International Standard ISO 6887

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Microbiology — General guidance for the preparation of dilutions for microbiological examination

Microbiologie — Directives générales pour la préparation 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.

International Standard ISO 6887 was developed by Technical Committee ISO/TC 34, *Agricultural food products,* and was circulated to the member bodies in November 1981.

It has been approved by the member bodies of the following countries:

Australia	Iraq	South Africa, Rep. of
Brazil	Israel	Sri Lanka
Canada	Italy	Tanzania
Chile	Mexico	Thailand
Czechoslovakia	Netherlands	United Kingdom
Egypt, Arab Rep. of	New Zealand	USSR
Ethiopia	Philippines	Venezuela
France	Poland	Yugoslavia
Germany, F.R.	Portugal	
India	Romania	

No member body expressed disapproval of the document.

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Microbiology — General guidance for the preparation of dilutions for microbiological examination

0 Introduction

This International Standard is intended to provide general guidance for the preparation of dilutions for microbiological examination of products not dealt with by existing International Standards and for the consideration of bodies preparing reference microbiological methods of test for application to food products or to animal feeding stuffs. In view of the large variety of products within this field of application, these guidelines may not be appropriate for some products in every detail, and for some other products it may be necessary to use different methods. Nevertheless, it is hoped that, in all cases, every attempt will be made to apply these guidelines as far as possible and that deviations from them will only be made if absolutely necessary for technical reasons.

When this International Standard is next reviewed, account will be taken of all information then available concerning the extent to which the guidelines have been followed and the reasons which necessitated deviation from them in the case of particular products.

The harmonization of test methods cannot be immediate, and, for certain groups of products, International Standards and/or national standards, that do not comply with these guidelines, may already exist. In cases where International Standards already exist for the product to be tested, they should be followed, but it is hoped that, when they are reviewed, they will be aligned with this International Standard so that, eventually, the only remaining departures from these guidelines will be those necessary for well established technical reasons.

1 Scope and field of application

This International Standard lays down general guidelines for the preparation of dilutions for aerobic microbiological examinations of products intended for human or animal consumption. (At present, the guidelines should be used in conjunction with the methods described in ISO 4831, ISO 4832, ISO 4833 and ISO 6579.)

This International Standard is applicable to the products mentioned, provided that the appropriate subclauses of clause 9 are respected.

2 References

ISO 4831, *Microbiology — General guidance for the enumeration of coliforms — Most probable number technique at* 30 °C.

ISO 4832, *Microbiology — General guidance for the enumeration of coliforms — Colony count technique at* 30 °C.

ISO 4833, *Microbiology — General guidance for the enumeration of micro-organisms — Colony count technique at* 30 °C.

ISO 6579, *Microbiology — General guidance on methods for the detection of* Salmonella.

3 Definitions

For the purpose of this International Standard, the following definitions apply:

3.1 initial suspension (primary dilution): 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 note to clause 9), 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. This should be taken into account for subsequent operations and/or in the expression of results.

3.2 further decimal dilutions: The suspensions or solutions obtained by mixing a determined volume of the initial suspension (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.

3.3 specific standard: An International Standard or guidance document describing the examination of a specific product (or group of products) for the detection or enumeration of a specific micro-organism (or group of micro-organisms) and/or describing the general characteristics of sampling and/or the preparation of test samples.

4 Principle

Preparation of the initial suspension (3.1) in such a way as to obtain as uniform a distribution as possible of the micro-organisms contained in the test portion.

Preparation, if necessary, of decimal dilutions (3.2) in order to reduce the number of micro-organisms per unit volume to allow, after incubation, observation of their growth (in the case of tubes or bottles) or colony counting (in the case of plates).

The appropriate number of micro-organisms is generally:

a) for the most probable number technique using 3 tubes: 1 micro-organism in 10 ml of the highest decimal dilution;

b) for the colony count technique: 30 to 300 colonies (for some groups, for example coliforms, 15 to 150 colonies).

5 Diluent

5.1 Basic materials

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.

Chemical products shall be of recognized analytical quality.

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

5.2 Composition

Unless there is irrefutable evidence (for example authoritative data or comparative tests) that other diluents are better suited for the preparation of particular products, use diluent with the following composition[1]:

peptone	1,0 g
sodium chloride	8,5 g
water	1 000 ml

5.3 Preparation

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

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

5.4 Distribution of diluent

Dispense the diluent (5.3) into test tubes or bottles (6.5) (for decimal dilutions) or into flasks or bottles (6.4) (for the initial suspension; in the case of non-liquid products, see 9.1.2) of appropriate capacities, in quantities such that, after sterilization, each tube or bottle contains 9,0 ml of diluent or a multiple of 9,0 ml and each flask or bottle contains 90 ml of diluent or a multiple of 90 ml (or other required quantities). Stopper the tubes, flasks, or bottles.

Sterilize by autoclaving at 121 ± 1 °C for 20 min.

If the diluent is not to be used immediately, store it in the dark at a temperature between 0 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 dilutions (or some of them) in quantities greater than 9,0 ml. The size of the test tubes, flasks and bottles should be specified accordingly.

6 Apparatus and glassware

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

Usual microbiological laboratory equipment and in particular

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

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

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

6.2 Blending equipment (for non-liquid products, see 9.1.2).

One of the following shall be used:

a) a rotary blender, operating at a rotational frequency between 8 000 and 45 000 min^{-1}, with glass or metal bowls preferably 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 sample plus diluent.

6.3 Mixer, capable of mixing 1 or 2 ml of the sample (in the case of liquid products), or of a higher dilution, in a tube of adequate dimensions, with 9 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 (Vortex mixer).

1) Subclause 7.2 of ISO 4831, ISO 4832 and ISO 4833 will be amended accordingly.

6.4 Flasks or bottles, of sufficient capacity to contain the 90 ml of diluent used for the initial suspension, or multiples of 90 ml (in the case of non-liquid products, see 9.1.2).

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 sample (if liquid) or of the initial suspension (in other cases), or further decimal dilutions.

6.6 Pipettes (plugged with cotton wool), of nominal capacity 1 ml (or if necessary 2 ml; see the note to 5.4) and having an outlet of diameter 2 to 3 mm.

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

6.8 pH meter, accurate to ± 0,1 pH unit.

6.9 Balance, of sufficient capacity, capable of weighing to the nearest 0,01 g (in the case of non-liquid products).

7 Sampling

Carry out sampling in accordance with the specific standard appropriate to the product concerned. If such a specific standard is not available, it is recommended that agreement be reached on this subject by the parties concerned.

8 Preparation of the test sample

See the specific standard appropriate to the product concerned. If such a specific standard is not available, it is recommended that agreement be reached on this subject by the parties concerned.

9 Procedure

NOTE — For a number of products, it may be necessary to take special precautions when preparing the initial suspension, for example:

— use of elevated temperatures to suspend cocoa, gelatine, milk powder;

— adjustment of the pH of the sample;

— reconstitution of dehydrated products and resuscitation of micro-organisms damaged during the various treatments and storage of the food product.

These precautions should be mentioned in the specific standard appropriate to the product concerned.

9.1 Test portion and initial suspension (primary dilution)

Use the procedure described in 9.1.1 in the following cases:

— for non-viscous liquid samples (water, milk, soft drinks, etc.) in which the distribution of micro-organisms is homogeneous or readily rendered homogeneous by mechanical means (shaking, etc.);

— for the liquid part of a heterogeneous mixture which is considered to be sufficiently representative of the sample as a whole (for example the aqueous phase of animal or vegetable fats).

In all other cases and in case of doubt, use the procedure described in 9.1.2.

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 (see clause 8).

9.1.1 Liquid samples (which can be taken by pipette)

Shake the test sample (see clause 8) manually by performing 25 up-and-down movements of amplitude about 30 cm in 7 s, or preferably use a standardized mechanical device to ensure uniform distribution of micro-organisms. Take 1 ml with a pipette (6.6) and add this test portion to 9 ml of diluent (5.4) avoiding contact between the pipette and the diluent (see the note to 5.4).

Carefully mix the test portion and diluent, either by aspirating ten times with a different pipette, or in the mechanical mixer (6.3) for 5 to 10 s. The frequency of rotation of the latter shall be chosen so that the liquid, as it swirls, rises to within 2 or 3 cm of the rim of the vessel.

NOTE — If it is known that, for certain products, clusters of micro-organisms are likely to be more efficiently dispersed by mechanical mixing than by the pipette procedure, thus giving significantly different results, the specific standard appropriate to the product concerned should only recommend one of these procedures, preferably that using the mechanical mixer. The conditions of use of the mixer should be precisely specified.

9.1.2 Other samples

Weigh, to the nearest 0,01 g, into a bowl [in the case of the rotary blender (6.2a)] or in a plastic bag [in the case of the stomacher (6.2b)], a mass m (generally 10 g or a multiple of 10 g) of the test sample (see clause 8), of sufficient size so that all the tests and all the further dilutions required by the specific standard appropriate to the product concerned can be performed.

Add a volume, in millilitres, equal numerically to $9 \times m$ of the diluent (5.4) (see the note to 3.1), at the appropriate temperature.

Operate the rotary blender for a sufficient time to give a total number of 15 000 to 20 000 revolutions. Even with the slowest blender, this time shall not exceed 2,5 min.

Operate the stomacher for 1 to 2 min, according to the nature of the product (see note 2).

Allow large particles to settle, if necessary for up to 15 min, then transfer a certain quantity from the top layer of the suspension to a culture tube, flask, or bottle (6.5) using a large pipette (6.7) (if there is a fat layer, take the sample from the aqueous part).

This quantity shall be of sufficient size so that all the tests and further dilutions can be performed. If only one portion has to be taken from the initial suspension for inoculation or further dilution, this transfer may be omitted.

NOTES

1 The stomacher may not be suitable for some products (for example those with sharp particles, or with constituents that are not easily broken down). It should only be used when there is evidence (published data or comparative tests) that the results obtained do not differ significantly from those obtained using a rotary blender.

2 Attention is drawn to the fact that, for certain products, in particular cereals, the times given above are not appropriate for micro-organisms such as yeasts and moulds.

In this case, the stomacher allows greater recovery rates than the rotary blender. Operate the stomacher for 10 min and avoid separation, as some yeasts and moulds could be lost from the supernatant liquid.

9.2 Further decimal dilutions

NOTE — In the case of a presence or absence test for 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 (or, if the mixture of the initial suspension was obtained using a pipette, use the same pipette), 1 ml of the initial suspension [primary 1 + 9 (10^{-1}) dilution] (9.1.1 or 9.1.2) into another tube containing 9 ml of sterile diluent at the appropriate temperature, avoiding contact between the pipette and the diluent (see the notes to 3.1 and 5.4).

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

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

9.3 Repetition of the different operations

Carry out the series of operations described in 9.1 and 9.2 as many times as is required (once, twice, etc.), in accordance with the specific standard appropriate to the product concerned.

NOTE — It has been established statistically that, in order to reduce the variability of the results when using the colony count technique, it is preferable to repeat the different operations with separate portions of test sample rather than to double the number of plates inoculated from each tube of a single dilution series.

9.4 Duration of the procedure

In general, the dilutions shall be prepared from the test sample immediately prior to the analysis; they shall be used for inoculating culture media within 30 min of preparation.