disinfecting solution daily.

6 Apparatus and glassware

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

6.1 Glassware

Disposable glassware is an acceptable alternative to re-usable glassware if it has suitable specifications. Re-usable glassware shall be capable of undergoing repeated sterilization and shall be chemically inert.

6.2 Loop inoculation equipment

6.2.1 Components

The total equipment assembly cannot be purchased as a unit, but may be made up from the components listed in 6.2.1.1 to 6.2.1.3. Automatic equipment may also be used.

6.2.1.1 Platinum inoculating loop, calibrated to hold 0,001 ml, soldered to a Luer-lock hypodermic needle. Make a 30° bend about 3 mm to 4 mm from the loop, with the loop opening towards the hub.

NOTE Suitable platinum inoculating loops are available from Gerber Instruments K. Schneider & Co. AG, Switzerland[1)].

Alternatively, use a loop, calibrated to hold 0,001 ml, made of platinum, platinum-rhodium or platinum-iridium wire of diameter 0,4 mm to 0,5 mm, attached to a wire of length 60 mm to 70 mm. Make a 30° bend about 3 mm to 4 mm from the loop. Kink the opposite end of the wire in several places. Insert the kinked end of the wire in a Luer-lock hypodermic needle, 13 gauge, sawn off 24 mm to 26 mm from the point where the barrel enters the hub, to a point where the bend is about 12 mm to 14 mm from the end of the barrel.

Regularly check the calibration of the loop and thus the accuracy of the procedure by analysing 25 samples in duplicate by both the standard plate count (see ISO 4833) and the plate-loop technique.

The samples should give plate counts of between 10 and 300 according to the standard plate count (third decimal dilution). The averages of the counts, obtained by the two methods, should not differ by more than ± 10 %.

6.2.1.2 Continuous pipette, of capacity 2 ml, with Luer-lock fitting (e.g. Socorex or Cornwall[1)] self-refilling syringe), adjustable to deliver 1,0 ml.

6.2.1.3 Silicone rubber tubing, of internal diameter 3,0 mm, of sufficient length to facilitate the inoculation process, attached to the syringe and extending into a capped diluent container.

NOTE A piece of silicone rubber tubing, a sinker and feed needle are delivered as standard accessories with some commercially available syringes.

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1) Platinum inoculating loops from Gerber Instruments, Socorex or Cornwall self-refilling syringes and Schott-Duran bottles with polypropylene screw-caps are examples of products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO or IDF of these products.

6.2.2 Assembly and sterilization of loop inoculation equipment

Attach the hypodermic needle with the platinum inoculating loop (6.2.1.1) to the continuous pipette (self-refilling syringe) (6.2.1.2). Connect the silicone rubber tubing (6.2.1.3) to the intake valve of the self-refilling syringe. Extend the distal end with attached sinker into the diluent container (6.3).

Fill the diluent container (6.3) to between 50 % and 80 % of its capacity with a suitable diluent (5.2).

Sterilize the assembled pipetting outfit for 15 min in an autoclave (6.11) set at 121 °C. Allow to cool down.

6.3 Capped diluent container, of max. capacity 1 000 ml (e.g. Schott-Duran[1)] bottle with polypropylene screw-cap). A small hole shall be made in the cap for the silicone rubber tubing to pass through. It is recommended to attach a test tube or other suitable device to the container to hold and protect the loop during sterilization.

6.4 Incubator, capable of operating at 30 °C ± 1 °C.

6.5 pH meter, having an accuracy of calibration of ± 0,1 pH unit at 25 °C.

6.6 Water bath, capable of operating at between 44 °C and 47 °C.

6.7 Colony-counting equipment, consisting, for example, of an illuminated base with a dark background, fitted with a magnifying lens to be used at a magnification of 1,5× and a mechanical or electronic digital counter. Alternatively, an automated microbiological analyser (image analyser) may also be used.

6.8 Test tubes, of approximate capacity 20 ml, with suitable stoppers.

6.9 Flasks or **bottles**, of appropriate capacity but not greater than 500 ml, with suitable stoppers.

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

6.11 Autoclave, capable of operating at 121 °C ± 1 °C.

7 Sampling

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

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707.

8 Preparations

8.1 Preparation of test samples

8.1.1 Prepare test samples while avoiding direct sunlight and taking the normal aseptic precautions whenever necessary.

8.1.2 Warm the refrigerated test samples to between 15 °C and 20 °C prior to testing.

8.1.3 Shake the test samples thoroughly and vigorously. To ensure adequate mixing, the sample bottles should not be too full (approx. 10 mm headspace is sufficient).

8.1.4 Allow 5 min for foam to disperse then remix by carefully inverting the sample bottles two or three times just before the next step (9.2.1). The time elapsing between the start of the warming procedure (8.1.2) and the inoculation step (9.2.1) shall not exceed 20 min.

8.2 Preparation of loop inoculation equipment

8.2.1 Rapidly depress the syringe plunger of the sterile loop inoculation equipment (6.2.2) several times to prime the glass syringe (adjusted to deliver 1 ml).

8.2.2 Before initial transfer is made in examining a series of samples, submerge the loop for 30 s in the disinfecting solution (5.4). Discharge at least five 1 ml portions of diluent to waste and then discharge a 1 ml portion into a sterile Petri dish (6.10). Label this dish “Sterile instrument control”.

9 Procedure

9.1 General

Do not carry out the operations described in 9.2 in direct sunlight. Take the normal aseptic precautions whenever necessary.

9.2 Inoculation and incubation

9.2.1 Dip the freshly prepared loop (see 8.2.2) into the prepared test sample (see 8.1.4) up to the bend in the wire shank (the bend serves as a graduation mark and also permits vertical removal of the loop). Draw the loop through the milk at the same depth over a distance of at least 20 mm and remove slowly and evenly at a speed of approx. 2 cm/s.

The speed of removal of the loop from the surface of the milk should be controlled carefully as this affects the volume of the test portion. Removing the loop too slowly causes less than 0,001 ml to adhere; jerking the loop out rapidly causes more than 0,001 ml to adhere. It is necessary to use wide-mouth sample bottles and good illumination to facilitate the process.

9.2.2 Raise the cover of a sterile Petri dish (6.10). Insert the loop and depress the plunger causing 1 ml sterile diluent to flow across the charged loop, thus washing a measured amount of sample into the dish. Do not depress the plunger so rapidly that diluent fails to follow the shank and flow across the loop.

NOTE Normally, the residue remaining on the loop after discharging the sample is not significant. However, imperfections in the welding of the loop or in the smoothness of the metal surface as in the case of old, damaged loops can lead to incomplete rinsing.

Replace badly damaged loops. As a precaution, sterilize the loop after every set of 20 test samples by submerging it in a disinfecting solution (5.4). Do not sterilize the loop in a flame as this will significantly reduce its life. Discharge at least five 1 ml portions of diluent to waste before proceeding.

9.2.3 Immediately after every set of test samples and before flushing or sterilizing the loop, discharge 1 ml of sterile buffer into a Petri dish. Label this dish “Rinsing control”. No more than two colonies should develop on this plate during incubation (9.2.6). Additionally, pour one sterile Petri dish with agar medium (5.3) only. Label this dish “Agar control”.

9.2.4 Add 10 ml to 12 ml of melted medium (see 5.3.2.3) at a temperature of between 44 °C and 47 °C to each inoculated dish. The time elapsing between discharging the loop and pouring the media into the dishes shall not exceed 15 min.

9.2.5 Mix immediately after pouring by rotating the dishes sufficiently to obtain evenly dispersed colonies after incubation. Allow the mixture to solidify by leaving the Petri dishes to stand on a cool horizontal surface.

9.2.6 Invert the prepared dishes. Place them for 72 h ± 3 h in the incubator (6.4) set at 30 °C. Do not stack the dishes more than six high. Separate stacks of dishes from one another and from the walls and top of the incubator (see ISO 7218).