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Milk and milk products — Preparation of test samples and dilutions for microbiological examination

Lait et produits laitiers — Préparation des échantillons pour essai et des dilutions en vue de l'examen microbiologique

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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. They are approved in accordance with ISO procedures requiring at least 75 % approval by the member bodies voting.

International Standard ISO 8261 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, in collaboration with the International Dairy Federation (IDF) and the Association of Official Analytical Chemists (AOAC), and will also be published by these organizations.

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Introduction

This International Standard is based on ISO 6887 : 1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination*. The necessary adaptations to microbiological laboratory practice in the dairy industry and instructions specific to dairy products, especially in relation to sample preparation, have been introduced.

The question of which diluent or diluents to specify has been the subject of discussion for some time. In this International Standard the peptone/saline solution, adopted in ISO 6887, has been specified but three other commonly used diluents are also specified for general use, these being diluents commonly used in dairy microbiological laboratories.

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Milk and milk products — Preparation of test samples and dilutions for microbiological examination

1 Scope

This International Standard lays down general guidelines for the preparation of

- test samples,
- primary dilutions, and
- further dilutions

for microbiological examination of milk and milk products.

2 Normative reference

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

ISO 707 : 1985, *Milk and milk products — Methods of sampling.*

3 Definitions

For the purposes of this International Standard, the following definitions apply.

3.1 primary dilution (initial suspension) : The suspension, solution or emulsion obtained after a weighed or measured quantity of the product under examination (or of a test sample prepared from the product) has been mixed, if necessary using a blender and observing appropriate precautions (see the notes to clause 8), with a ninefold quantity of dilution fluid (diluent, see clause 5), allowing large particles, if present, to settle.

NOTE — It may be necessary in certain cases, particularly for products giving an initial 1 + 9 suspension which is too viscous or too thick, to add more diluent. In some other cases, for the results of the tests to relate to certain specification criteria, a primary dilution more concentrated than 1 + 9 may be required. These factors should be taken into account for subsequent operations and/or in the expression of results.

3.2 further decimal dilutions : The suspensions, solutions or emulsions obtained by mixing a specific volume of the primary dilution (3.1) with a ninefold volume of diluent (see the note to 3.1), and by repeating this operation with every dilution thus prepared until a decimal dilution series, suitable for the inoculation of culture media, is obtained.

4 Principle

Preparation of the primary dilution (initial suspension) (3.1) and, if necessary, of further decimal dilutions (3.2) to reduce the number of micro-organisms per unit volume to facilitate microbiological examination.

5 Diluents

5.1 Basic materials

In order to improve the precision 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.

Chemical reagents shall be of recognized analytical grade.

The water used shall be water distilled from glass apparatus, or deionized water. It shall be free from substances that might influence the growth of micro-organisms under the test conditions. This shall be periodically checked, particularly in the case of deionized water.

NOTE — Tests to determine the suitability of water for microbiological applications have been published in MARTH, E.H. (editor), *Standard methods for the examination of dairy products*, 15th edition, 1984, Washington D.C., USA : American Public Health Association.

Solutions of sodium hydroxide or hydrochloric acid (approximately 0,1 mol/l) shall be used to adjust the pH of diluents, unless otherwise specified.

5.2 Diluents for general use

5.2.1 Peptone/saline solution

Composition

Peptone	1,0 g
Sodium chloride (NaCl)	8,5 g
Water	1 000 ml

Preparation

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

Adjust the pH so that, after sterilization, it is 7,0 ± 0,1 at 25 °C.

5.2.2 Quarter-strength Ringer's solution

Composition

Sodium chloride (NaCl)	2,25 g
Potassium chloride (KCl)	0,105 g
Calcium chloride, anhydrous ($CaCl_2$)	0,06 g
Sodium hydrogencarbonate ($NaHCO_3$)	0,05 g
Water	1 000 ml

Preparation

Dissolve the salts in the water.

Adjust the pH so that, after sterilization, it is 6,9 ± 0,1 at 25 °C.

5.2.3 Peptone solution

Composition

Peptone	1,0 g
Water	1 000 ml

Preparation

Dissolve the peptone in the water.

Adjust the pH so that, after sterilization, it is 7,0 ± 0,1 at 25 °C.

5.2.4 Phosphate buffer solution

Composition of stock solution

Potassium dihydrogenphosphate (KH_2PO_4)	42,5 g
Water	1 000 ml

Preparation

Dissolve the salt in 500 ml of water.

Adjust the pH using 1,0 mol/l sodium hydroxide or hydrochloric acid solution so that, after sterilization, it is 7,2 ± 0,1 at 25 °C.

Dilute to 1 000 ml with water. Store the stock solution under refrigeration.

Before use, add 1 ml of this stock solution (at 20 °C) to 1 000 ml of water for use as diluent.

5.3 Diluents for special purposes

5.3.1 Sodium citrate solution (for cheese, processed cheese and roller-dried milk)

Composition

Trisodium citrate dihydrate ($Na_3C_6H_5O_7.2H_2O$)	20,0 g
Water	1 000 ml

Preparation

Dissolve the salt in the water by heating at 45 °C to 50 °C.

Adjust the pH so that, after sterilization, it is 7,5 ± 0,1 at 25 °C.

5.3.2 Dipotassium hydrogenphosphate solution (for cheese, processed cheese, acid casein and lactic casein powders, caseinates, acid whey powder and sour cream)

Composition

Dipotassium hydrogenphosphate (K_2HPO_4)	20,0 g
Water	1 000 ml

Preparation

Dissolve the salt in the water by heating at 45 °C to 50 °C.

Adjust the pH. For primary dilution of acid casein, lactic casein and acid whey powder, the pH at 25 °C after sterilization shall be 8,4 ± 0,1; for caseinates, cheese, processed cheese and sour cream, it shall be 7,5 ± 0,1.

NOTE — The most suitable diluent for rennet casein remains to be established.

5.4 Distribution, sterilization and storage of diluent

Dispense the diluent (5.2 or 5.3) for the primary dilution into flasks or bottles (6.4). Dispense the diluent for decimal dilutions (5.2) into the test tubes (flasks or bottles) (6.5). The quantities dispensed shall be such that, after sterilization, each flask or bottle (6.4) contains 90 ml of diluent or a multiple of 90 ml (or other required quantities) and each test tube (flask or bottle) (6.5) contains 9,0 ml of diluent or a multiple of 9,0 ml (or other required quantities).

Stopper the test tubes, flasks or bottles.

Sterilize by autoclaving at 121 °C ± 1 °C for 15 min (a longer period may be necessary for larger volumes).

If the diluent is not to be used immediately, store it in the dark at a temperature between 0 °C and 5 °C, for no longer than 1 month, in conditions which do not allow any change in its volume or composition.

NOTE — If it is necessary to count several groups of micro-organisms using different culture media, it may be necessary to distribute all the diluents (or some of them) in quantities greater than 9,0 ml; the size of the test tubes, flasks and bottles (6.4 and 6.5) should be specified accordingly.

6 Apparatus and glassware

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

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

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, the test sample, or the dilutions, except for apparatus that is supplied sterile (particularly plastic apparatus) shall be sterilized by one of the following methods :

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

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

Pipettes, however, shall not be sterilized in an autoclave because moisture will condense on the interior surfaces on cooling and affect the accuracy of delivery.

6.2 Blending equipment

One of the following shall be used :

a) a rotary blender, operating at a rotational frequency between 8 000 min^{-1} and 45 000 min^{-1}, with glass or metal bowls fitted with lids, resistant to the conditions of sterilization;

b) a peristaltic-type blender (stomacher), with sterile plastic bags.

NOTE — The bowls or plastic bags should have sufficient capacity to allow the sample to be properly mixed with the appropriate amount of diluent. In general, the volume of the container should be equal to about twice the volume of the test sample plus diluent.

6.3 Mixer, capable of mixing 1 ml or 2 ml of the test sample (in the case of liquid products), or the decimal dilutions, in a tube of adequate dimensions with 9 ml or 18 ml of diluent, in order to obtain a homogeneous suspension, and working on the principle of eccentric rotation of the contents of the test tube (e.g. Vortex mixer).

6.4 Flasks or **bottles**, of sufficient capacity to contain, and leave adequate head-space for mixing, the 90 ml of diluent used for the initial suspension, or multiples of 90 ml.

6.5 Test tubes (flasks or **bottles)**, of sufficient capacity to contain, and leave adequate head-space for mixing, 10 ml (or a multiple of 10 ml, if necessary) of the test sample (if it is liquid) or of the primary dilution (in other cases) or further decimal dilutions.

6.6 Pipettes (plugged with cotton wool), of nominal capacity 1 ml and having an outlet of diameter 1,75 mm to 3 mm.

NOTE — Use only pipettes with unbroken tips and, when appropriate, having graduations distinctly marked to contrast sharply with the contents.

6.7 Graduated pipettes (plugged with cotton wool), of relatively large capacity, for example 10 ml or 20 ml.

NOTE — Use only pipettes with unbroken tips and, when appropriate, having graduations distinctly marked to contrast sharply with the contents.

6.8 Glass beads, of diameter about 6 mm.

6.9 pH meter, with temperature compensation, accurate to 0,1 pH unit.

6.10 Balances, with sufficient weighing capacity and accurate to within 1 % of the net mass being weighed.

6.11 Water-bath, capable of operating at 45 °C ± 1 °C.

6.12 Water-bath, capable of operating at 37 °C ± 1 °C.

7 Sampling

See ISO 707.

8 Procedure

NOTES

1 For some specific investigations (e.g. *Salmonella*) special techniques or precautions may be necessary. In such cases the special techniques are mentioned in the standard for the method in question.

2 The operations described in 8.1 and 8.2 should not be carried out in direct sunlight.

3 Normal aseptic precautions should be taken.

8.1 Preparation of the test sample and primary dilution

To avoid damaging the micro-organisms by sudden changes in temperature, the temperature of the diluent during the operations described below shall be approximately the same as that of the test sample, unless otherwise specified.

8.1.1 Milk and liquid milk products

Agitate the test sample thoroughly, so that the micro-organisms are distributed as evenly as possible, by rapidly inverting the sample container 25 times. Avoid foaming or allow foam to disperse. The interval between mixing and removal of the test portion shall not exceed 3 min.

Remove 1 ml of the test sample with a sterile pipette (6.6) and add to 9 ml of diluent (5.2) (or 10 ml of test sample to 90 ml of diluent, or 11 ml of test sample to 99 ml of diluent).

Shake this primary dilution (for example, 25 times, with a movement of about 300 mm, in 7 s). A 10^{-1} dilution is thus obtained.

Prepare further dilutions in accordance with 8.2.

8.1.2 Dried milk, dried sweet whey, dried buttermilk and lactose

Thoroughly mix the contents of the closed container by repeatedly shaking and inverting. If the test sample is in the original unopened container, too full to permit thorough mixing, transfer it to a larger container. Mix. Open the container and remove the test portion required with a spatula, proceeding as indicated below. Immediately close the container again.

Warm a bottle containing 90 ml of a suitable diluent (5.2 or, if necessary, for roller-dried milk, 5.3.1 at pH 7,5 ± 0,1) to 45 °C ± 1 °C in the water-bath (6.11).

Weigh 10 g of the test sample into a suitable glass vessel (for example a beaker) and drop the powder into the dilution bottle containing the diluent chosen. Alternatively, weigh 10 g of the test sample directly into the bottle with the diluent.

In order to dissolve, swirl slowly to wet the powder then shake the bottle 25 times, with a movement of about 300 mm, in about 7 s. A peristaltic-type blender [6.2 b)] may be used as an alternative to shaking.

Replace the bottle in the water-bath for 5 min, shaking occasionally.

Prepare further dilutions in accordance with 8.2.

NOTE — For better reconstitution, particularly with roller-dried milk, glass beads (6.8) can be helpful. If used they should be added to the bottle before sterilization.

8.1.3 Cheese and processed cheese

Either weigh 10 g of the test sample in a dish and transfer it to the container of a rotary blender [6.2 a)] or a peristaltic-type blender [6.2 b)] or weigh 10 g of the test sample directly into the container.

When a rotary blender or a peristaltic-type blender is used, add 90 ml of diluent (5.2, 5.3.1 or 5.3.2 at pH 7,5 ± 0,1). Blend until the product is thoroughly dispersed (1 min to 3 min). In the case of a rotary blender, operate the equipment for a sufficient time to give a total of 15 000 revolutions to 20 000 revolutions. Even with the slowest rotary blender this time shall not exceed 2,5 min. Ideally, ensure that the temperature of the dispersion does not exceed 40 °C, and in any case do not allow it to exceed 45 °C. Allow any foam to disperse.

Prepare further dilutions in accordance with 8.2.

8.1.4 Acid casein, lactic casein and acid whey powder

Weigh 10 g of the test sample in a dish. Transfer to a dilution bottle with glass beads containing 90 ml of dipotassium hydrogenphosphate diluent (5.3.2) at pH 8,4 ± 0,1 for acid and lactic caseins.

Leave for 15 min at ambient temperature and then raise the temperature to 37 °C ± 1 °C in a water-bath (6.12).

Keep the bottle at 37 °C for a further 15 min and shake vigorously at intervals.

NOTE — Avoid using a rotary blender [6.2 a)] or a peristaltic-type blender [6.2 b)] because of the formation of foam that ensues.

Prepare further dilutions in accordance with 8.2.

8.1.5 Caseinate

Either weigh 10 g of the test sample in a dish and transfer it to the container of a rotary blender [6.2 a)] or a peristaltic-type blender [6.2 b)] or weigh 10 g of the test sample directly into the container. Add 90 ml of dipotassium hydrogenphosphate diluent (5.3.2) at pH 7,5 ± 0,1 at ambient temperature. Mix for about 2 min. In the case of a rotary blender, operate for a sufficient time to give a total of 15 000 revolutions to 20 000 revolutions. Even with the slowest rotary blender this time shall not exceed 2,5 min.

Raise the temperature to 37 °C ± 1 °C in a water-bath (6.12). In the case of a rotary blender, transfer into a sterile dilution flask. Keep at 37 °C for a further 15 min. Allow foam to subside before proceeding.

Prepare further dilutions in accordance with 8.2.

8.1.6 Butter

Weigh 10 g of the test sample into a container and place the container in a water-bath (6.11) at 45 °C ± 1 °C. Keep it in the water-bath until the whole test portion has just melted. Add 90 ml of diluent (5.2). Mix. This operation is more easily carried out in a peristaltic-type blender [6.2 b)].

Alternatively use only the aqueous phase for dilution, as follows.

Take a test portion of 50 g (containing about 8 ml of water) and add 42 ml of diluent (5.2.3) warmed to 45 °C. Place the container in a water-bath (6.11) at 45 °C ± 1 °C until the butter melts. Shake well and allow to separate for no longer than 15 min.

If necessary, to separate the phases, transfer the melted test portion to a sterile centrifuge tube (or melt the test portion directly in the tube) and centrifuge at a rotational frequency of 1 000 min^{-1} to 2 000 min^{-1}.

Remove the fatty (upper) phase aseptically with a sterile tube connected to a vacuum pump. Pipette from the bottom layer.

Prepare further dilutions in accordance with 8.2.

8.1.7 Frozen milk products (including edible ices)

Proceed as in the case of butter (8.1.6) (first alternative) but using a water-bath (6.12) at no more than 37 °C ± 1 °C. The temperature of the test sample shall not be allowed to exceed the temperature of this water-bath.

Prepare further dilutions in accordance with 8.2.

8.1.8 Custard, desserts, fermented milk and cream

Weigh 10 g of the test sample into a flask (6.4) containing glass beads (6.8).

For custards, desserts and sweet cream, add 90 ml of diluent (5.2) and shake to disperse. For fermented milk and sour cream, use diluent 5.3.2 at pH 7,5 ± 0,1. A peristaltic-type blender [6.2 b)] may be used.

Prepare further dilutions in accordance with 8.2.

8.2 Further decimal dilutions

NOTE 1 — In the case of a test for the presence or absence of a micro-organism in 0,1 ml or 0,1 g of product, it is not necessary to prepare the following dilutions.

Transfer, by means of a fresh pipette, 1 ml of the primary dilution (for example 8.1.1 or 8.1.2) into another tube containing 9 ml of sterile diluent (5.2), avoiding contact between the pipette and the diluent (see the notes to 3.1 and 5.4). Use a fresh pipette for each dilution.

If larger volumes are required, transfer 10 ml of the primary dilution to a bottle containing 90 ml of sterile diluent (5.2), or transfer 11 ml of primary dilution to 99 ml of sterile diluent (5.2).

NOTE 2 — In a routine procedure, if a 10^{-3} dilution is required, 1 ml of primary dilution should be transferred to 99 ml of sterile diluent (5.2).

Mix carefully, either by aspirating 10 times with a fresh pipette, or in the mechanical mixer (6.3) for 5 s to 10 s, to obtain a 10^{-2} dilution. The rotational frequency of the mixer shall be chosen so that the liquid, as it swirls, rises to within 2 cm or 3 cm of the rim of the vessel.

If necessary, repeat these operations with sterile diluent (5.2) using the 10^{-2} and further dilutions to obtain 10^{-3}, 10^{-4}, etc. dilutions until the appropriate number of micro-organisms per millilitre has been obtained (see clause 4).

When 10 ml plus 90 ml or 11 ml plus 99 ml have been taken, shake manually as described in 8.1.1.

8.3 Duration of the procedure

The time between the end of the preparation of the primary dilution and the mixing of dilutions and media (described in the specific methods of examination) shall be not more than 15 min.