INTERNATIONAL STANDARD

ISO 6887-1

Second edition
2017-03

Microbiology of the food chain — 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

Microbiologie de la chaîne alimentaire — Préparation des échantillons, de la suspension mère et des dilutions décimales en vue de l'examen microbiologique —

Partie 1: Règles générales pour la préparation de la suspension mère et des dilutions décimales

Reference number
ISO 6887-1:2017(E)

COPYRIGHT PROTECTED DOCUMENT

ISO copyright office
Ch. de Blandonnet 8 • CP 401
CH-1214 Vernier, Geneva, Switzerland
Tel. +41 22 749 01 11
Fax +41 22 749 09 47
copyright@iso.org
www.iso.org

Contents

Page

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 procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

This second edition cancels and replaces the first edition (ISO 6887-1:1999), which has been technically revised.

A list of parts in the ISO 6887 series can be found on the ISO website.

Introduction

Because of the large variety of food and animal feed products, this horizontal method might not be appropriate in every detail for certain products. In this case, different methods which are specific to these products can be used if absolutely necessary for justified technical reasons.

When this document is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from this method 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 may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed, they will be changed to comply with this document so that eventually, the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

This document defines the general rules for the preparation of samples, initial suspensions and subsequent dilutions for microbiological examination. The remaining parts of ISO 6887 give specific rules for the preparation of samples and initial suspensions, each covering the variety of food and feed products and environmental samples to which ISO 6887 applies.

For a number of products, it is necessary to take special precautions, especially when preparing the initial suspension, because of the physical state of the product (such as dry products, highly viscous products) or the presence of inhibitory substances (such as spices, high salt content) or the acidity, etc. These are covered in general terms in this document.

Any special diluents or practices required for particular products or microorganisms in specific standard methods take priority over the general rules listed in the ISO 6887 series. These can include the following:

— specific rehydration procedures for foods of low water activity to minimize osmotic shock;

— the use of adequate temperatures to aid suspension of cocoa, gelatine, milk powder, etc.;

— resuscitation procedures for the improved recovery of stressed microorganisms resulting from food processing and storage;

— homogenization procedures and duration specific to certain products (e.g. cereals) and/or to certain determinations (e.g. yeasts and moulds).

Microbiology of the food chain — 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

WARNING — The use of this document may involve hazardous materials, operations and equipment. It is the responsibility of the user of this document to establish appropriate safety and health practices and to determine the applicability of regulatory limitations before use.

1 Scope

This document defines general rules for the aerobic preparation of the initial suspension and of dilutions for microbiological examinations of products intended for human or animal consumption.

This document is applicable to the general case and other parts apply to specific groups of products as mentioned in the foreword. Some aspects might also be applicable to molecular methods where matrices can be associated with inhibition of the PCR steps and consequently affect the test result.

This document excludes preparation of samples for both enumeration and detection test methods where preparation instructions are detailed in specific International Standards.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

3.1
laboratory sample
sample prepared for sending to the laboratory and intended for inspection or testing

[SOURCE: ISO 7002:1986, A.19]

3.2
composite sample
mixed sample of a number of the same items of food, animal feed, animals or environment, from which a test portion is taken for examination in the laboratory

Note 1 to entry: See illustration of a composite sample in Annex A.

3.3
pooled sample
mixed sample of a number of the same items of food, animal feed, animals or environment, where the complete mixture is the test portion and is taken as a whole for examination in the laboratory

Note 1 to entry: See illustration of a pooled sample in Annex A.

3.4
test sample
sample prepared from the *laboratory sample* (3.1) according to the procedure specified in the method of test and from which *test portions* (3.5) are taken

Note 1 to entry: Preparation of the laboratory sample before the test portion is taken is infrequently used in microbiological examinations.

[SOURCE: ISO 7002:1986, A.47]

3.5
test portion
measured (volume or mass) representative sample taken from the *laboratory sample* (3.1) for use in the preparation of the *initial suspension* (3.6)

Note 1 to entry: Sometimes preparation of the *laboratory sample* (3.1) is required before the test portion is taken, but this is infrequently used in microbiological examinations.

3.6
initial suspension
primary dilution
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 with, normally, a nine-fold quantity of diluent, allowing large particles, if present, to settle

Note 1 to entry: Nine-fold quantities of diluent are normally used to produce a decimal dilution series, but other ratios may be required for specific purposes, such as to enumerate low numbers.

3.7
further dilution
suspension or solution obtained by mixing a measured volume of the *initial suspension* (3.6) with an *x*-fold volume of diluent and by repeating this operation with further dilutions until a dilution series, suitable for the inoculation of culture media, is obtained

Note 1 to entry: Ten-fold dilutions are normally used to produce a decimal dilution series, but other ratios may be required for specific purposes.

3.8
pooled test portions
mixture of test portions from a number of the same items of food, animal feed, animals or environment, where the complete mixture is the test portion examined

Note 1 to entry: See illustration of pooled test portions in Annex A.

3.9
pooled (pre-)enriched test portions
individually (pre-)enriched test portions from a number of the same items of food, animal feed, animals or environment, from which specified volumes are combined for further examination

Note 1 to entry: See illustration of pooled (pre-)enriched test portions in Annex A.

3.10
specific standard
International Standard or guidance document describing the examination of a specific product (or group of products) for the detection or enumeration of a specific microorganism (or group of microorganisms)

4 Principle

Preparation of the initial suspension (3.6) in such a way as to obtain as uniform a distribution as possible of the microorganisms contained in the test portion (3.5).

Preparation, if necessary, of further dilutions (3.7) in order to reduce the number of microorganisms per unit volume to allow, after incubation, observation of their growth or not (in the case of tubes or bottles) or colony counting (in the case of plates), as stated in each specific standard.

NOTE In order to restrict the range of enumeration to a given optimum interval, or if high numbers of microorganisms are foreseen, it is possible to inoculate only the necessary (decimal) dilutions needed to achieve the enumeration according to the calculations described in ISO 7218.

5 Diluents

5.1 Basic materials

To improve the reproducibility of test results, it is recommended that either ready-made diluents or dehydrated basic components or a dehydrated complete preparation should be used. In all cases, the manufacturer's instructions shall be followed rigorously.

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

The water used shall be distilled water or of equivalent quality (see ISO 7218 or ISO 11133).

For more detailed rules on preparation and performance testing of culture media, see ISO 11133.

5.2 Diluents for general use

5.2.1 Peptone salt solution

5.2.1.1 Composition

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

5.2.1.2 Preparation

Dissolve the components in the water in flasks, bottles or test tubes (6.4) by heating, if necessary.

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

5.2.2 Buffered peptone water

5.2.2.1 Composition

Peptone[a]	10,0 g
Sodium chloride	5,0 g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)[b] ‡	9,0 g
Potassium dihydrogen phosphate (KH_2PO_4) ‡	1,5 g
Water	1 000 ml

[a] For example, enzymatic digest of casein.

[b] If disodium hydrogen phosphate with a different water content is used, amend the mass of the ingredient accordingly. For example, in case of anhydrous disodium hydrogen phosphate (Na_2HPO_4), use 3,57 g.

‡ Buffer ingredients, see 5.2.3.

5.2.2.2 Preparation

Dissolve the components in the water in flasks, bottles or test tubes (6.4), by heating if necessary.

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

5.2.3 Double-strength buffered peptone water

This diluent may be necessary for high acid samples (see 8.6) and is prepared by dissolving double the quantities of a complete dehydrated medium in 1 000 ml of water and processing in the same manner.

If the diluent is prepared from individual ingredients, only double the quantities of the two buffer ingredients (marked ‡) are required.

5.3 Diluents for special purposes

See the specific standard or part of ISO 6887 appropriate to the product concerned.

5.4 Distribution and sterilization of the diluent

Dispense the diluent in volumes as necessary for the preparation of the initial suspensions into vessels (6.4) of appropriate capacity.

Dispense further diluent in volumes as necessary for the preparation of the (decimal or other ratio) dilutions into vessels (6.4) of appropriate capacity.

The tolerance allowable on final diluent volumes, after sterilization, shall not exceed ±2 %.

In order to enumerate several groups of microorganisms using different culture media, it may be necessary to distribute all the diluents (or some of them) in quantities greater than 9,0 ml into vessels (6.4) of appropriate size.

Stopper the vessels loosely to allow for expansion on heating.

Sterilize in the autoclave at 121 °C ± 3 °C for 15 min (see ISO 7218).

After autoclaving, check that the volumes from a proportion of the batch of diluent prepared are within the permitted tolerance of ±2 %. This may be achieved either destructively by emptying the contents

of the vessels into a tared container after autoclaving or non-destructively by marking and weighing vessels positioned through the autoclave both before and after autoclaving. For small batches of less than 100 units, check at least one unit; for larger batches, check 3 % to 5 % by either method.

To ensure diluent volumes meet the permitted tolerance, autoclaving bulk volumes and dispensing the required amounts into sterile vessels aseptically may also be used.

5.5 Performance testing for diluents

Test all diluents before use, according to Table 1, by the method given in ISO 11133.

The productivity target for diluents requires that the number of colonies counted after the specified incubation time at laboratory ambient temperature (18 °C to 27 °C) shall be within ±30 % of the number counted initially.

Table 1 — Test microorganisms and productivity criterion for diluents

Media	Incubation	Test microorganisms	WDCM number[a]	Reference medium	Control method	Criteria
Peptone salt diluent Buffered peptone water (single and double strength) Peptone solution Phosphate buffered diluent	45 min to 1 h at laboratory ambient temperature (18 °C to 27 °C)	*Escherichia coli*[c] *Staphylococcus aureus*	00012 or 00013 00034[b]	TSA	Quantitative	±30 % of original count

[a] Refer to the reference strain catalogue available at http://www.wfcc.info for information on culture collection strain numbers and contact details.

[b] Strain to be used as a minimum.

[c] Free choice of strain; one of the strains must be used as a minimum.

6 Apparatus

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

6.1 Apparatus for dry sterilization (oven) and wet sterilization (autoclave), see ISO 7218.

6.2 Homogenizer.

6.2.1 Rotary homogenizer (blender).

See ISO 7218. If a large sample is to be homogenized, the equipment should include a sterile 1 litre bowl.

6.2.2 Peristaltic homogenizer.

See ISO 7218. With sterile bags or filter bags to retain particulate material where necessary.

6.3 Mechanical stirrer, see ISO 7218.

6.4 Flasks, test tubes or screw-cap bottles, of appropriate capacities.

6.5 Total-delivery graduated pipettes, of nominal capacities 1 ml and 10 ml, graduated in 0,1 ml and 0,5 ml divisions, respectively. Mechanical pipettors of suitable accuracy may also be used (see ISO 7218).

6.6 pH-meter, capable of being read to the nearest 0,01 pH unit at 25 °C, enabling measurements to be made which are accurate to ±0,1 pH unit (see ISO 7218).

6.7 Balances and gravimetric diluters, capable of weighing to 1 % of the mass (see ISO 7218).

6.8 Sterile sample trays, of appropriate dimensions.

6.9 Sterile scissors, forceps or tongs, straight scalpels or **knives**, and **spatulas**.

6.10 Special opening equipment, such as bottle and can openers.

6.11 Equipment for collecting test portions from frozen laboratory samples.

6.11.1 Variable speed electric drill, with maximum speed in use of 900 r/min, or **hand drill**.

6.11.2 Sterile wood bit for electric drill, of 14 mm or 16 mm diameter.

6.11.3 Sterile wood chisel, of 20 mm width, and **hammer** or **plastic mallet**.

6.11.4 Other apparatus that does not cause overheating or contamination of the sample.

6.11.5 Equipment for cauterization of sample surfaces, e.g. portable gas blowtorch.

6.11.6 Template for surface sampling, metallic frame of appropriate dimensions to delineate the surface to be sampled, sterilized by flaming after immersion in 70 % (volume fraction) alcohol.

6.12 Water bath, capable of being maintained at 44 °C to 47 °C or as stated for specific purposes.

6.13 Sterile wide-necked bowls, containers or **plastic bags**, of 500 ml capacity.

6.14 Sterile glass beads or **balls**, to disperse samples such as swabs.

7 Sampling

Carry out sampling in accordance with the specific standard appropriate to the product concerned or see ISO/TS 17728. If specific sampling instructions are not available, it is recommended that agreement be reached on this subject by the parties concerned.

Some guidance is given in other parts of ISO 6887 on sampling specific to certain products (see, for example, ISO 6887-3).

8 Preparation of samples

8.1 General

Requirements for the different general categories of products subjected to microbiological examination are given in this clause. For specific requirements on certain product types, see the appropriate part of ISO 6887 for further information.

In other cases, 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.

8.2 Frozen products

8.2.1 General

Frozen products are considered under two headings:

— small laboratory samples that may be defrosted before testing;

— large pieces or blocks from which laboratory samples or test portions are taken without defrosting.

8.2.2 Small samples defrosted before testing

These samples include packaged retail products of all types (generally under 2 kg), including cuts and portions of meat and fish, vegetables, desserts and prepared multi-component products.

All such products stored and submitted to the laboratory frozen should be brought to a consistency that allows sampling in the original packaging. This may be achieved by standing at 18 °C to 27 °C (laboratory ambient temperature) for a maximum of 3 h, or at 5 °C ± 3 °C for a maximum of 24 h. Store thawing samples on separate trays (6.8) to prevent cross-contamination from any "drip" (thaw liquid) leaking through the packaging.

Samples shall be tested as quickly as possible after this, even if the product is still partially frozen when taking the test portion, as addition of the diluent at ambient laboratory temperature will facilitate full defrosting.

Defrosting in a temperature-controlled water bath or under running cold water is not recommended as this can result in contamination of the sample if the packaging is not completely watertight.

8.2.3 Large pieces or blocks sampled while frozen

8.2.3.1 General

These samples include large pieces or blocks of frozen products (generally over 2 kg), including carcases and joints of meat, and fish that has been block frozen in bulk.

Separate the sample from any packaging using scissors or a scalpel (6.9), and place it on a tray (6.8) with a flat side facing upwards.

Three options for sampling exist depending on the purpose of the testing and requirements of the customer. If sampling requirements are not known or specified, these should be discussed between all parties.

8.2.3.2 **Total sample** (surface and depth)

Using an electric drill (6.11.1) equipped with the appropriate bit (6.11.2) or any other apparatus (6.11.4), or failing this, the hand drill (6.11.1), make holes in the specified points (see Annex B). Set the speed of the drill or other apparatus to not more than 900 r/min to avoid fusion or dispersion of the shavings.

Using a sterile spatula (6.9), collect the resultant shavings and place them in a tared container or plastic bag (6.13) to be used for homogenization. If the mass is greater than 50 g, mix the shavings thoroughly in another container or plastic bag to provide a test sample, and then remove the final homogeneous test portion for testing.

The entire sampling operation shall not cause a significant increase in the temperature of the sample that would damage any microorganisms present.

8.2.3.3 Sample at depth only

Using the wood chisel and hammer (6.11.3), remove a surface strip 2 mm to 3 mm thick from an area of approximately 6 cm by 6 cm.

Cauterize the exposed area with the blowtorch (6.11.5) until the cleared surface is carbonized. Then proceed according to 8.2.3.2, making holes in the cauterized area without penetrating through to the lower surface of the block.

8.2.3.4 Surface only sample

Sterilize the template (6.11.6) and the wood chisel (6.11.3) by dipping in 70 % (volume fraction) alcohol and flaming. While the template is still hot, apply it to the surface of the frozen product.

Using the sterile chisel and hammer (6.11.3), chip off the upper layer of product within the template to a depth of 2 mm to 3 mm. Collect the resultant pieces and place them in a tared container or plastic bag (6.13) to be used for homogenization.

8.3 Hard and dry products

Do not homogenize hard or dry products in a rotary homogenizer for more than 2,5 min at one time to avoid an excessive rise in temperature.

For some hard and dry products, it may be necessary to mince or to grind the laboratory sample. In this case, to avoid an excessive rise in temperature, do not mince or grind for more than 1 min.

8.4 Dehydrated and other low-moisture products

Mix powdered products thoroughly in their container and then weigh out using aseptic techniques.

Other products may require breaking or cutting up into small pieces with sterile tools (6.9) before sampling.

For dehydrated and other low-moisture products, it is important to weigh the diluent and then add the test portion to reduce osmotic shock on any microorganisms present.

Low-moisture products may require a period (up to 1 h) soaking in the diluent used for the initial suspension to soften them before homogenization and subsequent manipulations. See ISO 6887-4 for detailed requirements for the different types of low moisture products.

See ISO 6887-4 for further information on preparation of low-moisture products before testing for specific groups of microorganisms, such as yeasts and moulds.

8.5 Liquid and non-viscous products

Before taking the test portion, the laboratory sample should be shaken by hand (e.g. 25 times through an arc of 25 cm) or by mechanical means in order to ensure that the microorganisms are uniformly distributed.

8.6 Acidic products

It is important when preparing a suspension of acidic products that the pH is brought back to near neutrality (pH 7,0 ± 0,5).

The use of buffered peptone water (5.2.2) is sufficient for most products with pH greater than or equal to 4,5. More acidic products (greater than or equal to pH 3,5) may be brought back to the required pH using double-strength buffered peptone water (5.2.3), but the pH of such products should be checked when these are tested for the first time to ensure the required range is achieved.

Samples which continue to acidify during incubation of non-selective (pre-)enrichment cultures, e.g. "live" yogurts and similar cultured products, may reduce the culture pH during incubation and should be monitored to check the pH remains above 4,5. Increased buffering capacity may be used, but the modified (pre-)enrichment of such products shall be verified to ensure conditions are satisfactory for growth of the specific microorganisms sought.

See ISO 6887-4 for further information on preparation of acidic products before testing for specific groups of microorganisms such as acidophiles.

8.7 High-fat (over 20 %) foods

The use of a diluent with between 1 g/l and 10 g/l of added polysorbate 80 [polyoxyethylene (20) sorbitan monooleate], according to approximate fat levels, may improve emulsification during suspension.

Generally, 1 g/l per 10 % fat content is sufficient (e.g. for fat content of 40 %, add 4 g/l).

See ISO 6887-4 for further information on examining high-fat foods.

8.8 Multi-component products

For multi-component products (which contain pieces of different foods), sampling should be carried out by taking amounts of each component representative of their proportions in the initial product.

Homogenizing the whole laboratory sample is also possible as this will provide a more homogeneous test sample for subsequent examination of a test portion (see Annex C).

It may be necessary to mince or to grind the laboratory sample. In this case, do not mince or grind for more than 1 min to avoid an excessive rise in temperature.

8.9 Packaged products

Packaged products submitted to the laboratory are of various types but these are considered under two headings as follows:

— soft packaging: to be removed or opened aseptically using scissors, knives or scalpels (6.9);

— rigid packaging (cans, glass containers, etc.) to be opened using appropriate implements (6.10) under aseptic conditions.

All operations, before and after opening packaged foods, shall be carried out aseptically to avoid external contamination.

If it is possible to remove the contents aseptically after opening without risk of external contamination, cleaning and disinfecting of the packaging is not necessary.

Clean the surface of rigid or semi-rigid packaging using mild detergent in water, then dry with a clean towel or fresh absorbent paper. When packaging is very thin and could be damaged by wetting (e.g. pieces of food packaged in films or flexible containers), omit this step and disinfect only.

Disinfect the outside of packaging carefully with 70 % (volume fraction) alcohol or aseptic wipes to avoid contamination when opening.

Open film-wrapped portions of food on trays carefully by peeling off the packaging film so the food can be exposed for sampling.

For foods packed in a controlled atmosphere and vacuum-packed foods, open the sealed packaging using a sterile knife, scalpel or scissors and forceps or tongs (6.9).

8.10 Surface samples (swabs and other devices)

Mix the swabs, or other devices such as small cloths or wipes, in the same diluent as that used to saturate them for sampling and/or to transport them, to disperse the microorganisms that adhere (see ISO 18593).

To achieve this, break the handles off the swabs so the swabs may be shaken in the specified quantity of diluent for the initial suspension. Small glass beads or balls (6.14) may be used to aid dispersion of retained organisms on the swabs or other devices.

Use the resulting suspension as the initial suspension.

For surface samples, the initial dilution should be recorded. For example, from a sample (swab or other) from a 25 cm^2 surface and diluted in a total volume of 25 ml of diluent, 1 ml of this initial suspension represents 1 cm^2.

9 Specific procedures

9.1 Test portion and initial suspension (primary dilution)

Weigh or measure the test portion, to a tolerance of ±5 %, into a sterile container or plastic bag (6.13). A mass of m g or a volume of V ml (minimum 10 g or 10 ml, unless otherwise stated) representative of the laboratory sample shall be used (see Clause 8).

NOTE Using larger test portions than the minimum specified above will increase the reliability of enumeration test results[6] (see example data in Annex C).

Add a quantity of diluent equal to 9 × m g or 9 × V ml to prepare a primary decimal dilution. This quantity shall be measured, preferably by mass, to a tolerance of ±2 %, but measurement by volume is also permitted provided that the tolerance is no more than ±2 %. Other primary dilutions of lower or higher ratios of diluent to test portion may be required for special purposes; these are prepared using the same tolerances.

Certain types of product result in viscous or thick initial suspensions when prepared with the usual 1 in 10 dilution and additional diluent may be necessary to facilitate further testing. In such cases, the diluent shall be added in other ratios (e.g. 1 in 20, 1 in 50, 1 in 100) until a satisfactory initial suspension for further operations is achieved. These non-standard ratios shall be taken into account in subsequent operations, particularly in the calculation and expression of results.

In other cases where low numbers of microorganisms are sought and the product being tested will produce a suitably liquid initial suspension, it may be desirable to prepare the suspension at lower ratios (e.g. 1 in 2 or 1 in 5). However, use of such lower ratio initial suspensions may result in imbalance of the inoculum to medium ratio in subsequent selective plating in or on solid media or into liquid media (e.g. inhibition of microbial growth by the increased concentration of food components) and this approach shall be used with caution and verified on a case-by-case basis.

To avoid damage to microorganisms by sudden changes in temperature, the temperature of all diluents shall be approximately the same as the laboratory ambient temperature, except where otherwise specified for particular products (see specific standard).

Homogenize the mixture according to the requirements of ISO 7218, using equipment such as that listed in 6.2.

Allow large particles to settle, if necessary, for up to 15 min. Filtration systems giving equivalent results, such as plastic bags with integral filter liners, may also be used.

For enumeration of spores, heat treatment of the initial suspension (e.g. 10 min ± 1 min at 80 °C ± 5 °C) shall be performed immediately after preparation of the suspension, followed by rapid cooling (e.g. under running cold water) to minimize subsequent loss of the target spores.

9.2 Duration of the procedure

The time between the end of the preparation of the initial suspension (9.1) and the moment when the inoculum comes into contact with the final culture medium shall not exceed 45 min.

Additionally, the time between the preparation of the initial suspension (9.1) and the beginning of preparation of any subsequent dilutions (see Clause 10) shall not exceed 30 min.

If the ambient temperature of the laboratory is high and outside the recommended range (>27 °C), these two maximum durations should be reduced to minimize potential for microbial growth and consequently higher results.

If a resuscitation period to maximize recovery of damaged microorganisms is required by the specific International Standard, this shall be timed once the initial suspension has been prepared and the subsequent dilution steps commenced immediately after this period has ended.

9.3 Pooling and compositing procedures for qualitative tests

There are four options for combining examination steps for qualitative examinations of product or environmental samples of the same type from the same source or origin, to reduce workload when a large number of samples is to be examined (see Clause 3 for definitions). This may be necessary to reflect microbiological quality of a large batch of product or environmental samples or is sometimes required by national or regional legislation.

A number of items of the same type may be composited or pooled at the sampling stage and the client shall make this clear when the laboratory sample is received to ensure the subsequent test procedure is carried out correctly. Compositing of individual samples may also be undertaken at the laboratory if the client has made clear that this is required. These two procedures are illustrated in Figure A.1 and Figure A.2 (see Annex A).

Pooling of test portions may also be carried out in the laboratory and the examination continued in larger quantities of pre-warmed media as illustrated in Figure A.3 (see Annex A). Temperature monitoring and maximum incubation times from the range permitted should be used to avoid false negative results caused by temperature lag in the larger volumes. Alternatively, the (pre-)enrichment cultures from individual test portions may be pooled (see Figure A.4) and carried through as a single test.

All these procedures shall be verified before use to show that the risk of false-negative results has not been increased; an example of a suitable verification protocol is given in Annex D.

10 Further dilutions

10.1 Decimal dilution series

For a decimal dilution series for use in enumeration tests, transfer, using a pipette (6.5), 1 ml ± 0,02 ml of the initial suspension into a tube containing 9 ml ± 0,2 ml of sterile diluent at laboratory ambient temperature (unless otherwise stated in the specific standard). Avoid any contact between the pipette containing the inoculum and the sterile diluent to minimize cross-contamination potential.

NOTE If a greater volume is needed to perform a large number of tests or replicate tests, a determined volume (more than 1 ml) of the initial suspension, with a tolerance of ±2 %, can be added to a tube containing a nine-fold volume of sterile diluent.

For optimal precision, do not introduce the pipette more than 1 cm into the initial suspension and avoid withdrawing particles of the food product in the inoculum.

Mix thoroughly, preferably by using a mechanical stirrer (6.3) for 5 s to 10 s, to obtain a 10^{-2} dilution.

If necessary, repeat these steps using the 10^{-2} and subsequent dilutions and a new sterile pipette or tip for each operation, to obtain sufficient (10^{-3}, 10^{-4}, etc.) dilutions to enumerate the appropriate number of microorganisms at the optimum range for counting (see Clause 4). The sequence of operations for

preparing multiple decimal dilutions suitable for different anticipated levels of contamination and using the pour plate technique is shown in Figure 1.

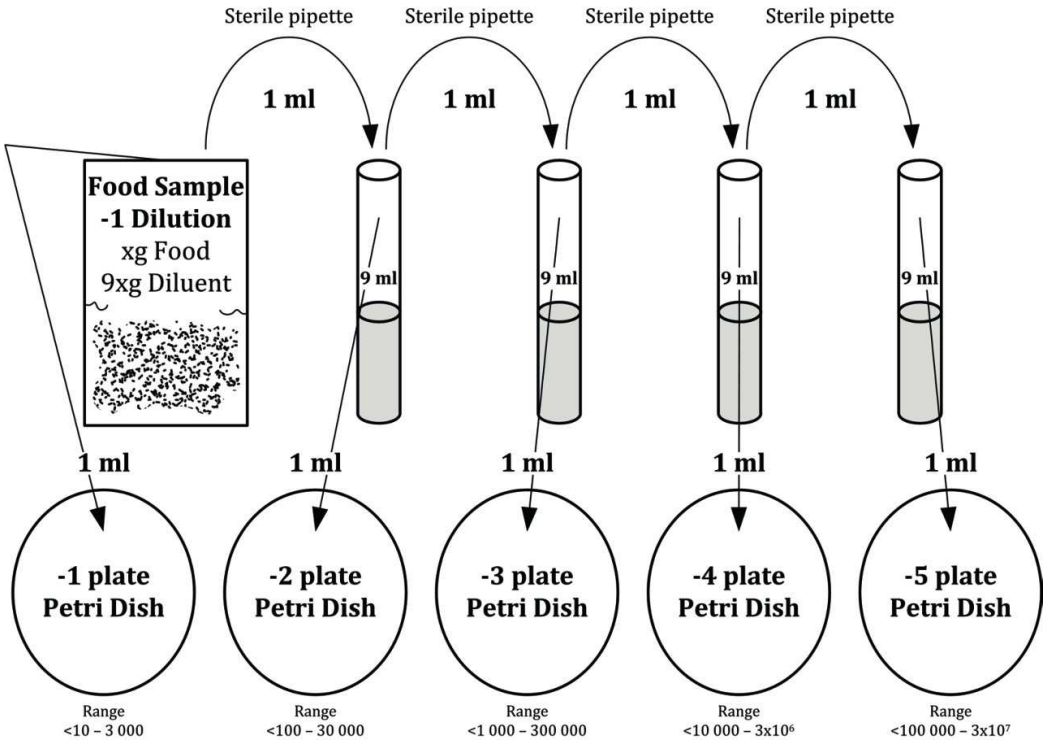


Figure 1 — Sequence for preparation of multiple decimal dilutions — Example for pour plate technique

10.2 Other dilution series

Prepare any other dilution series required for special purposes, e.g. 1 in 2 (1 ml to 1 ml), 1 in 5 (1 ml to 4 ml), in an identical way by using different ratios of the initial suspension to diluent. Record the ratio and take it into account in subsequent steps, such as the calculation and expression of results.

Annex A
(informative)

Illustrations of pooling and compositing procedures

A.1 General

These illustrations show pooling and compositing procedures, as defined in Clause 3, which may be used for qualitative testing only. The test procedure given in ISO 6579-1 for detection of *Salmonella* is shown as an example, but the same procedures may also be applied to other detection methods.

It is possible to composite (see Figure A.1) or pool (see Figure A.2) samples of the same type, to reduce workload when a large number of samples are required to be examined. This may be necessary to reflect microbiological quality of a large batch of product or environmental samples or required by national or regional legislation.

Similarly, a number of test portions may be pooled (see Figure A.3) and examined together in larger quantities of media, or the (pre-)enrichment cultures from the test portions may be pooled (see Figure A.4) and carried through as a single test.

All these procedures shall be verified before use to show that the risk of false-negative results has not been increased. An example of a suitable verification protocol is given in Annex D.

A.2 Composited samples

A number of items are composited into one sample and mixed before the test portion is taken as shown in Figure A.1.

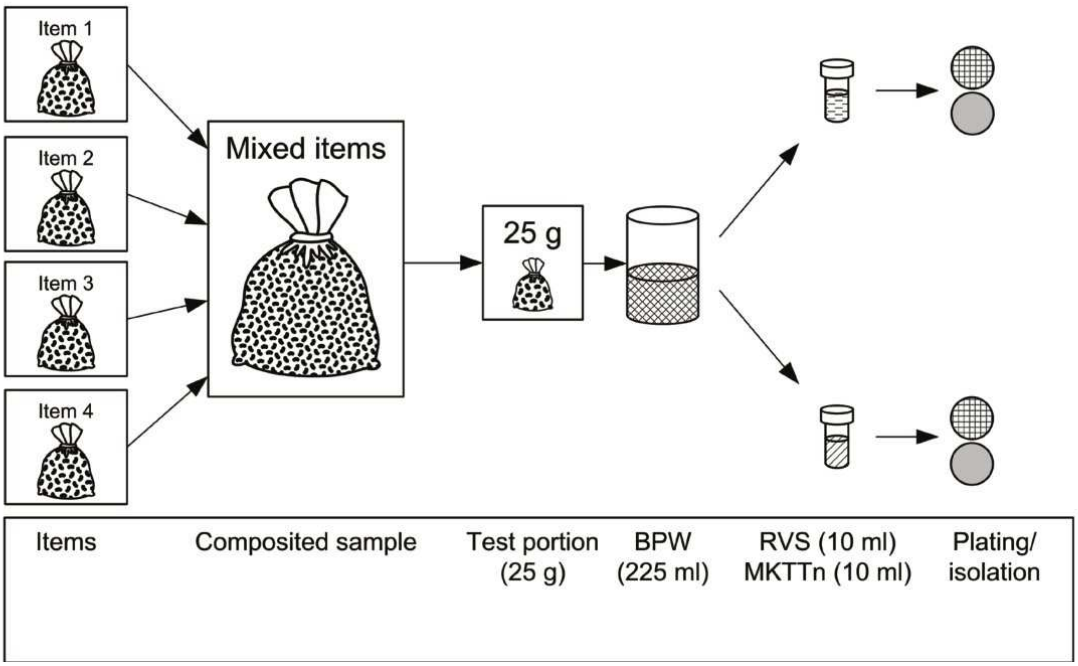


Figure A.1 — Compositing samples

A.3 Pooled samples

A number of items of the same type are pooled into one sample and the whole mixture is used as the test portion (see Figure A.2).

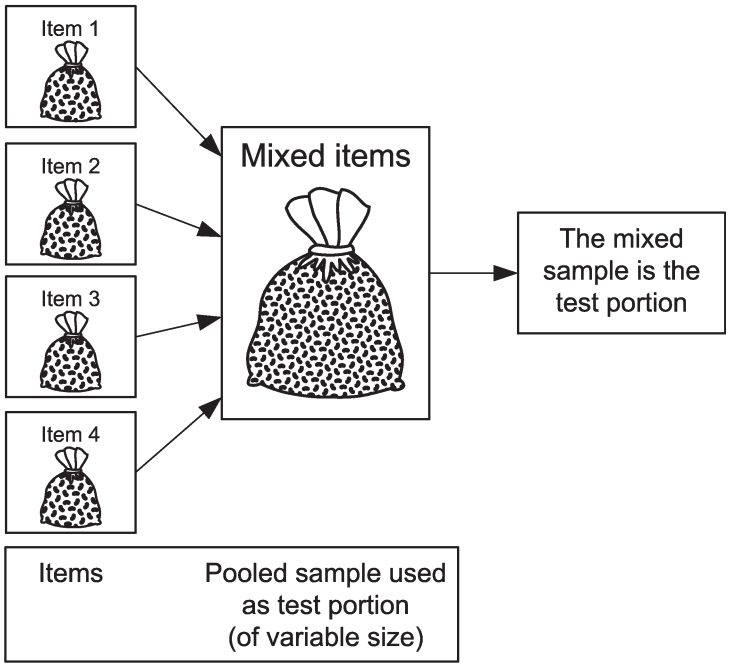


Figure A.2 — Pooling samples

A.4 Pooled test portions

The test portions from a number of items of the same type are mixed and the whole mixture is used as the test portion for subsequent examination (see Figure A.3).

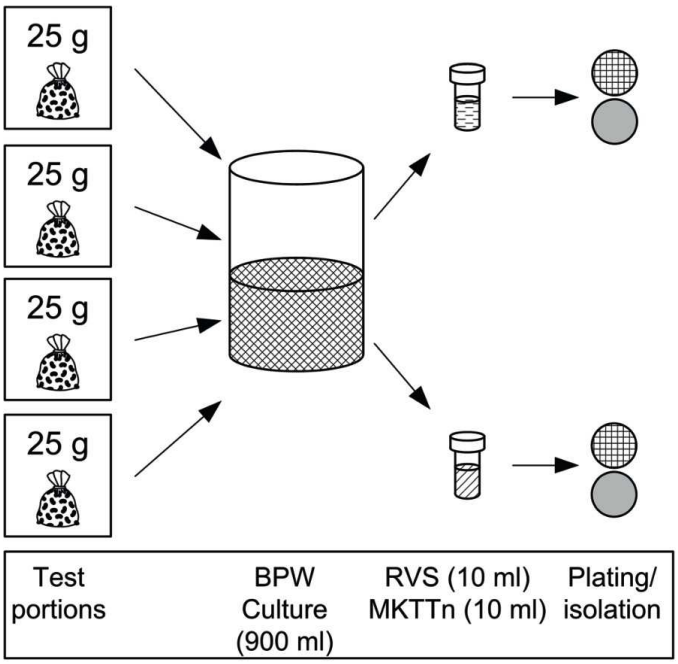


Figure A.3 — Pooling test portions

A.5 Pooled (pre-)enriched test portions

The test portions of a number of items of the same type are (pre-)enriched and then a specified volume from each culture is combined for subsequent examination (see Figure A.4).

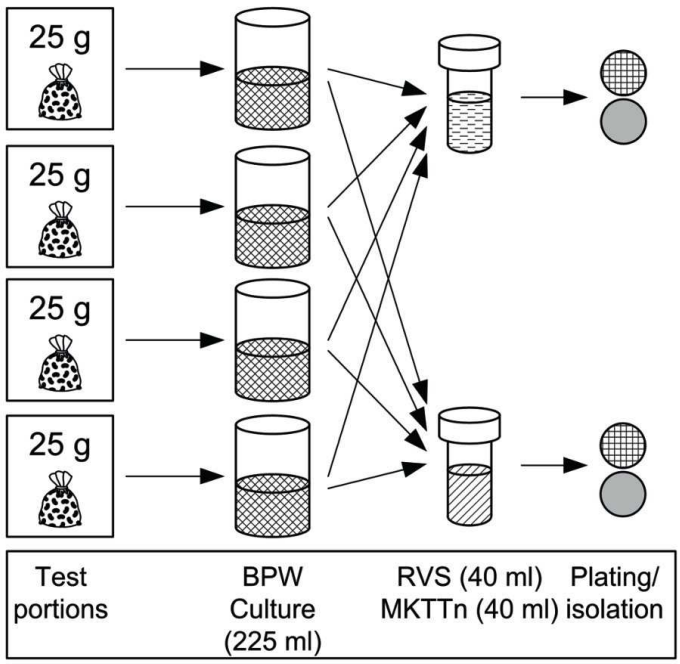


Figure A.4 — Pooling (pre-)enriched test portions

Annex B
(informative)

Method for sampling frozen test pieces or blocks

B.1 Non-homogeneous blocks

For non-homogenous blocks (compressed, conglomerated, frozen or deep-frozen pieces) of mass 25 kg to 30 kg, the perforation points shall be as shown in Figure B.1.

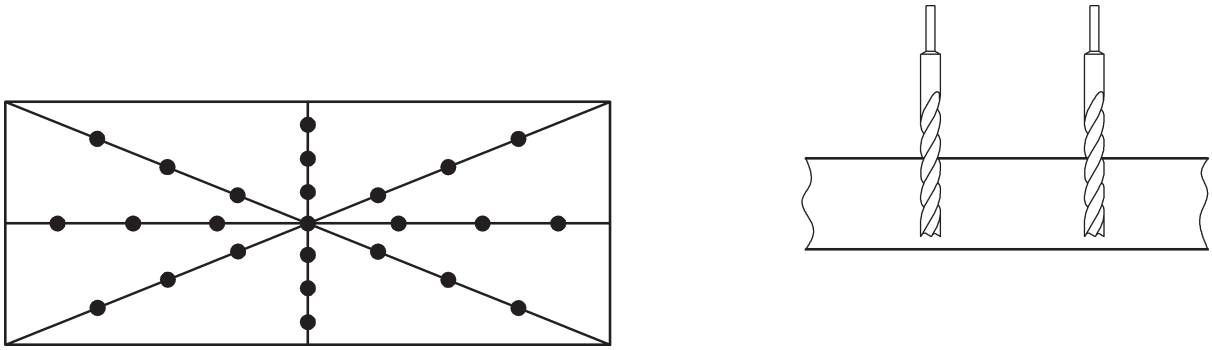
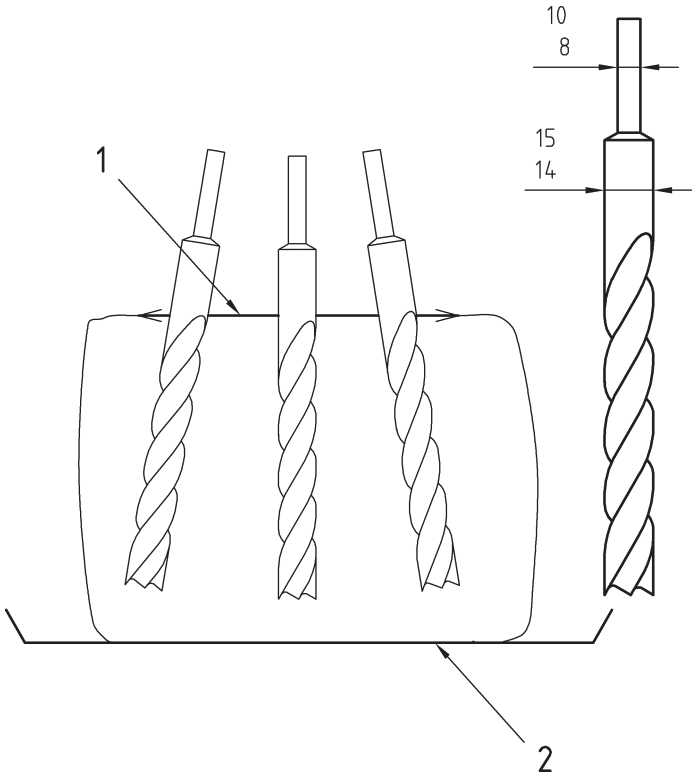


Figure B.1 — Procedure for non-homogeneous blocks

B.2 Homogeneous test pieces

For homogeneous pieces, the perforation points and depth limits shall be as shown in Figure B.2.

Dimensions in millimetres



Key

1 cauterized area

2 tray

Figure B.2 — Procedure for homogeneous samples

Annex C
(informative)

Data showing reliability of test results according to size of test portions

C.1 General

Data are available to show that the larger the test portion used, the less variance occurs between replicate test results of the same sample types.[6] The data presented in this annex are based on an experimental protocol developed and used in The Netherlands and France.

C.2 Dutch study comparing different sample sizes and effects of sample homogenization

Samples of 600 g from three sample types (pre-cut vegetables, Chinese rice dish and milk shake or soft ice cream) were used to compare differences in the homogeneity of the individual sample types, together with four different sample preparation procedures. The test portions for two samples were taken without prior homogenization, while two test samples were homogenized before taking the test portions (see Table C.1).

Table C.1 — Study design to show effects of sample preparation method and test portion size

Sample preparation method	Homogenization of sample	Test portion	Test portion dilution
T1	No	10 g	1 in 10
T2	Yes (100 g)	10 g	1 in 10
T3	Yes (100 g, 1 to 1 with diluent)	20 g	1 in 5
T4	No	35 g	1 in 10

For samples prepared without homogenization, two test portion sizes were used: the minimum of 10 g specified for enumeration tests in many specific standards (T1) and a larger test portion of 35 g (T4).

For the two samples prepared with homogenization, test samples of 100 g were taken and homogenized: one (T2) was homogenized directly, while the other (T3) was diluted 1 to 1 with diluent before homogenization.

All sample preparations were tested for aerobic colony count, using a final dilution factor of 1 in 10.

The results of the study were tested for variance using the F-test and these are shown in Table C.2.

Table C.2 — Effect of four sample preparation techniques on the variance of test results from three sample types

Matrix (no. of samples)	Results	Sample preparation T1 (10 g)	Sample preparation T2 (100 g)	Sample preparation T3 (100 g)	Sample preparation T4 (35 g)
Pre-cut vegetables (18)	Mean	0,150	0,164	0,111	0,172
	Standard deviation	0,128	0,179	0,071	0,183
	Variance	0,016	0,032	0,005	0,003
Chinese rice dish (22)	Mean	0,285	0,218	0,104	0,216
	Standard deviation	0,261	0,239	0,072	0,237
	Variance	0,068	0,057	0,005	0,056
Milk shake or soft ice cream (8)	Mean	0,094	0,064	0,069	0,115
	Standard deviation	0,142	0,035	0,042	0,092
	Variance	0,020	0,001	0,002	0,008
TOTALS	Mean	0,201	0,171	0,101	0,182
	Standard deviation	0,212	0,200	0,068	0,199
	Variance	0,045	0,040	0,005	0,040

These data are in agreement with other published work[6] showing that the least variance for all three sample types of differing homogeneity was obtained when the largest samples (100 g) were homogenized, with or without prior 1 to 1 dilution.

C.3 French study comparing seven different sample preparation techniques

Samples of three different sample types (pâté, cheese and mixed salad) were prepared using seven different techniques (T1 to T7) involving variations of sample size and homogenization as shown in Table C.3.

Table C.3 — Study design to show effects of sample preparation method and test portion size

Sample preparation method	Homogenization of sample	Test portion	Test portion dilution
T1	No	10 g from 1 area	1 in 10
T2	No	10 g taken from 5 areas	1 in 10
T3	Yes (whole sample)	10 g	1 in 10
T4	Yes (whole sample, 1 to 1 with diluent)	20 g	1 in 5
T5	No	35 g from 1 area	1 in 10
T6	No	35 g taken from 5 areas	1 in 10
T7	Yes (100 g, 1 to 1 with diluent)	20 g	1 in 5

The study was similar to that described in C.2, with a further variation of taking the test portions from a single area of the sample (T1 and T5) or from five different areas (T2 and T6) across the sample. Technique T7 was similar to technique T4 except that a sample of 100 g was used rather than the whole sample.

All sample preparations were tested for aerobic colony count, using a final dilution factor of 1 in 10.

The results of the study were tested for variance and these are shown in Table C.4.

Table C.4 — Effect of seven sample preparation techniques on the variance of test results from three sample types

Technique (test portion size)		T1 (10 g)	T2 (10 g)	T3 (10 g)	T4 (20 g)	T5 (35 g)	T6 (35 g)	T7 (20 g)
Sample size		Whole	Whole	Whole	Whole or ≈100 g	Whole	Whole	100 g
Homogenized (Y/N)		N	N	Y	Y (1 to 1 with diluent)	N	N	Y (1 to 1 with diluent)
No. of samples		129	124	130	135	6	10	16
Sample types		Pâté, cheese	Pâté, cheese	Pâté, cheese	Pâté, cheese	Pâté	Pâté, mixed salad	Pâté, mixed salad
RESULTS (550 samples in duplicate)	sd	0,81	0,59	0,36	**0,17**	0,33	0,30	**0,15**
	Variance	0,66	0,35	0,13	**0,03**	0,11	0,09	**0,02**

These data also illustrate that the least variance for all three sample types of differing homogeneity was obtained when the largest samples (100 g) were homogenized, with or without prior 1 to 1 dilution.

Annex D
(informative)

Verification protocol for pooling samples for qualitative tests

D.1 Pooling tests

D.1.1 General

Two procedures for pooling test portions at different stages of qualitative testing are detailed in the main text (see 9.3). This annex describes a protocol suitable for verifying that the pooling procedure has no effect on the number of false-negative results obtained after examination of different matrices by qualitative methods.

Only test portions from the same type of product or environmental sample are to be pooled and the chosen pooling procedure is to be verified before subsequent use on routine samples. Only samples from the same origin/source (e. g. same batch, lot) are to be pooled and then only at the request of the client. Samples from different origin/sources, such as from different clients, are not to be pooled.

D.1.2 Inocula

Use a standard suspension of an appropriately stressed strain (see ISO 16140-2) of the test microorganism appropriate to the method being investigated. Inoculate test portions of the matrix at a level of approximately 5 cfu per 25 g (or ml), of which not more than 2 of 300 replicate tests should contain no detectable organisms per 25 g.

NOTE The size of the test portion is normally 25 g (or 25 ml) for qualitative tests, but alternative quantities can be used provided this is taken into account.

The stress conditions applied should mimic the type of stress encountered by the target microorganism when present in a naturally contaminated sample of the product or environmental sample.

Certified or other reference materials with a known range of the appropriate microorganism may be used for preparation of the inocula.

D.1.3 Sample preparation

Add or mix an appropriate quantity of the suspension of microorganisms to a known quantity of uncontaminated product or environmental sample to provide sufficient material for the series of tests to be undertaken. If samples are inoculated in bulk, ensure that this is sufficiently homogeneous. Alternatively, inoculate replicate pre-weighed 25 g (or ml) quantities of matrix to give sufficient test portions containing the defined level of microorganisms.

Retain a suitable quantity of the uninoculated matrix to use as sterile controls and to mix with the inoculated samples if preliminary tests show that the inoculum level is too high.

D.1.4 Pooling test portions

D.1.4.1 Preliminary reference test

Add the inoculum (D.1.2) containing approximately 5 cfu of the test microorganism to the initial suspension of the 25 g (or ml) test portion in 225 ml (pre-)enrichment broth, incubate and complete the examination according to the method being investigated.

A “detected” result confirms that the inoculum was recovered from the inoculated test portion of 25 g (or ml).

D.1.4.2 Verification protocol

The example described is pooling of test portions from 10 samples of the same type for the detection of *Salmonella* (by ISO 6579-1) but the protocol may be used for different numbers of samples, other microorganisms and other procedures.

Mix 10 test portions of 25 g (or ml) from each sample (total 250 g or ml) with 2 250 ml of pre-warmed pre-enrichment medium. Add the inoculum (D.1.2) containing approximately 5 cfu *Salmonella* and incubate the entire culture in accordance with the standard procedure, ensuring that the incubation temperature is achieved within a suitable time.

After incubation, subculture and complete the examination according to the method being investigated.

If *Salmonella* is detected, pooling of 10 × 25 g (or ml) test portions of the sample type is verified for the selected test strain and the stress conditions applied.

If *Salmonella* is not detected, repeat the pooling using fewer test portions until a positive result is obtained.

D.1.5 Pooling (pre-)enrichment portions

D.1.5.1 Preliminary reference test

Carry out the reference test according to D.1.4.1.

D.1.5.2 Verification protocol

The example described is pooling of pre-enrichment portions from 10 samples of the same type for the detection of *Salmonella* (by ISO 6579-1) but the protocol may be used for different numbers of samples, other microorganisms and other procedures.

Add the inoculum (D.1.2) containing approximately 5 cfu *Salmonella* to one of ten initial suspensions of 25 g (or ml) of the sample in 225 ml of pre-enrichment medium and incubate all ten suspensions in accordance with the standard procedure.

Inoculate a defined volume of the inoculated pre-enrichment culture and equal volumes of the other uninoculated pre-enrichment cultures into an appropriate volume of the selective medium, ensuring that the specified ratio for this sub-culturing step is maintained. Incubate the selective medium and complete the examination according to the method being investigated.

D.1.5.3 Interpretation of results

If *Salmonella* is detected, pooling of 10 pre-enrichment cultures from 25 g (or ml) test portions of the sample type is verified for the selected test strain and the stress conditions applied.

If *Salmonella* is not detected, repeat the pooling using fewer pre-enrichment cultures until a positive result is obtained in all replicate tests.

D.2 Confirmation of findings

Evidence to suggest that one or other of the pooling procedures described provides a satisfactory means of testing multiple samples requires confirmation.

Little reliance should be placed on the results of a single trial and the chosen protocol should be repeated at least five times, and ideally eight to ten times, using different samples of the same matrix

type/target microorganism combination to ensure, with reasonable precision, that the test is capable of detecting the target organism at the lower effective concentration.

Bibliography

[1] ISO 7002, *Agricultural food products — Layout for a standard method of sampling from a lot*

[2] ISO/TS 17728, *Microbiology of the food chain — Sampling techniques for microbiological analysis of food and feed samples*

[3] ISO 18593, *Microbiology of food and animal feeding stuffs — Horizontal methods for sampling techniques from surfaces using contact plates and swabs*

[4] ISO 16140-2, *Microbiology of the food chain — Method validation — Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method*

[5] ISO 6579-1, *Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of* Salmonella *— Part 1: Detection of* Salmonella *spp.*

[6] CORRY. J.E.L.C., JARVIS B., HEDGES A.J. Minimising the between-sample variance in colony counts on foods. *Food Microbiol*. 2010, **27** pp. 598–603

ICS 07.100.30

Price based on 26 pages