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*भारतीय मानक*

*Indian Standard*

सूक्ष्मजैविक विश्लेषण के लिए खाद्य पदार्थों के नमूने लेने,

परिवहन, भंडारण और नमूने तैयार करने की विधि

(*दूसरा पुनरीक्षण* )

Sampling, Transport, Storage and Sample Preparation of

Food Samples for Microbiological Analysis

(*Second Revision*)

ICS 07.100.30

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**B U R E A U O F I N D I A N S T A N D A R D S**

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Food Microbiology Sectional Committee, FAD 31

**FOREWORD**

This Indian Standard (First Revision) was adopted by the Bureau of Indian Standards, after the draft finalized by the Food Microbiology Sectional Committee had been approved by the Food and Agriculture Division Council.

Sampling is an operation that requires most careful attention; emphasis should be strongly laid on the necessity of obtaining a properly representative sample. When sampling is performed by laboratories, written sampling procedures are demanded by IS/ISO/IEC 17025 ‘General requirements for the competence of testing and calibration laboratories’. Written procedures are also required for subsampling steps in the laboratory, that is the preparation of test/analytical portions. The sampling procedure is a part of the measurement procedure, but not of the measurement itself.

A wide variety of food categories of either plant or animal origin are available in the market, which include fresh, processed precooked, canned, frozen and dehydrated foods that are sold to consumers in bulk packages and in containers of different types and sizes. The microorganisms that are inherently present in the food or which gain entry into the products during production, processing and packaging can grow at favorable temperatures during storage. These organisms, may be pathogenic or spoilage types. The pathogenic organisms and toxin-producing types give rise to food poisoning and food-borne infections. The spoilage type may not cause infection but affect quality by spoilage of foodstuffs.

Microbiological examination of the foods provides information on the hygienic environment of their production and handling, processing efficiency, defects due to microbial growth and the organisms responsible for the same, and on the presence of specific pathogens and food poisoning organisms.

The objective of this standard is to enable the user to obtain representative samples of a food lot, submit the samples to the laboratory in a condition that is unchanged microbially from the time of sampling and prepare the samples for analysis. The procedure describes in this standard apply generally to collecting, labelling, transporting, storing and preparing samples for analysis.

The procedures described in this Indian Standard are recognized as a good practice to be followed whenever practicable. However, it is impossible to lay down fixed rules to be followed in every case, and, however explicit, they cannot fully take the place of judgement, skill and experience. In particular, unforeseen circumstances may render some modifications desirable. Whenever special requirements are given for sampling and/or arise from a specific analysis to be performed, these requirements should be followed.

This document also defines the general rules for the preparation of samples, initial suspensions and subsequent dilutions for microbiological examination. 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.

Any special diluents or practices required for particular products or microorganisms in specific standard methods take priority over the general rules listed in this method which can include the following:

* 1. specific rehydration procedures for foods of low water activity to minimize osmotic shock;
  2. the use of adequate temperatures to aid suspension of cocoa, gelatin, milk powder, etc.;
  3. resuscitation procedures for the improved recovery of stressed microorganisms resulting from food processing and storage;
  4. homogenization procedures and duration specific to certain products and/or to certain determinations.

This standard was first published in 1969 with a view to providing general guidance in regard to the practices to be followed and precautions to be observed in the sampling of different types of foods and in handling of the samples for microbiological analysis with the title ‘Methods for drawing and handling of food samples for microbiological analysis’. The standard was first revised in 1984 making general requirements of sampling more exhaustive and modifying the scale of sampling so as to bring it in line with the corresponding provisions given in other Indian Standards on foods.

The second revision of the standard has been brought out to incorporate following modifications keeping in view the technological advancements in this area alongwith the editorial changes to align the standard as per the current Indian Standard format:

1. Detailed sample preparation procedures for various commodities of food products for microbiological analysis have been incorporated in the document.
2. The standard has been revised thoroughly, making it inclusive with respect to the sampling, transport, storage and sample preparation of various commodities, hence, the title of this Indian Standard has been modified reflecting this change, accordingly.
3. Milk and milk foods and special foods, like infant foods have also been included in the standard which have been excluded in the scope pf previous version of this standard.
4. ‘Performance testing of diluents’, ‘Carcass sampling for microbiological analysis’ and ‘Classification of major taxa of fish’ have been added in the standard in the Annex A, Annex B and Annex C, respectively.

The list of documents referred while preparing this standard are included in Bibliography.

The composition of the committee responsible for the formulation of this standard is listed in Annex D.

In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS 2 : 2022 ‘Rules for rounding off numerical values (second revision)’.

|  |  |  |
| --- | --- | --- |
| **CONTENTS** | | Page |
| **Foreword** | |  |
|  | **Scope** |  |
|  | **References** |  |
|  | **Terms and definitions** |  |
|  | **Sampling** |  |
|  | **Transport and storage**  5.1 Transport  5.1.1 General  5.1.2 Apparatus and equipment  5.1.3 Transportation protocol  5.1.4 Transportation by the laboratory  5.1.5 Transportation by a contractor or courier  5.1.6 Storage during transport  5.2 Receipt of Samples  5.3 Storage of Samples |  |
|  | **Sample preparation**  6.1Diluents and general sample preparation procedure  6.2 General preparation of samples  6.3 Sample preparation – meat and meat products including  poultry meat 6.4 Sample preparation – milk and milk products6.5 Sample preparation – miscellaneous products6.6 Eggs and egg products6.7 Bakery goods, pastry and cakes, doughnuts, Ready to Eat (RTE) foods6.8 Fresh fruit and vegetables (pre-packed)6.9 Fermented products (idli, uttapam etc.) or other products containing viable microorganisms6.10 Beverages (alcoholic and non-alcoholic drinks and bottled waters, still carbonated)6.11 Sample preparation - fish & fish products6.12 Sample preparation - crustaceans, molluscs, tunicates and echinoderms6.13 Salted or pickled products (including fish eggs/roe such as caviar)6.14 Fermented, marinated and breaded products6.15 Frozen fish, Crustacea, Molluscs, Tunicates and Echinoderms6.16 Additional Guidance for Small Fish, Crabs and Lobsters |  |
| **Annex A Performance testing for diluents** | |  |
| **Annex B Carcass sampling for microbiological analysis** | |  |
| **Annex C Classification of major taxa of fish**  **Bibliography** | |  |

*Indian Standard*

**Sampling, Transport, Storage and Sample Preparation of**

**Food Samples for Microbiological Analysis**

( *First Revision* )

1. **SCOPE**

**1.1** This standard prescribes the procedures for sampling, transport, storage and sample preparation of food samples for the purpose of microbiological analysis.

**1.2** The procedure for sample preparation of following important types of foods and food categories are covered in this standard:

1. Meat & Meat Products
2. Milk & Milk Products
3. Egg & egg products
4. Bakery goods, pastry and cakes, doughnuts and ready to eat products
5. Fresh fruits and vegetables (pre packed)
6. Fermented products (idli, uttapam etc.) or other products containing viable microorganisms
7. Beverages (alcoholic and non-alcoholic drinks and bottled waters, still carbonated)
8. Fish and fish Products (Raw fishery products, including fish, crustaceans, molluscs, tunicates and Echinoderms)
9. Miscellaneous products [Spices & inhibitory food materials, cocoa and cocoa containing products, confectionery (bars or sweets), flours, cereal grains and by-products, animal feed, gelatin, margarine and spreads]

**1.3** Because of the large variety of food products, this 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.

**2 REFERENCES**

The standards given below contain provisions which, through reference in this text, constitute provisions of this standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of these standards.

|  |  |
| --- | --- |
| *IS No.* | *Title* |
| IS 10232 : 2020/ ISO 6887-1 : 2002 | Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — general rules for the preparation of initial suspension and decimal dilutions ( s*econd revision* ) |
| IS 17383 : 2020  ISO 11133 : 2014 | Microbiology of food chain carcass sampling for microbiological analysis ( *first revision* ) |
| IS 16122 : 2013  ISO 7218 : 2007 | Microbiology of food and animal feeding stuffs - General requirements and guidance for microbiological examinations |

1. **TERMS AND DEFINITIONS**
   1. **Acceptance Criteria -** Sample characteristics required upon arrival at the laboratory and before the acceptance
   2. **Bulk Products -** Products that are not separated into individual items or units.
   3. **Bulk Sample**
2. collection of increments or groups thereof intended for separate investigation (raw bulk sample); or
3. combined aggregation of the items or portions of items taken from a lot of pre-packed products (bulked sample).
4. composite of the increments taken from a bulk lot (bulk sample in a proper sense); or
   1. **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.

* 1. **Further Dilution -** Suspension or solution obtained by mixing a measured volume of the *initial suspension* 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.
  2. **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.
  3. **Laboratory Sample -** Amount or units of product that arrive in the laboratory to be analyzed.
  4. **Lot/Batch -** A lot is a predefined quantity of food product, produced under similar, or uniform, conditions so that the units in the lot are similar in their microbiological status.

* 1. **Packaged Products -** Products separated into units or items, sealed or wrapped by the manufacturer.
  2. **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.
  3. **Random Sample -** A random sample is one in which all elements in the lot have an equal and independent chance of being included in the sample.
  4. **﻿Receipt -** Procedures adopted by the laboratory when the samples arrive.
  5. **Refrigeration/Cold Chain -** Maintenance of samples at cold temperatures to minimize changes in microbial load.
  6. **Representative Sample -** Sample drawn so as to reflect, as accurately as possible, the properties of interest of the lot from which it is taken.
  7. **Sample (General Term) -** One or more items (or a proportion of material) selected in some manner from a population (or from a larger quantity of material) intended to provide information representative of the population, and possibly, to serve as a basis for a decision on the population or on the process which had produced it.
  8. **Sample Size (n) -** Sampling size is the number of sample units comprising the total sample drawn from a lot or production.
  9. **Sample Unit -** Sample unit is one of a number of individual containers, or a portion of a food or primary container examined or evaluated as a single unit.
  10. **Sampling Plan -** Predetermined procedure for the selection, withdrawal, and preparation of samples from a lot to yield the required information so that a decision can be made regarding the acceptance of the lot.
  11. **Sampling Technique -** Appropriate procedures using sampling devices to obtain and describe samples of food chain or feed material, either in the field or food industries or during transportation and in laboratory.
  12. **Sampling -** It is the selection of a certain portion, number of container/pack of the product units from a particular lot of the same food.
  13. **Test Portion -** Measured (volume or mass) representative sample taken from the laboratory samplefor use in the preparation of the initial suspension.
  14. **Test Sample -** Sample prepared from the laboratory sample (**3.7**)according to the procedure specified in the method of test and from which test portions (**3.21**)are taken.
  15. **Transport -** Care and handling of the sample from when it was taken until arrival at the laboratory to ensure that microbiological integrity is maintained.

1. **SAMPLING**
   1. **General**
      1. The presence of certain microorganisms in foods can affect public health and the quality of foods consumed. For this reason, the sampling followed by testing of foods for a variety of microorganisms is a common part of most food safety and quality systems. The adequacy and condition of the sample received for testing are of primary importance. If samples are improperly collected and mishandled or are not representative of the sampled lot, the laboratory results will be misleading as interpretations about a large consignment of food are based on a relatively small sample of the lot, established sampling procedures must be applied uniformly.
      2. The number of units that comprise a representative sample from a designated lot of a food product must be statistically significant. The composition and nature of each lot affects the homogeneity and uniformity of the total sample mass. The proper statistical sampling procedure, according to whether the food is solid, semisolid, viscous, or liquid, must be determined by the collector at the time of sampling by using the suitable sampling plan. Sampling plans are required which ensure that fair and valid procedures are used when food is being controlled for compliance. No sampling plan can ensure that every item in a lot conforms. These sampling plans are nevertheless useful for guaranteeing an acceptable quality level.
      3. Most of the sampling procedures involve the selection of a sample (or samples) from a lot (**3.8**), the inspection or analysis of the sample, and the classification of the lot (as ‘acceptable’ or ‘not acceptable’) based upon the result of the inspection or analysis of the sample.
   2. **Sampling Procedure**

For the purpose of detailed sampling procedure, the general guidelines issued by Food Safety and Standards Authority of India for the sampling of food products for microbiological analysis may be referred (Refer Bibliography for the link to download the guidelines).

1. **TRANSPORT AND STORAGE**
   1. **Objectives -** The main objectives of transport and storage are to:
2. deliver food product samples to the laboratory for analysis within the stipulated time period
3. protect food from potential sources of contamination
4. protect food from damage likely to render the food unsuitable for analysis;
5. retain the integrity of the sample and prevent further changes in the level of target microorganisms.
   1. **General Conditions:** Where necessary, conveyances and bulk containers should be designed and constructed so that they:
6. do not contaminate foods or packaging;
7. can be effectively cleaned and, where necessary, disinfected;
8. permit effective separation of different foods or foods from non-food items where necessary during transport;
9. provide effective protection from contamination, including dust and fumes;
10. can effectively maintain the temperature, humidity, atmosphere, and other conditions necessary to protect food from harmful or undesirable microbial growth and deterioration likely to render it unsuitable for consumption; and
11. allow any necessary temperature, humidity, and other conditions to be checked.
    1. **Transport**

**5.3.1** *General*

* + - 1. All samples collected should be transported to the laboratory at the earliest, not more than 36 h and preferably 24 h after collection. Chilled samples shall be transported to the laboratory for testing as quickly as possible at the temperature (2-8 °C) (but not frozen). For highly perishable fresh and refrigerated food products, freezing temperatures must be avoided during transport and storage. Pre-packed food should be stored at or below the storage temperature given on the label.
      2. Frozen or refrigerated products shall be transported in insulated containers of rigid construction under frozen conditions and the prescribed temperature of the products. Samples that were not frozen before sampling shall not be frozen after sampling.
      3. Dehydrated foods may be shipped and stored without refrigeration and should not be allowed to absorb any atmospheric moisture. These shall be stored in a clean, cool, and dust-free place. The samples should be protected from direct sunlight or other sources of heat.
      4. Samples having different storage temperatures shall be transported in separate transport containers. If cool packs/ ice gel packs are used, laboratory samples shall not be in direct contact.

**5.3.2** *Apparatus and Equipment*

**5.3.2.1** *Refrigerators, freezers, cool boxes, boxes or containers, cold packs*

1. *Refrigerated Vehicles*: These vehicles are equipped with a refrigeration unit to maintain the storage area below 8 °C and monitor temperatures during transport.
2. *Vehicle refrigerator*: Portable refrigerator for use in a vehicle to keep refrigerated samples below 8 °C. The refrigerator may be equipped with an integral battery or function from the vehicle battery.
3. *Vehicle Freezer*: Portable freezer for use in a vehicle to keep frozen samples at –15 °C or below. The freezer may be equipped with an integral battery or function from the vehicle battery.
4. *Cool Box*: Insulated container equipped with cold packs. The cool box shall be used to maintain the temperature at –15 °C or below for frozen products or below 8 °C for chilled products.
5. *Boxes and Containers*: Made of cardboard, polystyrene, or other plastic as appropriate.
6. *Cool Pack/ Ice Gel packs*: These are purpose-made packs that are frozen before use to maintain low temperatures when placed in sample transport containers.
   * + 1. *Temperature-Monitoring Equipment*
7. Thermometers, temperature probes, and integrated temperature recorders/ data logger which is battery-operated with associated software for programming the recorder and downloading the temperature records.
8. All devices shall be calibrated and capable of recording temperatures from –20 °C to +10 °C.

**5.3.3** *Transportation Protocol*

When sampling is carried out by the laboratory, samples may be transported by laboratory personnel using laboratory equipment or by a suitable transport service provider. In other cases, transport conditions are the responsibility of the sampler and/or client after discussion of suitable transport and delivery methods for the sample types with the laboratory. In the transportation protocol, the following critical factors shall be considered:

* + 1. duration of the transportation;
    2. nature of the samples, temperature and method of recording the temperature (i.e. before and after or throughout the transport);
    3. packaging and secondary containment to protect sample integrity;
    4. an arrangement in the transport boxes or other equipment to prevent the mixing of frozen, refrigerated, and non-perishable products. The sample may be labelled accordingly before transportation. For bivalve mollusks, gastropods, echinoderms, and tunicates, transport temperature shall be between 0 °C and 10 °C and these samples shall not be frozen.

**5.3.4** *Transportation by the Laboratory*

Immediately after collection, sample bottles, bags, or boxes shall be placed in a protective container at an appropriate temperature, such as a cool box containing cool packs/ ice gel packs. It is essential that samples, other than those already frozen, do not come into direct contact with frozen surfaces as this may affect the intrinsic microbial flora. If the laboratory vehicle is refrigerated or it is equipped with a portable refrigeration unit, the samples may be transferred from the portable container used for sampling. The refrigeration unit shall be switched on long enough before use to ensure the required temperature is achieved. If the vehicle is not refrigerated, a cool box shall be available in the vehicle and shaded from direct sunlight to minimize heat gain during transport. The temperature range permitted during transport and the maximum duration of transport shall be documented in the client contract. The permitted temperature range will depend on the food type (ambient, refrigerated or frozen product) and should be related to the transport duration, for example, >2 °C to ≤8 °C for chilled samples over longer transport times or >8 °C to <10 °C for shorter transport times of less than 4 h. The vehicle refrigerator or cool box should include a thermometer or a temperature recorder. If not, the temperature of the products or cool box should be recorded each time the cool box is opened and upon final receipt at the laboratory. If a temperature recorder is used, the monitoring unit should be placed in contact with the sample, however, the integrity of the samples should not be altered. Some ambient-stable products do not require refrigerated transport (e.g. powdered products, cans, etc.); however, in high ambient temperatures, it may be useful to record the vehicle and/or container temperature to check that excessively high temperature (e.g. >40 °C) have not affected the samples.

**5.3.5** *Transportation by a Contractor or Courier*

When the laboratory is not able to their use its own transport system, it may avail the service of suitable service providers following the above-mentioned transportation conditions. Conditions for transporting the samples shall be carefully documented and agreed upon before the contract is placed. Record the time the container is given to the contractor and also ensure that the time of arrival in the laboratory is recorded. Whenever the maximum agreed transport temperature or time is exceeded, the client shall be informed, and if testing is still required, the maximum transport temperature and/or time shall be recorded on the test report.

**5.3.6** *Storage During Transport*

The storage condition will be determined by the temperature control required for  
individual products.

**a)** *Perishable* -The sample should be stored under chilled or frozen conditions as per the requirement. Cool packs/ ice gel packs can be used for refrigerated products during transportation and temperature is to be maintained between 2-8 oC. For frozen products, the transportation temperature of -20 oC to -15 oC is to be maintained.

**b)** *Non-Perishable* **-** Storage of non-perishables should maintain the originality of the  
sample as is during the sampling conditions. Transportation should be done at  
temperatures not more than 40 oC.

**5.4 Receipt of Samples**

As soon as the sample arrives at the laboratory, its general physical condition should be noted. If the sample cannot be analyzed immediately, it should be stored as described in **5.5**. Check sampling containers for gross physical defects. Carefully inspect plastic bags and bottles for tears, pinholes, and puncture marks. If sample units were collected in plastic bottles, check bottles for fractures and loose lids. If plastic bags were used for sampling, be certain that twist wires did not puncture surrounding bags.  Each sample shall be checked for labels for appropriate information regarding the sample. Store samples at the appropriate temperature until analysis.

**5.5 Storage of Samples**

The samples for microbiological analysis shall be stored at a temperature mentioned below in order to minimize any alteration in the number of microorganisms present:

1. stable products: ambient temperature (18 °C to 27 °C);
2. frozen or deep-frozen products: below −15 °C, preferably below −18 °C;
3. other products not stable at ambient temperature (perishable products), including spoiled foods, unless otherwise stated in specific standards: 5 °C ± 3 °C.
4. **SAMPLE PREPARATION**
   1. **Diluents and General Sample Preparation Procedure**

## *Diluents*

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.

## *Diluents for General Use*

### *Peptone Salt Solution*

* + - * 1. *Composition:*

|  |  |
| --- | --- |
| Enzymatic digest of casein | 1.0 g |
| Sodium chloride | 8.5 g |
| Water | * 1. ml |

* + - * 1. *Preparation:*Dissolve the components in the water in flasks, bottles or test tubes by heating, if necessary. Adjust the *p*H if necessary so that, after sterilization, it is 7.0 ± 0.2 at 25 °C. The solution may be stored at a temperature of 5 °C ± 3 °C for a maximum of one month.

1. *Buffered Peptone Water*
   * + - 1. *Composition:*

|  |  |
| --- | --- |
| Peptonea | 10.0 g |
| Sodium chloride | 5.0 g |
| Disodium hydrogen phosphate dodecahydrate (Na2HPO4·12H2O)b | 9.0 g |
| Potassium dihydrogen phosphate (KH2PO4) | 1.5 g |
| Water | 1000 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 (Na2HPO4), use 3.57 g.

* + - * 1. *Preparation:*Dissolve the components in the water in flasks, bottles or test tubes by heating if necessary. Adjust the *p*H, if necessary, so that after sterilization, it is 7.0 ± 0.2 at 25 °C. The solution may be stored at a temperature of 5 °C ± 3 °C for a maximum of one month.

### *Double-Strength Buffered Peptone Water*

This diluent may be necessary for high acid samples like apple juiceand 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 are required.

**6.1.1.2** *Diluents for Special Purposes*

1. *Peptone-Salt Solution with Bromocresol Purple*
2. *Composition:*

|  |  |
| --- | --- |
| Peptone salt solution | 1000 ml |
| Bromocresol purple (0.04 % alcohol solution, e.g. ethanol solution) | 0.1 ml |

1. *Preparation***:** Add 0.1 ml of Bromocresol purple to 1000 ml of peptone salt solution.
2. *Application***:** This solution may be used in certain acidic products like fruit beverages so that adjustment of the *p*H can be carried out without the use of a sterile *p*H probe. Bromocresol purple is yellow at acidic *p*H, changing to purple at *p*H above 6.8. The solution may be stored at a temperature of 5 °C ± 3 °C for a maximum of one month.

##### *Sodium Citrate Solution*

1. *Composition:*

|  |  |
| --- | --- |
| Trisodium citrate dihydrate (Na3C6H5O7⋅2H2O) | 20.0 g |
| Water | 1. ml |

##### *Preparation***:** Dissolve the salt in water by heating, if necessary, on a hotplate at a temperature between 45 °C and 50 °C. Adjust the *p*H, if necessary, so that after sterilization it is 7.5 ± 0.2 at 25 °C. The solution may be stored at a temperature of 5 °C ± 3 °C for a maximum of one month.

##### *Application:*This solution is used for cheese and (roller-)dried milk, and some caseinates.

##### **c)** *Dipotassium Hydrogen Phosphate Solution*

1. *Composition:*

|  |  |
| --- | --- |
| Dipotassium hydrogen phosphate (K2HPO4) | 20.0 g |
| Water | 1. ml |

##### *Preparation:*Dissolve the salt in the water by heating, if necessary, on a hotplate at a temperature between 45 °C and 50 °C. For acid whey powder, adjust the *p*H so that for the primary dilution after sterilization it is 8,4 ± 0,2 at 25 °C. For cheese, roller-dried milk, fermented milk, yogurt, caseinates and sour cream, adjust the *p*H so that after sterilization it is 7,5 ± 0,2 at 25 °C. The solution may be stored at a temperature of 5 °C ± 3 °C for a maximum of one month.

##### *Application:*This solution is used for cheese, (roller-)dried milk, fermented milk, yogurt, some caseinates, dehydrated acid whey, and sour cream.

##### **d)** *Dipotassium Hydrogen Phosphate Solution with Antifoam Agent*

1. *Composition:*

##### 

##### Dipotassium hydrogen phosphate solution - Prepare according to **6.1.1.2 c)**

##### Antifoam stock solution - Dissolve 1 g polyethylene glycol 2000 in 100 ml water by mixing.

##### *Preparation:*Add 1 ml of the antifoam stock solution to 1 liter of the K2HPO4 solution. Adjust the *p*H so that for the primary dilution of both acid and lactic casein, after sterilization, it is 8,4 ± 0,2 at 25 °C, and for rennet casein, after sterilization, it is 7,5 ± 0,2 at 25 °C.

##### *Application***:** This solution is used for acid casein, lactic casein and rennet caseins.

##### **e)** *Tripolyphosphate Solution*

1. *Composition:*

|  |  |
| --- | --- |
| Sodium tripolyphosphate pentabasic (Na5O10P3) | 20.0 g |
| Water | 1000 ml |

##### *Preparation:*Dissolve the salt in the water by heating slightly on a hotplate, if necessary. The solution may be stored at a temperature of 5 °C ± 3 °C for a maximum of one month.

##### *Application:*This solution is used as alternative diluent for rennet caseins that are difficult to dissolve.

##### *Diluent for General Use with α-Amylase Solution*

1. *Composition:*

|  |  |
| --- | --- |
| α-amylase | 1.0 g |
| Water | 100 ml |

* + - 1. **2)** *Preparation:*Dissolve the α-amylase in the water and sterilize the solution by passing through a 0.2 µm membrane filter. The enzyme solution can be stored for up to 1 month at 5 °C ± 3 °C or up to 3 months at ≤ −20 °C.
      2. Final composition of the α-amylase solution may need to be adjusted depending on the enzymatic activity of the commercial α-amylase used and the thickening properties of the test sample.
      3. **3)** *Application:*This enzyme solution is added at the rate of 10 ml to 1 000 ml of diluent (1 percent volume fraction) to improve solubility of swelling starch products, cereals and cereal-containing products.

### g) *Cellulase Solution*

* + - 1. **1)** *Composition:*

|  |  |
| --- | --- |
| Cellulase | 1.0 g |
| Water | 100 ml |

* + - 1. **2)** *Preparation:*Dissolve the cellulase in the water and sterilize the solution by passing through a 0,2 µm membrane filter. The enzyme solution can be stored for up to two weeks at 5 °C ± 3 °C or up to 1 month at ≤−20 °C.

NOTE - Use of fresh solution will ensure maximum enzyme activity.

* + - 1. **3)** *Application:*This enzyme solution is added at the rate of 10 ml to 1 000 ml of diluent (1 percent volume fraction) to improve solubility of carboxymethyl cellulose, locust beans, carob, guar and cassia gums.

### h) *Papain Solution*

**1)** *Composition:*

|  |  |
| --- | --- |
| Papain | 5.0 g |
| Water | 100 ml |

* + - 1. **2)** *Preparation:*Dissolve the papain in the water and sterilize the solution by passing through a 0.2 µm membrane filter. The enzyme solution can be stored for up to 1 month at 5 °C ± 3 °C.

NOTE - Use of fresh solution will ensure maximum enzyme activity.

* + - 1. **3)** *Application:*This enzyme solution is added at the rate of 20 ml to 1 000 ml of diluent (2 percent volume fraction) to improve solubility of gelatin.

### *Phosphate Buffered Diluent*

1. *Composition:*

|  |  |
| --- | --- |
| Disodium hydrogen phosphate dodecahydrate (Na2HPO4 · 12H2O) | 9.0 g |
| Potassium dihydrogen phosphate (KH2PO4) | 1.5 g |
| Water | 1000 ml |

1. *Preparation:*Dissolve the components in the water, by heating, if necessary., Adjust the *p*H, if necessary, so that, after sterilization, it is *p*H 7,0 ± 0,2 at 25 °C.
2. *Application:*Phosphate buffered solution is used as a diluent for gelatine.

## 6.1.2 *Sterilization of the Diluent*

**6.1.2.1** Dispense the diluent in volumes as necessary for the preparation of the initial suspensions into flasks/bottles/test tubes of appropriate capacity. Dispense further diluent in volumes as necessary for the preparation of the (decimal or other ratio) dilutions into flasks/bottles/test tubes of appropriate capacity.

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

**6.1.2.3** 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 test tubes of appropriate size. Stopper the lid loosely to allow for expansion on heating. Sterilize in the autoclave at 121 °C ± 3 °C for 15 min.

**6.1.2.4** After autoclaving, check that the volumes from a proportion of the batch of diluent prepared are within the permitted tolerance of ±2 percent. For small batches of less than 100 units, check at least one unit; for larger batches, check 3 percent to 5 percent 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.

**6.1.3** Performance testing of diluents may be carried out as per procedure given in Annex A.

## 

* 1. **General Preparation of Samples**

## 6.2.1 *Frozen products*

Samples of frozen products may be of two types, namely, small laboratory samples that may be defrosted before testing, and large pieces or blocks from which laboratory samples or test portions are taken without defrosting.

### *Small Samples Defrosted Before Testing*

* + - * 1. 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.
        2. 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 period of 18-24 h. If rapid thawing is desired, thaw the sample at less than 45°C for not more than 15 min. Store thawing samples on separate trays to prevent cross-contamination from any “drip” (thaw liquid) leaking through the packaging.
        3. 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.

### 6.2.1.2 *Large Pieces or Blocks Sampled While Frozen*

* 1. These samples include large pieces or blocks of frozen products (generally over 2 kg), including carcasses and joints of meat, and fish that has been block frozen in bulk. Separate the sample from any packaging using scissors or a scalpel and place it on a tray with a flat side facing upwards.
  2. 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:

**1)** *Total Sample (Surface and Depth)*

1. Using an electric drill equipped with the appropriate bit or any other apparatus or failing this, the hand drill makes holes in the specified points Set the speed of the drill or other apparatus to not more than 900 r/min to avoid fusion or dispersion of the shavings.
2. Using a sterile spatula collect the resultant shavings and place them in a tared container or plastic bag 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.

**2)** *Sample at Depth Only*

Using the wood chisel and hammer 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 until the cleared surface is carbonized. Then proceed according to, making holes in the cauterized area without penetrating through to the lower surface of the block.

**3)** *Surface Only Sample*

Sterilize the template and the wood chisel 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 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 to be used for homogenization.

## *Hard and Dry Products*

Do not homogenize hard or dry products (For example, Beetle nut) 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.

## *Dehydrated and Other Low-Moisture Products*

**a)** Mix ‘powdered products’ (For example, Soy flour) 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 before sampling.

**b)** 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.

**c)** ‘Low-moisture products’ (For example, Dry fish) may require a time period (up to 1 h) soaking in the diluent used for the initial suspension to soften them before homogenization and subsequent manipulations.

## *Liquid and Non-Viscous Products*

For liquid and non-viscous products (For example, Non-alcoholic beverages), before taking the test portion, the laboratory sample should be shaken by hand (For example, 25 times through an arc of 25 cm) or by mechanical means in order to ensure that the microorganisms are uniformly distributed.

## *Acidic Products*

It is important when preparing a suspension of acidic products (For example, Fruit juices) that the *p*H is brought back to near neutrality (*p*H 7.0 ± 0.5). The use of buffered peptone water is sufficient for most products with *p*H greater than or equal to 4.5. More acidic products (greater than or equal to *p*H 3.5) may be brought back to the required *p*H using double-strength buffered peptone water but the *p*H 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 *p*H during incubation and should be monitored to check the *p*H 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.

## *High-Fat 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 of high fat foods (For example, Butter).

Generally, 1 g/l per 10 percent fat content is sufficient (For example, fat content of 40 percent, add 4 g/l).

## *Multi-Component Products*

For multi-component products (which contain pieces of different foods like pizza, RTE 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. 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.

## *Specialized Packaged Products (For Example, Canned, Multi Layered laminated Packs, Retort Pack etc.)*

* + - 1. 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
* Rigid Packaging: to be opened using appropriate implements under aseptic conditions
  + - 1. 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.
      2. 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 percent (volume fraction) alcohol or aseptic wipes to avoid contamination when opening.
      3. Open film-wrapped portions of food on trays carefully by peeling off the packaging film so the food can be exposed for sampling.
      4. 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.
      5. Record the particulars of the sample, namely, its size, make of container/tetra pack/retort pack, code marks, etc. Note the condition of the container/tetra pack/retort pack, mechanical defects, perforations, rust spots, dents and container/tetra pack/retort pack abnormalities.
      6. *Canned Foods*

1. *Preparation of Cans for Opening*— Clean the can with soap and water. If the can is greasy, petroleum ether, alcohol or naphtha may be applied at the site of opening. The cans should be preferably opened at a suitable place so as to preserve the packer’s code and also avoid disturbance of the seaming compound at the packer’s end of the can. The site of the opening may be sterilized by holding the can over the frame of a burner and distributing the heat with a circular motion over the previously cleaned top. The burner should not be played down on the top of the can as this will result in a concentration of heat at the top causing scorching of the material and it might lead to spurting of the contents when the opening is made. If the containers are badly swollen they should not be flamed but sterilized by applying mercuric chloride solution (0.2 percent) or 70 percent alcohol.
2. *Opening of Container* — If the can contains solid or semi-solid products, cut a circular disc around a central puncture using a sterile can opener. In the case of liquid products, cut a hole about 1.5 cm in diameter with a sterilized tapered punch.
3. *Removal of Sample* — Solid products may be removed by using sterile cheese trier, spoon or cork-bore. In the case of semi-solid and liquid products the samples may be drawn by using sterile un tapered glass tubes or pipettes.
4. The material may be transferred to dilution bottles or directly into different media in test-tubes or petri dishes for determining numbers and types of micro-organisms in the canned material.
5. In the case of concentrated acidic products, for example, tomato paste or fruit pulp, the spoilage may be highly localized. The top bottom and side of the product should be carefully examined separately for growth of micro-organisms.

**6.3 Sample Preparation - Meat and Meat Products Including Poultry Meat**

**6.3.1** *General Case for Acidic Products (Cured/Pickled Meat)*

## Sample preparation procedures as given in 6.2.5 may be followed for acidic meat products (having *p*H between 3.5 and 4.5) for acidic meat products, such as, cured/pickled meat.

## 6.3.2 *High-Fat Products*

The general case for preparation of high-fat products is given in **6.2.6** and there are no additional specific requirements for meat and meat products.

**6.3.3** *Fresh Meat/Chilled Meat*

Sample preparation requires taking sample into dilution buffer at ratio of 1:9 and homogenization in a suitable homogenizer. No additional specific requirements for meat and meat products.

NOTE - Use the whole laboratory sample for preparation of the initial suspension if the sample mass is equal to or less than 50 g.

### 6.3.4 *Blocks, Large Pieces, Meat Cuts*

For meat cuts, take the test portion at depth and/or a surface sample and prepare the initial suspension at the ratio of 1:9.

**6.3.5** *Slices or Pieces of Meat or Cooked Meat*

Take strips from the center of the slices or pieces to prepare the initial suspension at the ratio of 1:9.

**6.3.6** *Fragments, Shavings and Trimmings*

Homogenize these thoroughly before removing the test portion for preparation of the initial suspension at the ratio of 1:9.

### 6.3.7 *Meat Products in “Skins” (Sausages)*

If the skin is not intended for consumption, disinfect the cooked or raw sausages at the point of incision by wiping the surface with 70 percent (volume fraction) alcohol or by cauterizing using a blowtorch, pull to remove the skin with sterile forceps or tongs. Slice the sausages and cut into small pieces before homogenizing. Do not remove edible skins from raw sausages, but slice and homogenize including the skin. percent

### 

### 6.3.8 *Cooked Meats*

For packaged cooked meats, open the packaging as given in **6.2.8**of this standard and prepare test portions as for raw products.

### 6.3.9 *Chicken and Duck Feet*

Cut several units of chicken or duck feet (including all parts) with a pair of sterile scissors along the joints into smaller pieces. Mix and weigh out the test portion in a tared sterile plastic bag. Add nine times this mass of an appropriate diluent and massage by hand for 1 min to 2 min to make the 1 in 10 initial suspension using dilution buffer.

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**6.3.10** *Procedure for Pre-Packed Products*

For general instructions on sample preparation of packaged products, *see* **6.2.8** of this standard.

**6.3.11** *Procedure for Non-Frozen Products (Fresh Meat/Chilled Meat)*

### 6.3.11.1 *Sample Preparation from Depth Within the Test Material*

1. Such test portions are used to examine only the deep tissue and sampling is carried out after cauterization of the surface. Use scalpels and forceps to remove an appropriate area of skin from cuts of meat presented with skin on.
2. If packaged, remove the sample aseptically and place on a sterile tray. Remove a surface layer 2 mm to 5 mm thick from the upper surface to expose an area of approximately 5 cm by 5 cm with a sterile scalpel or knife. Cauterize this exposed surface using a blowtorch until charring occurs. Using a fresh sterile knife or scalpel, remove a layer about 4 cm by 4 cm and 1 cm deep from below the charred area. Using sterile forceps and scalpel, remove the required test portion from the exposed area and place it in a tared sterile container or plastic bag.
3. Weigh the test portion and add nine times this mass of an appropriate diluent to make the 1 in 10 initial suspension.

### *Sample Preparation from the Surface of Meat (Excision/Destructive Method)*

1. Samples are taken without cauterization of the exposed surface.
2. If packaged, it may be necessary to remove the meat aseptically and place it on a sterile tray with the test surface uppermost. Use a sterilized or disinfected template and apply to the designated area. Using a sterile scalpel, cut along the inside edges of the template. Then, using sterile forceps to lift the test portion, cut across the whole area to a depth of 2 mm to 3 mm and place the pieces in a tared sterile container or plastic bag.
3. Weigh the test portion and add nine times this mass of an appropriate diluent to make the 1 in 10 initial suspension.For such surface samples, the initial dilution should be recorded. For example, from a sample from a 25 cm2 surface diluted in a total volume of 100 ml of diluent, 1 ml of this initial suspension represents 0.25 cm2.

### *Sample Preparation from Individual Slices*

1. Samples are taken without cauterization of the exposed surface. If packaged, it may be necessary to remove the meat aseptically and place it on a sterile tray with the test surface uppermost. Using a sterile scalpel and forceps, cut a strip 1 cm wide along the centre of the greatest length. Cut the strip into small pieces and place them into a tared sterile container or plastic bag.
2. Weigh the test portion and add nine times this mass of an appropriate diluent to make the 1 in 10 initial suspension.

### NOTE - For sample preparation of carcass samples, please refer Annex B.

## 6.3.12 *Sample Preparation for Frozen Products (Frozen Meat)*

Procedures for handling small samples of all types by defrosting before sampling and those for sampling larger blocks of meat and meat products without preliminary defrosting may be followed as given in **6.2.1** of this standard.

## 6.3.13 *Sample Preparation for Dried/Dehydrated Meat Products*

## Sample preparation procedures given in 6.2.2 and 6.2.3 may be followed for dried and partially dehydrated meats and meat extracts.

#### 6.4 Sample Preparation – Milk and Milk Products

**6.4.1** *General Procedures*

All preparations and manipulations should be carried out using an aseptic technique with sterile equipment to prevent microbial contamination of samples from all external sources.

**6.4.1.1** *Frozen Products*

## Sample preparation procedures as given in 6.2.1 may be followed for frozen milk products.

**6.4.1.2** *Hard and Dry Products*

Mix hard products in a peristaltic blender, place the sample and diluent in double- or triple-layered sterile bags to prevent puncturing and possible sample spillage, or alternately homogenize using a rotary blender when appropriate for hard, low moisture products.

## Sample preparation procedure as given in 6.2.2 may be followed for alternative preparation methods.

## 6.4.1.3 *Liquid and Non-Viscous Products*

## Sample preparation procedures as given in 6.2.4 may be followed for liquid and non-viscous milk products.

**6.4.1.4** *Multi-Component Products*

## Sample preparation procedures as given in 6.2.7 may be followed for multi-component milk products.

**6.4.1.5** *Acidic Products*

Samples of acidic milk products may be prepared using the procedure as given in **6.2.5** of this standard.

**6.4.1.6** *High-Fat Foods (Fat Content > 20 percent Mass Fraction)*

High fat milk products samples may be prepared using the procedure as given in **6.2.6** of this standard.

#### 6.4.2 Specific Procedures

**6.4.2.1** *Milk and Liquid Milk Products (Pasteurized/ Boiled Milk/ Flavored Milk/ Sterilized Milk/ UHT milk/ Evaporated Milk/ Sweetened Condensed Milk)*

1. Mix the test sample thoroughly so that the microorganisms are distributed as evenly as possible by rapidly inverting the sample container 25 times. Avoid foaming or allow any foam to disperse. The interval between mixing and removing the test portion shall not exceed 3 min.
2. Remove the test sample with a sterile pipette and prepare further dilutions or inoculate directly a medium or a broth in accordance with the procedure of the specific method of enumeration or detection.

**6.4.2.2** *Milk Powder, SMP, Partly SMP, Dairy Whitener, Cream Powder, Ice Cream Mix Powder, Dehydrated Milk, Dehydrated Sweet Whey, Dehydrated Acid Whey, Dehydrated Butter Milk and Lactose*

1. Thoroughly mix the contents of the closed container by repeatedly shaking and inverting it. If the test sample is in the original unopened container and this is too full to permit thorough mixing, transfer it to a larger container, then mix. Open the container, remove the test portion required with a spatula and proceed as indicated below. Immediately close the container again.
2. Prepare the initial suspension for dehydrated products and other low- moisture products, with a diluent for general use. For dehydrated acid whey, use dipotassium hydrogen phosphate solution at *p*H 8.4 ± 0.2 or, if necessary, for roller-dried milk use sodium citrate solution or dipotassium hydrogen phosphate solution at *p*H 7.5 ± 0.2 (For better reconstitution and in particular with roller-dried milk, glass beads can be helpful. If used, they are added to the bottle before sterilization).
3. Swirl slowly until the test portion has dispersed completely. Allow to stand for 5 min, swirling occasionally. A peristaltic blender may be used, if dispersion is not complete. The diluent may be pre-warmed to (44 to 47) °C in a water bath if a homogeneous suspension cannot be obtained even after blending.

**6.4.2.3** *Cheese and Cheese Products (Processed Cheese/ Cheese Spread/ All Other Cheeses Categories Including Fresh Cheeses/ Cheddar /Cottage / Soft/ Semi Soft Cheese, Chhena and Paneer*

Weigh the test portion into the container of a rotary blender or of a peristaltic blender. Add a diluent for general use, sodium citrate solution or dipotassium hydrogen phosphate solution at *p*H 7.5 ± 0.2. Blend until the cheese is thoroughly dispersed. Allow any foam to disperse. The diluent may be pre-warmed to (44 to 47) °C in a water bath if a homogeneous suspension cannot be obtained even after blending.

#### 6.4.2.4 Acid Casein, Lactic Casein, Rennet Casein and Caseinate

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##### **A)** *General Cases*

1. Thoroughly mix the contents of the closed container by repeatedly shaking and inverting it. Weigh the test portion into a sterile plastic bag for a peristaltic blender. Add the appropriate diluent at room temperature, as follows:
   * + - 1. for acid and lactic casein: dilute with dipotassium hydrogen phosphate solution with antifoam agent at *p*H 8.4 ± 0.2;
         2. for caseinate: dilute with peptone-salt solution, sodium citrate solution or dipotassium hydrogen phosphate solution at *p*H 7.5 ± 0.2;
         3. for rennet casein: dilute with dipotassium hydrogen phosphate solution with antifoam agent at *p*H 7.5 ± 0.2.
2. Mix well manually and allow to stand at room temperature for 15 min. Blend for 2 min in the peristaltic blender by using, if necessary, two sterile bags for granular products. Allow to stand for 5 min.

##### **B)** *Special Case: Rennet Casein*

1. Rennet casein can be difficult to dissolve. An alternative procedure to that described in may be used. Using dipotassium hydrogen phosphate solution with antifoam agent as diluent for rennet caseins may not be efficient to dissolve the grains. These casein grains hamper the enumeration of microorganisms at 30 °C. Therefore, the following alternative procedure is recommended.
2. If necessary, grind the dry casein before taking the test portion. Transfer approximately 20 g of the test sample into a suitable container. Grind it using an apparatus with blades able to rotate at approximately 20000 r/min and equipped with a device that prevents the sample from heating during grinding.
3. Weigh 5 g of the thus-prepared test sample in a sterile bottle of 250 ml with glass beads to facilitate mixing. Add 95 ml of the sodium tripolyphosphate solution preheated to (34 to 38) °C in a water bath. Mix by leaving the bottle on a mixing device for 15 min. Then, place it in the water bath set at (34 to 38) °C for 15 min while mixing from time to time.

#### 6.4.2.5 Pasteurized Butter

1. Weigh the test portion into a sample container. Place the container in a water bath set at (44 to 47) °C. Keep it in the water bath until the whole test portion has just melted. Add a diluent for general use warmed to (44 to 47) °C and mix in a peristaltic blender.
2. Alternatively, use only the aqueous phase for dilution, as follows:
3. Take a test portion of 50 g containing a volume-to-mass ratio of water of W percent (W ml/100 g). Add (50 − [50 × W/100]) ml of diluent for general use pre-warmed in the water bath at (44 to 47) °C. In these conditions, 1 ml of the aqueous phase corresponds to 1 g of butter.

Example: For 50 g butter containing a volume-to-mass ratio of water of about 16 percent (16 ml/100 g), the aqueous phase represents 8 ml of liquid. Add (50 – [50 × 16/100]) = 42 ml of diluent for general use pre- warmed in the water bath at (44 to 47) °C.

1. Place a container in the water bath set at (44 to 47) °C until the butter melts. Remove from the water bath, shake well, and allow phases to separate for no longer than 15 min. If necessary, remove the fat phase with a spatula or a glass rod.
2. 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 allowing phases to separate. It can be necessary to remove the fatty (upper) phase aseptically with a sterile tube connected to a vacuum pump. Pipette from the bottom layer.

**6*.*4.2.6** *Milk-Based Ice-Cream Ice Cream, Frozen Dessert, Milk Lolly, Ice Candy*

Weigh the test portion into a flask or into a sterile plastic bag for a peristaltic blender. Add a diluent for general use at room temperature and blend. The product melts during blending.

#### 6.4.2.7 Milk-Based Custard, Desserts and Sweet Cream (pH > 5), Pasteurized Cream

Weigh the test portion into a flask containing glass beads. Add a diluent for general use at room temperature and shake to disperse. Alternatively, a peristaltic blender may be used. In this case, the bag should not contain any glass beads.

#### 6.4.2.8 Milk-Based Fermented Milks, Yogurt, Probiotic Milk Products and Sour Cream (pH < 5)

Weigh the test portion into a flask containing glass beads, if necessary. Add buffered peptone water), double-strength buffered peptone water or dipotassium hydrogen phosphate solution at *p*H 7.5 ± 0.2 at room temperature and shake manually. Alternatively, a peristaltic blender may be used. In this case, the bag should not contain any glass beads.

**6.4.2.9** *Dehydrated Milk-Based Infant Foods (Infant Milk Food, Infant Formulae, Infant Milk Substitute, Follow Up Formula, Cereal Based Complimentary Food)*

1. Thoroughly mix the contents of the closed container by repeatedly shaking and inverting it. If the test sample is in an original unopened container that is too full to permit thorough mixing, transfer it to a larger container, then mix. Open the container. Remove the required test portion with a spatula and proceed as indicated below. Immediately close the container again.
2. Prepare the initial suspension for dehydrated products and other low- moisture products with a diluent for general use or, for samples with high starch content, a diluent for special purposes
3. The diluent may be pre-warmed to (44 to 47) °C in a water bath if a homogeneous suspension cannot be obtained even after blending. For better reconstitution, glass beads might be helpful. If used, add them to the bottle before sterilization. In order to dissolve the sample, swirl slowly to wet the powder, and mix manually or with a peristaltic blender. Allow to stand for 5 min, with occasional manual shaking. Samples with high starch content may cause problems because of the high viscosity of the primary dilution. In this case, use a diluent for general use with α-amylase solution or use a dilution of 1 in 20. In this case, record the ratio and take it into account in subsequent steps, such as the calculation and expression of results.
4. For sample preparation procedure of non-dehydrated milk-based infant foods with or without **6.4.1** of this standard may be referred.

### 6.4.2.10 *Further Decimal Dilutions*

To transfer a correct volume from a viscous initial suspension such as acid or rennet casein rinse the pipette with diluent by aspirating several times, using the diluent in the tube used for making the decimal dilution. Alternatively, if the initial suspension is too viscous, increase the proportion of the diluent of the initial suspension.

**6.5 Sample Preparation - Miscellaneous Products**

## 6.5.1 *General Sample Preparation*

All preparations and manipulations shall be carried out using aseptic techniques and sterile equipment.

## 6.5.1.1 *Acidic Products*

1. It is important to consider the end use of the product when testing acidic samples.
2. If the product is to be used as an ingredient in a final product of higher *p*H, then the *p*H of the initial suspension of the test portion shall be adjusted to *p*H 7.0 ± 0.5 with the diluents specified at or others with equivalent buffering capacity.
3. For *p*H adjustment of moderately acidic samples (*p*H ≥ 3.5 to *p*H < 4.5), use double-strength buffered peptone water.
4. If highly acidic (*p*H < 3.5) samples (For example, low *p*H fruits and juices or vinegars and pickles) are tested for acid-tolerant and acidophilic spoilage organisms using appropriate media, the *p*H of such samples shall not be adjusted.

## 6.5.1.2 *High-Fat Foods, Excluding Margarines and Spreads*

a) A diluent with between 1 g/l and 10 g/l of polysorbate 80 [polyoxyethylene (20) sorbitan monooleate] according to the estimated fat content shall be used to improve emulsification during suspension (For example, for a fat content of 20 percent, add 2 g/l).

b) Alternative surfactants and emulsifiers are available under various trade names, but the proportions to use should be determined by the laboratory.

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## 6.5.1.3 *Hard and Dry Products*

1. Do not homogenize hard or dry products in a rotary homogenizer for more than 2.5 min at a time to avoid excessive heating that may damage the microorganisms present.
2. Homogenize dry and hard heterogeneous products by mincing or grinding the laboratory sample. Avoid excessive heating during this process by homogenizing for periods of no more than 1 min at a time, with suitable rest intervals applied, depending on the product being processed. Mince or grind until the sample is visibly homogeneous.
3. Resuscitation at laboratory ambient temperature (18 °C to 27 °C) for up to 1 h is recommended to assist in the recovery of stressed organisms from all hard and dry products.
   * 1. *Specific Procedures*

## 6.5.2.1 *Dehydrated and Low aw Products*

The following are regarded as dehydrated products:

1. dehydrated meats and vegetables;
2. dried soups, bouillon cubes and gravy mixes;
3. powdered beverages (tea, cocoa and cocoa-based products, coffee, dehydrated fruit juice);
4. raw cellulose, soluble cellulose, dextrin, sorbitol, sugars, glucose, glutamate;
5. herbs, spices, flavourings and colourings;
6. polysaccharide gelling agents, alginates, gums, etc.;
7. coconut, partially dehydrated vegetable/yeast/meat/fish extracts;
8. chocolate and confectionery (bars or sweets);
9. dehydrated whole egg and dried egg white;
10. cereals, flours, animal feeds;
11. powdered or pelletized viable microorganisms (For example, yeasts for bakery);
12. Inclusion of ready to use cultures.

### 1) *Preparation of Samples*

i) Mix powdered products thoroughly in their container using a sterile spatula and then weigh out using aseptic techniques. Weigh accurately into a pre-dispensed volume of diluent to minimize osmotic shock to the microflora.

ii) Other products may require breaking or cutting up with sterile tools into small pieces before taking the test portion.

### 2) *Preparation of Initial Suspension*

**i)** *Powdered Products, Completely Soluble Including Flavors and Colors*

It is not necessary to homogenize fully soluble products mechanically as mixing by hand is adequate. Prepare the initial suspension in accordance with **6.2.2** and of this standard.

**ii)** *Other Less Soluble or Non-Powdered Products*

Prepare the initial suspension using a rotary blender or peristaltic homogenizer.

**6.5.2.2** *Products Which Swell in Water*

1. For all products that swell in water (e.g. polysaccharides and gum gelling agents, dehydrated parsley, isabgol) make further dilutions (1 in 20, 1 in 50 or 1 in 100, as appropriate) until a suitable suspension is obtained.
2. Record the use of additional diluent to ensure the correct calculation of enumeration test results.
3. Where greater dilutions are made, the number of inoculated plates for enumeration tests shall be increased to ensure a minimum of 0,1 g of the test portion is distributed between all plates when low counts are expected.
4. The solubility of some substances is improved by the addition of a specific enzyme solution to the initial suspension in buffered peptone water. Few examples of suitable enzymes are the following:
5. 1 percent (volume fraction) alpha-amylase for swelling starch products, cereals and cereal- containing products;
6. 1 percent (volume fraction) cellulase for carboxymethyl cellulose, locust beans, carob, guar and cassia gums;
7. percent (volume fraction) papain for gelatin.
   * + 1. *Spices and Inhibitory Food Materials*
8. **This category includes black pepper, white pepper, celery seed or flakes, chili powder, cumin, paprika, parsley flakes, coriander powder, sesame seed, potato flakes and vegetable flakes etc.**
9. Weigh the test portion accurately and add it to the required volume of diluent to make the initial suspension at a 1 in 10 dilutions.
10. For food materials that contain inhibitory substances (e.g. onion powder, garlic, oregano, peppers, certain teas and coffees, vitamin premixes and highly salted products), it is necessary to decrease the antimicrobial activity before testing by using special preparation procedures such as the following:
11. use of greater dilutions (e.g. 1 in 100 for cinnamon and oregano and 1 in 1 000 for cloves);
12. addition of potassium sulphite (K2SO3) to the buffered peptone water to achieve a final concentration of 0,5 % (w/v) for **onion flakes, onion powder, garlic flakes;**
13. use of diluent at 37 °C ± 1 °C, to aid dissolution, and higher dilutions (e.g. 1 in 50) for vitamin premixes;
14. use of higher dilutions for products containing more than 10 % (mass fraction) salt (sodium chloride) to ensure the total concentration in the initial suspension (not including any salt content of the diluent or enrichment broth) does not exceed 1 % (w/v).
15. If any of these techniques is used, spiked sample process controls shall be included at first use to verify the effectiveness of the neutralization process chosen.
    * + 1. *Cocoa and Cocoa-Containing Products*
16. Use either UHT milk or reconstituted non-fat dry milk powder (100 g/l water; sterilized after reconstitution) as the pre-enrichment broth for detection of the significant pathogens Salmonella spp. and STEC. BPW may be used as a general diluent for other tests.

NOTE - Milk is used to neutralize the bactericidal effect of cocoa or cocoa-containing products. The probable inhibitory factor in cocoa is anthocyanin

1. Preheat the diluent to 37 °C to 40 °C.
2. Weigh the test portion (e.g. 25 g) into a plastic bag add the warmed diluent (For example, 225 ml) to achieve a 1 in 10 initial suspension and mix by hand immediately.
3. Leave the suspension at laboratory ambient temperature (18 °C to 27 °C) for 20 min to 30 min to melt. Then, mix completely in a peristaltic homogenizer for 60 s ± 5 s.
4. For cocoa powder and any other samples which may be highly contaminated with Gram-positive bacteria as a result of inadequate thermal processing, addition of 0.45 ml 1 percent (w/v) aqueous brilliant green solution (1 g/100 ml water) to the initial suspension of 250 ml can reduce inhibition of low levels of Gram-negative target organisms during non-selective pre-enrichment. Whether this is used depends on the type of sample and laboratory experience with such samples.
5. When large portions of solid chocolate or other cocoa-containing materials which cannot be broken up easily are tested, it may be necessary to melt the chocolate at a temperature between 42 °C and 47 °C, for no longer than necessary, before taking the test portion.
6. For chocolate products containing >20 percent fat, unless the products already contain sufficient emulsifier, add sufficient polysorbate 80 [polyoxyethylene (20) sorbitan monooleate] or other emulsifier to the diluent.
   * + 1. *Confectionery (Bars or Sweets)*
7. Pre-heat the diluent to 37 °C to 40 °C.
8. Weigh out the test portion in a plastic bag and add the warmed diluent. Mix immediately by hand to distribute the test portion. Very hard sweets or candies may also be partially crushed with a heavy object, such as a hammer to aid dispersion.
9. Leave the suspension at laboratory ambient temperature (18 °C to 27 °C) for 20 min to 30 min to dissolve. Then, mix completely using the peristaltic homogenizer.

### *Resuscitation*: In general, leave the initial suspension of low-moisture products requiring resuscitation for up to 1 h at laboratory ambient temperature (18 °C to 27 °C) before preparing any further dilutions. Some specific cases are detailed in the relevant International Standards.

## *Flours, Cereal Grains and By-Products and Animal Feeds*

1. Mix dry powders well in the sample container, using a sterile tool before weighing out the test portion.
2. Weigh the test portion accurately and add it to the required volume of peptone salt solution to minimize osmotic shock to the microflora. This is the initial suspension at a 1 in 10 dilutions.
3. For flours, take a proportion of the required volume of diluent and add the test portion. Mix well by hand and then add the remainder of the diluent to obtain a 1 in 10 dilutions.
4. Before homogenization, leave to stand for 20 min to 30 min at laboratory ambient temperature (18 °C to 27 °C) to assist resuscitation of damaged organisms.
5. If the viscosity of the suspension increases so that it becomes too thick or viscous to mix well or to pipette, add a further equal volume of peptone salt solution to produce a 1 in 20 initial suspension and record this to ensure correct calculation of enumeration test results.
6. Mix for 60 s ± 5 s using a peristaltic homogenizer for flours or a rotary blender for cereal grains or animal feeds.
7. A test portion of 50 g is required when testing cereals and other heterogeneous products to improve the reliability of test results. In this case, use a 1 in 5 suspension, homogenize and then make a further 1 in 2 dilution to obtain the initial 1 in 10 suspension.

NOTE - Hard materials (e.g. grains and seeds) can puncture plastic bags double- or triple-bagging can help to prevent leakage and subsequent contamination. Test portions can also be partially crushed with a heavy object, such as a hammer before homogenizing.

1. Animal feeds are presented for testing in a variety of forms, but the general rules for cereal products above apply to many.
2. For pet chews, kibble (extruded product) and similar hard products, immerse in diluent at a 1 in 10 dilutions, then leave to soak for approximately 1 min before massaging by hand for 30 s ± 5 s. Mix when the products have softened using a peristaltic homogenizer or rotary blender for 60 s ± 6s, if necessary.

## 6.5.2.7 *Gelatin (Powdered and Leaf)*

### a) *Preparation of Samples*

Take a test portion of 20 g of the laboratory sample using aseptic techniques.

### b) *Preparation of Initial Suspension*

1. Transfer this test portion to a 500 ml sterile flask. Add 180 ml of phosphate buffered diluent and mix to disperse the granules in the liquid.
2. Leave the gelatin to adsorb the diluent for 60 min at laboratory ambient temperature (18 °C to 27 °C).
3. Place the flask in a water bath at 44 °C to 47 °C for a maximum of 30 min; mix frequently to dissolve the gelatin to obtain the initial 1 in 10 suspension.
4. Alternatively, papain may be used to dissolve the gelatin.

## 6.5.2.8 *Margarine and Spreads*

### a) *Sampling*

Samples may be taken from within the bulk product or from within and/or on the surface of packaged items ready for sale, using aseptic techniques throughout.

**b)** *Bulk or Pre-Wrapped Products of ≥1 kg*

1. To determine the microbiological quality of bulk product, examine only core samples. First, remove a slice of 3 mm to 5 mm thickness from the outer layer with a sterilized spatula or knife. Push a sterile metal corer into the product diagonally without going all the way through. Turn the corer in a full circle, then remove it with the cylindrical sample.
2. Transfer a portion of this core sample to a sterile container or plastic bag using a spatula or knife but retain the upper 25 mm to plug the hole made by the corer.
3. Take one or more core samples to obtain an adequate laboratory sample.

NOTE - Any other sampling method (such as taking one mass of at least 500 g) is permitted if the product is regarded as homogeneous.

**c)** *Pre-Wrapped Product of ≤1 kg*

1. The laboratory sample shall be made up of one or more pre-wrapped, intact items.
2. Remove the wrapping and take a representative test sample from one or more packs using aseptic techniques. Remove the outer 5 mm section before sampling if the packs are ≥500 g. Take the test sample using a sterile instrument or use a sterile corer to take a cylindrical portion through the laboratory sample.
3. If the customer requests testing of the product surface only, remove the test sample by scraping sufficient material from the surface with a sterile implement.

### d) *Preparation of Test Sample*

Weigh 50 g from the laboratory sample containing a volume-to-mass ratio of water of W percent into a sterilized flask or other container.

**e)** *Preparation of the Aqueous Phase (Primary Dilution)*

1. Pre-warm a volume of [50 − (50 x W/100)] ml of diluent in a water bath at 44 °C to 47 °C and add it to the test sample in the container. In these circumstances, 1 ml of the aqueous phase is equivalent to 1 g of the margarine or spread in the test sample.

Example: For a 50 g test sample of margarine with an 84 percent fat content and therefore a volume-to-mass ratio of about 16 percent, the aqueous phase represents 8 ml of water. Add [50 − (50 × 16/100)] = 42 ml of diluent. This can also be calculated by taking the sample weight of 50 g and multiplying by the fat content of 84 percent (i.e. 50 × 84 / 100 = 42 ml).

1. Place the container in the water bath at 44 °C to 47 °C until the product has completely melted. The time taken shall not exceed 20 min.
2. Mix in a peristaltic homogenizer for 60 s ± 5 s and then for further 30 s increments until an emulsion is produced. Leave the container at laboratory ambient temperature (18 °C to 27 °C) so that the fatty (upper) layer and the aqueous (lower) layer are separated fully.
3. Use the aqueous layer to take the test portion (1 ml corresponds to 1 g of the test portion) and prepare the initial suspension.

## Eggs and Egg Products

### *Fresh Whole Eggs*

* + - 1. *General*

1. Eggs used for routine microbiological examination shall not have any visible cracks in the shells.
2. Eggs may be examined singly or in batches according to the purpose of the testing.
3. Examination of whole eggs may be carried out with or without cleaning/disinfecting of the eggshell as required by the customer. To examine only the contents, always disinfect the eggs before opening. For detection of pathogens (which may also be found on the outside of the egg), disinfection of the shell may not be required, but agreement on the procedure to be used shall be reached between the parties.
   * + 1. *Disinfecting the Shell*
4. Remove any dirt or faeces with a damp tissue and blot dry.
5. Wearing sterile gloves and using a clean gauze or wipe soaked in a solution of either 70 % (volume fraction) ethanol or isopropanol to water, wipe the entire shell surface. This reduces the risk of contamination of the egg yolk and albumen when the egg is broken open to remove the contents.
6. Allow to dry completely without re-contaminating the shells before breaking the egg to take the

test portion.

### *Microflora of Whole Egg Shell*

* + - 1. *Method by Rinsing the Whole Egg Shell*

Place the whole intact egg in a peristaltic homogenizer bag or other sterile container and add a known volume of the diluent or culture medium required in the test method. Then, massage or rotate the egg carefully in the liquid. Remove the egg and use the liquid as the initial suspension to continue with the test method.

* + - 1. *Friction Method*

1. Use sterile gauze (or other equivalent fabric/paper) soaked in diluent or the required culture medium. Hold the gauze with sterile forceps and rub over the entire eggshell.
2. Place the pieces of gauze in the volume of diluent or culture medium required by the test method.
   * + 1. *Soaking method*

Break the egg aseptically and discard the contents into a bowl or beaker. Retain the shell and place it in a peristaltic homogenizer bag with the required volume of diluent or culture medium. Massage and crush the shell in the bag by hand and use this as the initial suspension.

### *Internal Microflora*

1. Using fresh sterile gloves for each egg, break the egg open aseptically into a sterile container. If the yolk and white are to be examined separately, separate them and place each in a different sterile container.
2. Add peptone salt solution to give a 1 in 10 dilutions for the yolk and 1 in 40 dilutions for the white to overcome inhibition by the naturally occurring lysozyme.
3. To examine the whole egg contents, place all of the yolk and white (of approximately 20 ml) in a sterile container with 180 ml of buffered peptone water or into the appropriate diluent or enrichment broth required by the specific International Standard and use this as the initial 1 in 10 suspension.

### *Bulk Whole Liquid Egg, Egg White and Egg Yolk*

These bulk products may or may not be pasteurized. For bulk whole liquid egg or egg yolks, dilute 1 in 10 with buffered peptone water. For bulk liquid egg whites, use a 1 in 40 suspension in buffered peptone water to overcome inhibition by the naturally occurring lysozyme.

### *Dehydrated Whole Egg and Dried Egg White*

### Procedure of sample preparation of dehydrated whole egg and dried egg white, 6.2.3 of this standard may be referred.

### *Whole Egg Microflora (Shell Plus Yolk Plus White)*

Using aseptic techniques, break the egg and place the shell and contents in a sterile plastic bag or other container. Crush and shake the mixture to homogenize it by hand. Take the required test portion to make the initial suspension.

## Bakery Goods, Pastry and Cakes, Doughnuts, Ready to Eat (RTE) Foods

### *General*

1. Bakery goods, including sweet pastries, doughnuts and cakes, are made from flour, butter, eggs and other ingredients and some also include dairy or fruit products. RTE food also includes multi ingredients. As such, they should be treated in the same way as other multi-component products (*see* **6.2.7**).
2. The test portion of relatively homogeneous products, such as loaves of bread, rolls and other finished items made from plain dough, should be taken according to the purpose of the testing. For example, surface samples may be required for investigations of mould spoilage.

### *Preparation of Samples*

1. For packaged pre-cooked products, open the packaging aseptically. Take pieces of each component in proportion to the amounts in the whole product.
2. Alternatively, homogenize the entire laboratory sample to reflect the microflora of the whole item and take a representative test portion.
3. Treat biscuits or cookies in the same way as dehydrated products if they are hard and low in moisture.

## Fresh Fruit and Vegetables (Pre-Packed)

* + 1. *Sample Preparation of Multi-Component Products*

1. For multi-component products (those containing pieces of different fruit or vegetables), take pieces of each component in proportion to the amounts in the whole product to provide the test portion.
2. Alternatively, homogenize the entire laboratory sample to reflect the microflora of the whole item and take a representative test portion.
3. Dilute the test portion 1 in 10 with buffered peptone water. Homogenize using a peristaltic homogenizer until a suitable initial suspension is obtained.
   * 1. *Pre-Packed Products of One Type of Fruit or Vegetable*

Weigh the test portion and dilute 1 in 10 with buffered peptone water. Homogenize using a peristaltic homogenizer until a suitable initial suspension is obtained.

* + 1. *Coconut*

Aseptically weigh test portion and add into suitable sterile diluent, mix well, and let stand 60 ± 5 min at room temperature. Mix well by swirling and determine *p*H with test paper. Add up to 2.25 ml steamed (15 min)[Tergitol Anionic 7](https://www.fda.gov/food/laboratory-methods/bam-r78-tergitol-anionic-7) and mix well. Alternatively, use steamed (15 min) [Triton X-100](https://www.fda.gov/food/laboratory-methods/bam-r86-triton-x-100). Limit use of these surfactants to minimum quantity needed to initiate foaming. For Triton X-100 this quantity may be as little as 2 or 3 drops.

* 1. **Fermented Products (Idli, Uttapam etc.) or Other Products Containing Viable Microorganisms**
     1. *General*

1. Such products are examined for contamination by microorganisms other than those used as starter cultures for fermentations or as the active constituent microflora of probiotic products.
2. To test these products for contaminants, use suitable inhibitors to suppress growth of the starter culture or probiotic organisms.
   * 1. *Diluent*

Use buffered peptone water routinely or at double-strength if the sample is highly acidic (*p*H < 4.5). In the case of yeast cultures or fermentations, add an anti-fungal agent (For example, cycloheximide or nystatin at a concentration of 50 mg/kg or amphotericin at 10 mg/kg) to the counting medium to reduce overgrowth by unwanted yeasts and moulds.

* 1. **Beverages (Alcoholic and Non-Alcoholic Drinks and Bottled Waters, Still Carbonated)**
     1. *General*

1. To detect contamination of these products, use membrane filtration of specified volumes through 0.45 µm sterile membranes.
2. For carbonated beverages, preliminary de-gassing is required to ensure accurate volumes are filtered or pipetted.
   * 1. *De-Gassing by Inversion and Mixing*

Invert the laboratory sample by hand (through an arc of 25 cm five times) and then loosen the cap carefully to release evolved carbon dioxide. Tighten the cap again and repeat the process until no more gas is evolved. Take the test portion by pipetting or filtering accurately. Use aseptic technique throughout.

* + 1. *De-Gassing Using Ultrasound*

1. Ultrasound may also be used to de-gas carbonated beverages.
2. Invert the container (through an arc of 25 cm five times) to mix and aseptically decant 10 percent of the contents into a sterile container.
3. Replace the lid of the container loosely and place it in an ultrasonic bath for 120 s ± 5 s. Check for any remaining gas and, if necessary, repeat the ultrasound treatment. Do not repeat this procedure more than twice to minimize potential damage to microorganisms in the sample.
4. Take the test portion by pipetting or filtering accurately. Use aseptic technique throughout.

### 6.11 Sample Preparation - Fish and Fish Products

**6.11.1** *Whole Fresh Fish (More Than 15 cm in Length)*

1. The gills and the anus shall be covered with sterile cotton wool, drenched in alcohol at a volume fraction of 70 percent. Disinfect the surface of the dorsal region (using cotton wool with alcohol at a volume fraction of 70 percent and remove and discard a section of the skin using sterile forceps and scalpel Take a cube-shaped sample of dorsal muscle, dice it and break up in an appropriate diluent. If the fish is eviscerated, the gills shall be covered with sterile cotton wool, drenched in alcohol at a volume fraction of 70 percent and a cube-shaped sample of dorsal muscle shall be removed from inside the body cavity.
2. Use sufficient material from the laboratory sample to give a representative test portion as specified in the test method.
3. Add diluent to make a 1 in 10 suspension and blend in a rotary or peristaltic homogenizer as necessary.

##### **6.11.2** *Whole Fresh Fish (Less Than 15 cm in Length)*

1. Using sterile scissors and forceps remove a portion of fish just anterior to the tail insertion by making two cuts to produce transverse sections, the first cut to remove the tail and tail insertion and the second to remove a steak.
2. Use sufficient material from the laboratory sample to give a representative test portion as specified in the test method.
3. Add diluent to make a 1 in 10 suspension and blend in a rotary or peristaltic homogenizer as necessary.

##### **6.11.3** *Sliced Fish, Fillets and Steaks*

### Procedure as given in 6.2 of this standard may be referred for sample preparation of sliced fish, fillets and steaks.

#### 6.11.4 Processed Fish Products

##### **6.11.4.1** *Whole Smoked Fish*

1. If the whole fish is eaten, then the skin shall be included in the sample. If the skin is not eaten then the skin shall be excluded. Use sufficient material from the edible portion of the laboratory sample to give a representative test portion as specified in the test method.
2. The test portion shall be taken from the dorsal area and the flesh cut, diced and homogenized using a rotary or peristaltic homogenizer as necessary in diluent to obtain a 1 in 10 suspension.

##### **6.11.4.2** *Smoked Fish Fillets and Slices, With or Without Skin*

1. Take pieces of the fillet and dice them, under sterile conditions, without removing the skin. Use sufficient material from the edible portion of the laboratory sample to give a representative test portion as specified in the test method.
2. Homogenize using either a rotary or peristaltic homogenizer as necessary in diluent to obtain a 1 in 10 suspension.

##### **6.11.5** *Fish and Fish-Based Multi-Component Products (For example, Pre-Prepared Fish Taco, Mixed Seafood Selections, Mixed Fish Ball)*

Take representative parts of each component in proportion to the amounts in the whole product. Use sufficient material from the edible portion of the laboratory sample to give a representative test portion as specified in the test method. Add the necessary quantity of diluent to give a 1 in 10 suspension. Blend in a rotary or peristaltic homogenizer.

##### **6.11.6** *Dried Fish Including Dried Salted Fish*

### Sample preparation procedure as given in 6.2.3 of this standard may be referred.

**6.12** **Sample Preparation - Crustaceans, Molluscs, Tunicates and Echinoderms**

##### **6.12.1** *Whole and Sliced Cephalopods*

1. Disinfect the surface of the skin and suckers (using cotton wool with alcohol at a volume fraction of 70 %). Remove the skin and suckers with sterile forceps and a scalpel and discard. Take cube-shaped samples of dorsal muscles and pieces from the tentacles. Use sufficient material from the laboratory sample to give a representative test portion as specified in the test method.
2. The flesh from cephalopods is relatively firm; grind up the test portion in diluent using a rotary homogenizer or cut it into fine pieces. Add further diluent to make a 1 in 10 suspension and blend in a rotary or peristaltic homogenizer as necessary.

##### **6.12.2** *Whole Crustacea Such as Crabs*

1. Disinfect the surface (using cotton wool with alcohol at a volume fraction of 70 %) and with sterile hammer pliers or forceps remove or break the carapace and claws to extract the maximum amount of flesh for testing. For large claws, an oyster cracker can be used to break open the shell before extracting the flesh.
2. Use sufficient material from the laboratory sample to give a representative test portion as specified in the test method.
3. Add diluent to make a 1 in 10 suspension and blend in a rotary or peristaltic homogenizer as necessary.

##### **6.12.3** *Shelled Crustacea Flesh*

Take the amount of flesh required in the test method, make the initial 1 in 10 suspension in a diluent and blend in a rotary or peristaltic homogenizer as necessary. Use sufficient material from the laboratory sample to give a representative test portion as specified in the test method.

##### **6.12.4** *Crustacea Such as Prawns, Crayfish, and Lobsters*

1. *Species Where Tails Only are Consumed*
2. Disinfect the surface (using cotton wool with alcohol at a volume fraction of 70 percent). Break the crustacean at the junction between the cephalothorax and abdomen. Using sterile forceps pull the edible portion of flesh from the cephalothorax and butt end of the abdomen.
3. Use sufficient material from the edible portion of the laboratory sample to give a representative test portion as specified in the test method. Add the necessary quantity of diluent to give a 1 in 10 suspension. Blend in a rotary or peristaltic homogenizer as necessary.

##### *Species Consumed Whole*

Take the entire animal for examination. Use sufficient material from the laboratory sample to give a representative test portion as specified in the test method. Add the necessary quantity of diluent to give a 1 in 10 suspension. Blend in a rotary or peristaltic homogenizer as necessary.

##### *Live Bivalve Molluscs*

**6.12.5.1** *General*

1. On arrival at the laboratory, the internal air temperature of the transport container shall be recorded. For samples where more than 4 h have elapsed between collection and receipt, the internal air temperature should be between 0°C and 10°C. If the internal air temperature of the transport container is greater than 10°C, the sample temperature should be measured; this should not exceed 10 °C. For samples where less than 4h have elapsed between collection and receipt, internal air/sample temperature should be less than the temperature recorded at the time of sampling.
2. Laboratory samples shall be stored at 3 °C ± 2 °C.
3. The animals shall be alive. Discard individuals with open or damaged shells. A representative test sample shall contain at least 10 individuals and shall be at least 50 g (25 g for small animals, for example, *Donax* spp.) as detailed in Table 1. Testing of bivalves includes both the flesh and intravalvular water. Open sufficient shellfish to yield the amount of flesh and intravalvular fluid specified in the test method.
4. Microbiological examination should be initiated within 24 h of collection of the sample. If testing cannot be initiated within 24 h or if sample temperatures of 0°C and 10°C cannot be achieved, data should be generated to verify that the use of alternate transport and storage conditions does not affect the microbiological content of the sample.

## Table 1

## (*Clause* 6.12.5.1)

## Recommended Number of Individual Live Bivalve Molluscs to be

## Submitted to the Laboratory

|  |  |  |
| --- | --- | --- |
| **Species** | | **Number** |
| **Scientific name** | **Common name (English)** |
| *Pecten maximus* | King scallop | 12 to 18 |
| *Aequipecten opercularis* | Queen scallop | 18 to 35 |
| *Crassostrea gigas* | Pacific oyster | 12 to 18 |
| *Ostrea edulis* | Flat oyster | 12 to 18 |
| *Mercenaria mercenaria* | Hard clams | 12 to 18 |
| *Tapes philippinarum* | Manilla clam | 18 to 35 |
| *Ruditapes decussatus* | Grooved carpet shells | 18 to 35 |
| *Spisula solida* | Thick trough shells | 35 to 55 |
| *Mya arenaria* | Sand gapers | 12 to 18 |
| *Ensis* spp. | Razor clams | 12 to 18 |
| *Mytilus* spp. | Mussels | 18 to 35 |
| *Cerastoderma edule* | Cockles | 35 to 55 |
| *Donax* spp. | Bean clams | 40 to 70 |

##### **6.12.5.2** *Methods Requiring a 1 in 10 Initial Suspension*

1. Wash and brush each shell under running water of potable quality, especially around the hinge or opening. Drain the cleaned bivalves and put them on a clean surface. If there is a byssus muscle, do not tear it away; cut it with sterile scissors, knife or scalpel before fully opening. As each shell is opened, collect the flesh and intravalvular water in a sterile container suitable for blending. Bivalves that have lost their intravalvular water may be used if they are still alive when the shell is opened.
2. Add one part of flesh and intravalvular water to two parts of diluent. Blend with a rotary homogenizer for 30 s to 2 min depending on the homogenizer used. A peristaltic homogenizer may be used but note that shell splinters can puncture plastic bags. Double- or triple-bagging can help to prevent leaking and the risk of contamination.
3. In this way, an approximate 1 in 3 suspension is obtained to which the required amount of diluent is added to obtain an accurate 1 in 10 initial suspension.

##### **6.12.5.3** *Methods Requiring a 1 in 2 Initial Suspension*

Proceed as in **6.11.8.2** but use one part of flesh and intravalvular water to one part of diluent to produce an accurate initial 1 in 2 suspension.

NOTE - An initial suspension of 1 in 2 is required for official control testing of bivalve shellfish, marine gastropods and echinoderms where a level of detection of ≤ 200 cfu per 100 g product is required.

##### **6.12.6** *Cooked or Precooked Shelled Bivalves*

### Sample preparation procedure as given in 6.2 of this standard may be referred.

**6.12.7** *Whole Cooked Molluscs in the Shell*

Different types of sample preparation are described below:

**6.12.7.1** *Cooked or Partially Cooked Gastropods*

Remove the operculum with a sterile scalpel then extract the body using forceps, a winkle picker or shellfish picker. Alternatively, carefully crush the shells open using a hammer without damaging the flesh. Remove any shell debris with sterile forceps and dice the flesh. Prepare an initial suspension of approximately 1 in 3 in diluent, homogenize, and then add the required amount of diluent to obtain an accurate 1 in 10 suspension.

**6.12.7.2** *Cooked or Partially Cooked Bivalves*

Extract the body from the shell using sterile forceps, scalpel and oyster knife or shellfish picker Dice the flesh. Prepare an initial suspension of approximately 1 in 3 in diluent, homogenize in a rotary or peristaltic homogenizer and then add the required amount of diluent to obtain an accurate 1 in 10 suspension.

**6.12.7.3** *Whole Cooked or Partially Cooked Crustacea*

Use sufficient material from the edible portion of the laboratory sample to give a representative test portion as specified in the test method. Add the necessary quantity of diluent to give a 1 in 10 suspension. Blend in a rotary or peristaltic homogenizer.

##### **6.12.8 Echinoderms**

**6.12.8.1** *Echinoderms Such as Sea Urchins*

1. Wash at least 10 individuals under running potable water, and place them on a sterile tray.
2. Hold the sea urchin with forceps or wear a strong clean glove and cut off a piece of the ventral surface with sterile sharp scissors to expose the flesh. Collect the whole flesh and fluid in a sterile container suitable for blending.
3. Prepare an initial suspension of approximately 1 in 3 in diluent, homogenize in a rotary or peristaltic homogenizer as necessary and then add the required amount of diluent to obtain an accurate 1 in 10 suspension.

##### **6.12.8.2** *Echinoderms Such as Holothurians (For example, Sea Cucumbers) and Tunicates*

1. Wash at least 10 individuals under running potable water, and place them on a sterile tray. Cut individuals into fine pieces with sterile scissors.
2. Prepare an initial suspension of approximately 1 in 3 in diluent, homogenize in a rotary or peristaltic homogenizer as necessary and then add the required amount of diluent to obtain an accurate 1 in 10 suspension.

##### **6.13 Salted or Pickled Products (Including Fish Eggs/Roe Such as Caviar)**

### Sample preparation procedure as given in 6.2.5 of this standard may be referred.

##### **6.14 Fermented, Marinated and Breaded Products**

### Sample preparation procedure as given in 6.2 of this standard may be referred.

#### 6.15 Frozen Fish, Crustacea, Molluscs, Tunicates and Echinoderms

##### **6.15.1** *Fish Fillets, Large Fish Pieces Frozen in Blocks, Frozen Small Parts and Single Portions*

1. Either take a test portion from the frozen block using a drill with a sterile bit or defrost at ambient temperature (18 °C to 27 °C) for approximately 60 min but no more than 3 h. Remove pieces with sterile pliers or forceps. Leave to defrost further if necessary until soft enough to cut into smaller pieces with a sterile knife and forceps
2. Use sufficient material from the edible portion of the laboratory sample to give a representative test portion as specified in the test method.
3. Blend the pieces in a rotary or peristaltic homogenizer with diluent to obtain a 1 in 10 suspension.

##### **6.15.2** *Shelled Crustacea (Such as Prawns) Frozen in Blocks*

1. Leave the laboratory sample to defrost for approximately 60 min but no more than 3 h at ambient temperature (18 °C to 27 °C) until the block breaks. Carefully separate the block into pieces using a sterile hammer or butcher’s knife and take pieces of flesh with sterile forceps or pliers Alternatively remove the test portion from the frozen block using a drill with a sterile bit
2. Use sufficient material from the edible portion of the laboratory sample to give a representative test portion as specified in the test method.
3. Homogenize using a rotary or peristaltic homogenizer in diluent to obtain a 1 in 10 suspension.

##### **6.15.3** *Whole Crustacea (Such as Prawns) Frozen in Blocks*

1. Leave to defrost for approximately 60 min but no more than 3 h at ambient temperature (18 °C to 27 °C) until the block breaks. Extract the individual animals with sterile pliers or forceps Allow to defrost so cephalothorax and abdomen may be separated and the edible portion removed with sterile forceps
2. Use sufficient material from the edible portion of the laboratory sample to give a representative test portion as specified in the test method.
3. Homogenize using a rotary or peristaltic homogenizer in diluent to obtain a 1 in 10 suspension.

##### **6.15.4** *Flaked Crustacean Flesh (Such as Crab Meats) Frozen in Blocks*

Remove the test portion from the frozen block using a drill with sterile bit or defrost at ambient temperature (18 °C to 27 °C) for approximately 60 min but no more than 3 h until the block breaks. Remove pieces of flesh with sterile pliers or forceps. Use sufficient material from the edible portion of the laboratory sample to give a representative test portion as specified in the test method. Homogenize using a rotary or peristaltic homogenizer in diluent to obtain a 1 in 10 suspension.

##### **6.15.5** *Molluscs (Whole Cephalopods, Bivalve Molluscs and Gastropods) Frozen in Blocks*

**6.15.5.1** *Whole Cephalopods Frozen in Blocks*

1. Remove material using a drill with a sterile bit or defrost at ambient temperature (18 °C to 27 °C) for approximately 60 min but no more than 3 h. Cut off pieces with sterile scissors or butcher’s knife.
2. Use sufficient material from the edible portion of the laboratory sample to give a representative test portion as specified in the test method.
3. Homogenize using a rotary or peristaltic homogenizer in diluent to obtain a 1 in 10 suspension.

##### **6.15.5.2** *Whole Gastropods and Bivalve Molluscs Frozen in Blocks*

1. Leave to defrost for approximately 60 min but no more than 3 h at ambient temperature (18 °C to 27 °C) until the block breaks. Extract the individual animals with sterile pliers or forceps Leave to defrost further if necessary until soft enough to extract the body from the shell using sterile forceps scalpel and oyster knife or shellfish picker
2. Alternatively crush the shells open using a sterile hammer without damaging the flesh. Remove any shell debris with sterile forceps and dice the flesh.
3. Use sufficient material from the edible portion of the laboratory sample to give a representative test portion as specified in the test method.
4. Homogenize using a rotary or peristaltic homogenizer in diluent to obtain a 1 in 10 suspension.

##### **6.15.5.3** *Cooked or Partially Cooked, Shelled Molluscs Such as Gastropods and Bivalve Molluscs Frozen in Blocks*

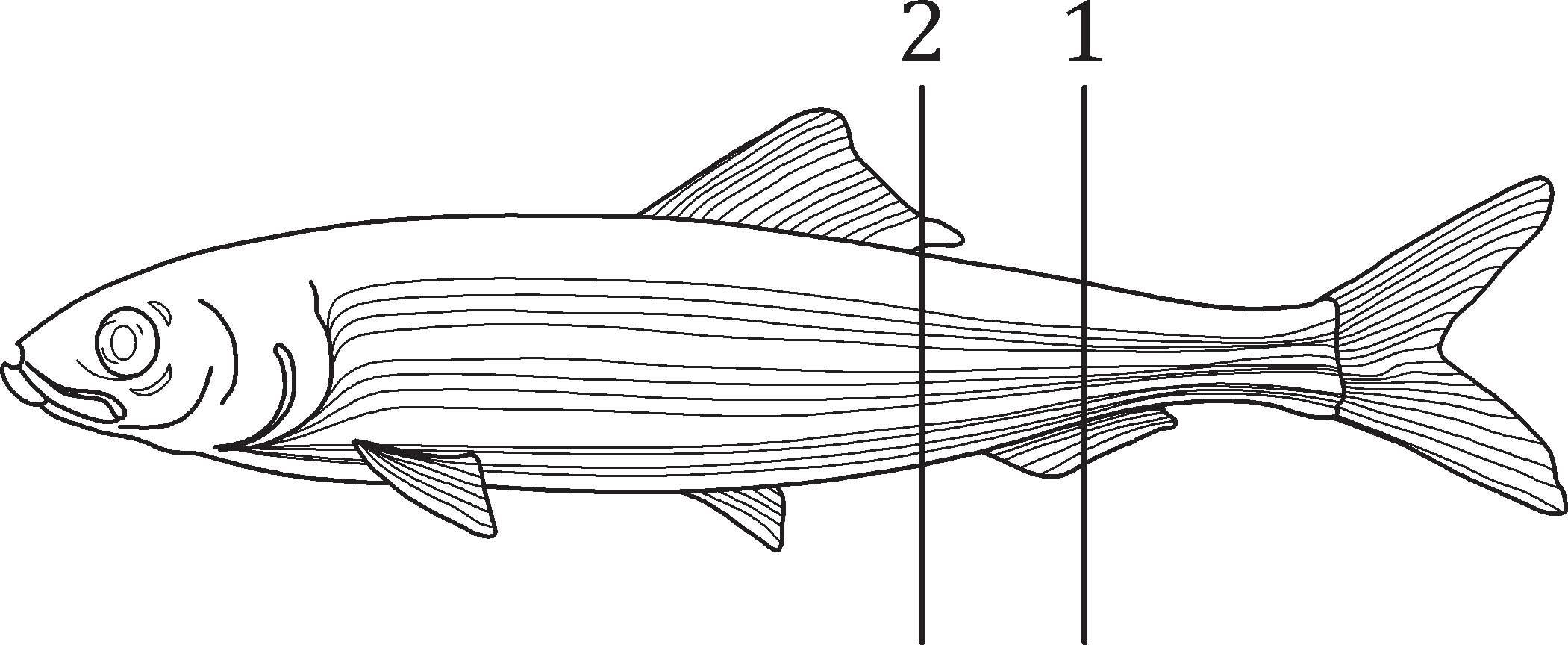
1. Leave to defrost for approximately 60 min but no more than 3 h at ambient temperature (18 °C to 27 °C) until the block breaks. Extract the individual animals with sterile pliers or forceps
2. Use sufficient material from the edible portion of the laboratory sample to give a representative test portion as specified in the test method. Homogenize using a rotary or peristaltic homogenizer in diluent to obtain a 1 in 10 suspension.

## NOTE: Annex C may be referred for classification of major taxa of fish

## 6.16 Additional Guidance for Small Fish, Crabs and Lobsters

### 6.16.1 *Small Fish (up to 15 cm Long)*

1. Using sterile scissors and forceps, remove a portion of the fish just anterior to the tail insertion by making two cuts to produce transverse sections; the first cut to remove the tail and the tail insertion and the second to remove a steak (*see* Figure 1).
2. Take care not to remove any viscera or gut contents.



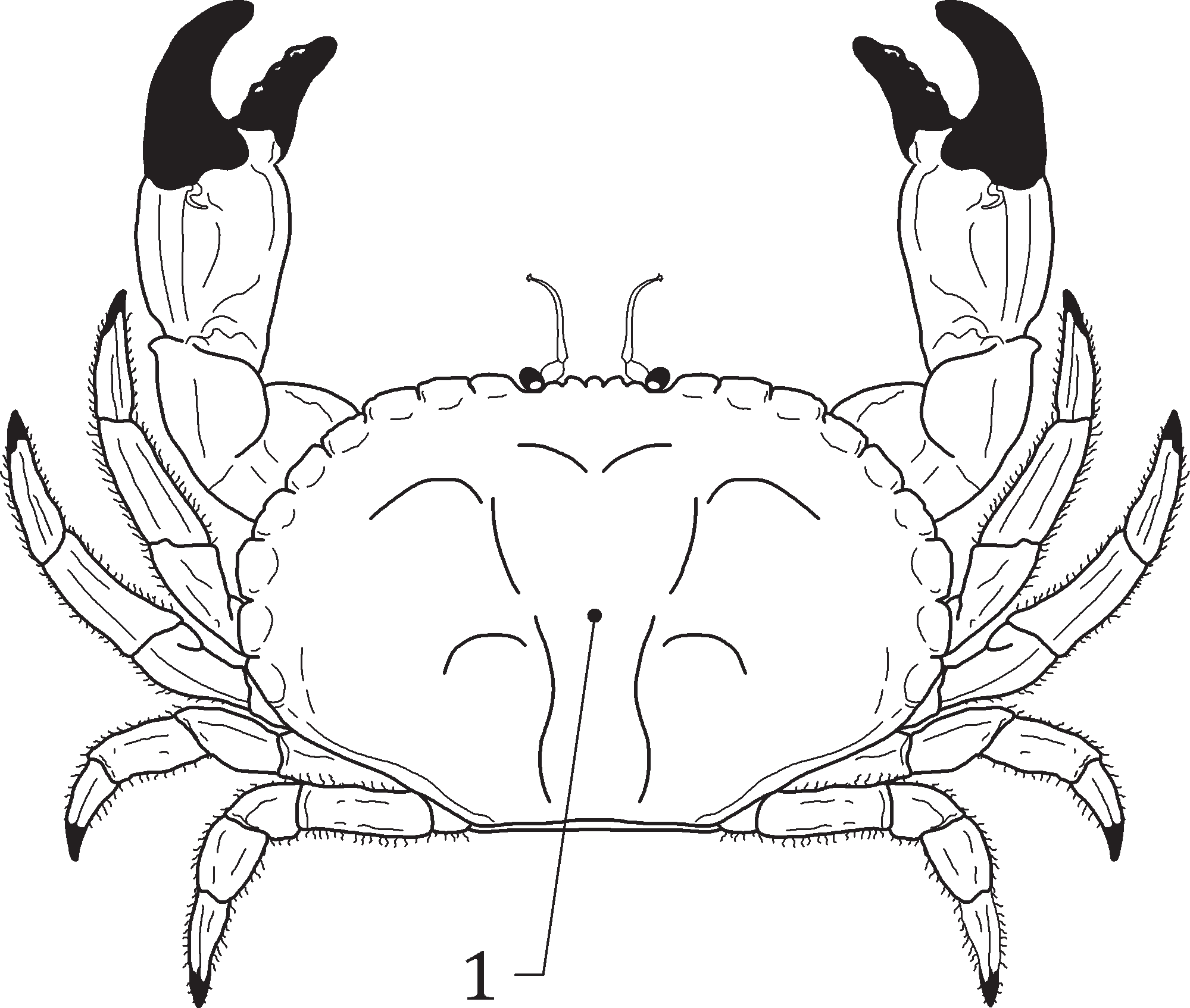
**Key**

|  |  |
| --- | --- |
| 1 | Cut 1 |
| 2 | Cut 2 |

**Figure 1 —** Example of test sampling of a fish up to 15 cm in length

### 6.16.2 *Crabs*

Lift off carapace (*see* Figure 2) with sterile forceps and crack claws. Using sterile forceps, take sufficient flesh to yield the amount specified in the test method.



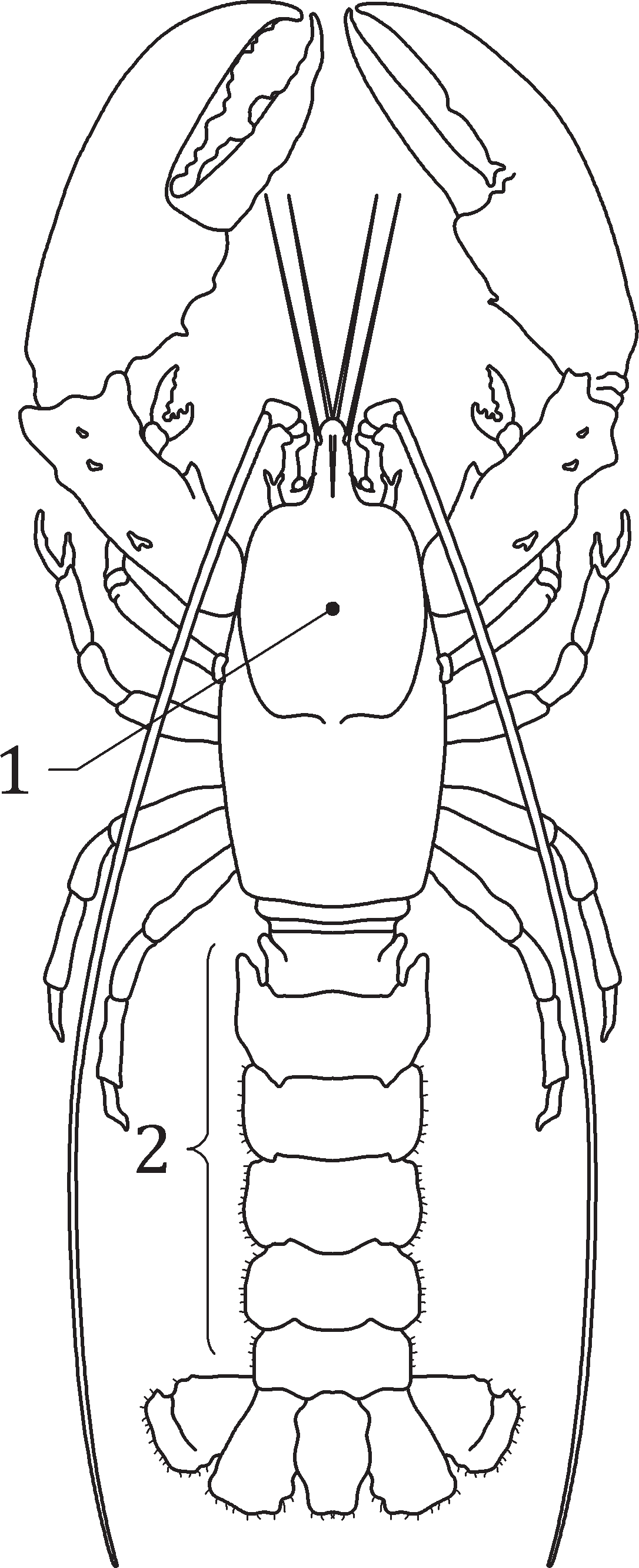
**Key**

|  |  |
| --- | --- |
| 1 | Carapace |

**Figure 2 —** Carapace of a crab

### 6.16.3 *Flesh from Lobsters and Crayfish*

1. Break the crustacean at the junction between the cephalothorax and abdomen (*see* Figure 3).
2. Using sterile forceps, pull the flesh from the cephalothorax and butt end of the abdomen (this includes a little gut, which is usually eaten).
3. Take sufficient flesh to yield the amount specified in the test method.



**Key**

|  |  |
| --- | --- |
| 1 | Cephalothorax |
| 2 | Abdomen |

**Figure 3 — Cephalothorax and abdomen of a lobster**

**Annex A**

( *Clause* 6.1.3 )

**Performance Testing for Diluents**

**A-1** 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 percent of the number counted initially. For detailed information please refer IS 17383**.**

**Table A-1**

**Test Microorganisms and Productivity Criterion for Diluents**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Media/**  **Diluent** | **Incubation** | **Test Microorganisms** | **ATCC No/ MTCC No** | **Reference Medium** | **Control Method** | **Criteria** |
| Peptone Salt | 45 min to 1 h at laboratory ambient temp | *Escherichia coli* | 25922/ 443 | TSA | Quantitative | ±30 % of original count |
| Buffered peptone water (single and double strength) | *Staphylococcus aureus* | 6538/  737 |
| Phosphate buffered diluent |

**A-2 Procedure**

**A-2.1** The method determines the ability of the diluent to support the survival of microorganisms without undue multiplication or reduction during the period of contact before plating on to agar or inoculation into liquid media.

**A-2.2** Inoculate a test portion (e.g. 9 ml) of the diluent with 1 ml of the test microorganism suspension containing around 104 cfu/ml and mix. Immediately remove 0,1 ml of inoculated diluent and spread over the surface of a non-selective agar (reference medium) such as TSA (*t*0).

**A-2.3** Hold the inoculated diluent at ambient temperature for the time lapse specified between the end of preparation of the initial suspension and the moment when the inoculum comes into contact with the culture medium (usually 45 min). Mix and then remove the same volume (0,1 ml) and plate again on the reference medium (*t*1). Incubate the reference medium at an appropriate temperature and time e.g. 30 °C/72 h.

**A-2.4** After incubation count the colonies on the plates (*t*0) and (*t*1).The number of microorganisms (*t*1) after incubation of the diluent shall be within ± 30 % of the initial count (*t*0).

**Annex B**

( *Clause* 6.3.11.3 )

**Carcass Sampling for Microbiological Analysis**

**B-1** **GENERAL**

**B-1.1** The determination of microbial counts and the prevalence and/or numbers of pathogenic microorganisms on carcasses is essential for validation and verification in risk-based slaughter hygiene assurance systems [e.g. those employing the hazard analysis critical control points (HACCP) principles and quality assurance systems]. Many laboratories are involved in (regional, national, and international) monitoring or surveillance programs on the prevalence and/or counts of pathogenic microorganisms to gather information for risk assessment. The design of such monitoring and surveillance programs will benefit from the use of this standardized sampling procedure.

**B-1.2** The sampling procedure includes the use of excision and swabbing techniques depending on the reason for sample collection. It also includes the use of carcass rinsing for the examination of carcasses of poultry and some small animals.

**B-1.3** The procedure specifies sampling methods for the detection and enumeration of microorganisms on the surface of carcasses or parts of carcasses of slaughtered meat animals. The microbiological sampling can be carried out as part of:

1. process hygiene control (to validate and or verify process control, e.g. total counts and  *Enterobacteriaceae*) in slaughter establishments for large mammals, poultry, and game,
2. risk-based assurance systems for product safety, and
3. monitoring or surveillance programmes for the prevalence and/or numbers of pathogenic microorganisms.

**B-2 TERMS AND DEFINITIONS**

**B-2.1 Carcass -** Body of an animal after slaughter and dressing

**B-2.2 Excision technique - R**emoval of measured areas of the surface tissue or skin by cutting

**B-2.3 Game -** Wild mammal or bird that is hunted for human consumption and farmed mammal or bird, includingratite (e.g. ostrich, emu), other than domestic ungulates and birds

**﻿**

**B-2.4 Large mammal - C**attle (including buffaloes, bison), sheep, pigs (including wild boar), various types of deer (includingreindeer, antelopes), horses (including donkeys and mules)

**B-2.5 Poultry -** Small-sized or medium-sized bird (eg.chicken, duck, goose, turkey, pigeon, quail, grouse.)

**B-2.6 Sampling point -** Stage on the production line where a sample is taken

**B-2.7 Sampling site -** Place on the carcass where a sample is taken

**B-2.8 Small mammal and other small animal -** Lagomorph (e.g. hare, rabbit), rodent, turtle, frog

**B-2.9 Swabbing method -** Technique using absorbent material attached to the end of a stick or wire, or a sponge or piece ofabsorbent material, to pick up microorganisms from surfaces

**B-3 GENERAL PRINCIPLES**

**B-3.1** The choice of sampling method depends mainly on the aim of the microbiological examination, the sensitivity required, and practical considerations. Excision, swabbing, and rinsing methods may be used, as described in this procedure.

**B-3.2** The excision of surface tissue generally harvests a higher number of microorganisms from the surface than other methods. Not all the microorganisms harvested will grow on the media and under the incubation conditions used. The repeatability and reproducibility of excision and rinsing methods are less variable than swabbing, because swabbing methods are more difficult to standardize. However, only a small proportion of the carcass is sampled by excision methods, which might result in significant inaccuracies when total contamination is low and heterogeneously distributed, or when the presence of the target microorganisms is sparse. Excision methods are destructive, and can sometimes affect the value of the meat, but are preferred when sampling frozen surfaces.

**B-3.3** Swabbing or rinsing techniques enable the examination of larger areas. Smaller areas targeting verified areas of greatest contamination may be examined using either excision or swabbing methods. Rinsing the whole carcass is an effective and practicable method for the examination of poultry (excluding large bird carcasses) and carcasses of some small mammals and other small animals.

**B-4 SAMPLING PLANS**

**B-4.1** Sampling plans should relate to the purpose of testing and be applied according to the circumstances. The process stage, the time since start-up of the slaughtering process, the frequency of sampling, and, if relevant, the following, should be taken into account:

1. the slaughterhouse practices for each animal;
2. the design of risk-based process control assurance or harmonized monitoring programmes;
3. the production volume;
4. previous results of monitoring (trend analysis);
5. the prevalence of relevant pathogenic microorganisms in the region from which the animal originates;
6. relevant national, regional and/or international regulations.

**B-4.2** In the case of process control, the time and frequency of sampling should relate to the level of slaughter hygiene.

**B-4.3** In the case of monitoring and surveillance for pathogens, the sampling time, sites on the carcass, and frequency should maximize the chance of detecting and/or counting the pathogens sought.

**B-5 SAMPLING POINTS ON THE PRODUCTION LINE**

**B-5.1** Sampling points should be selected according to risk-based principles, and relate, when relevant, to the higher probability of detecting contamination during the process or at points in the slaughter process, as appropriate to measure the hygiene of specific production steps or the entire slaughter process.

**B-5.2** Examples of sampling points are the following:

1. after the carcass polishing machine (pigs);
2. after the carcass washing machine (pigs and poultry);
3. after flaying (dehiding) (large mammals, game slaughtered in an abattoir, and others);
4. after evisceration (all animals);
5. immediately before chilling or freezing (all animals);
6. immediately after chilling (poultry, small mammals, and other small animals);
7. after chilling or freezing (all animals);
8. in the chill room (all animals).

During the chilling period, depending on the chill room conditions, microorganisms might become sublethally damaged or die, might be overgrown by psychrotrophic microorganisms, or they might become more firmly attached to the meat, resulting in underestimation. This effect will be reduced if the sampling is carried out as soon as possible after slaughter.

**B-6 SAMPLING SITES ON CARCASSES**

**B-6.1 Large Mammals -**The sampling sites chosen depend on the slaughterhouse practices, which can vary for different slaughterhouses and for different animals. The purpose is to examine the sites with the highest prevalence and/or level of contamination (see Table B.1). Figure B.1, Figure B.2, and Figure B.3 illustrate the sites most often identified as more highly contaminated. Other sampling sites may be selected depending on the contamination nature of carcass. Consistency in the sampling sites over time is important to detect changes in the pattern of observations over a period of time (trend analysis). It is usually preferable to sample more carcasses at the sites most likely to be contaminated, rather than more sites per carcass. Prevalence determinations in surveillance programmes will generally benefit from larger sampling areas.

**B-6.2 Poultry, Small Mammals, and Other Small Animals -** A common method is to rinse the whole of these small carcasses. If surface samples are taken, thesites chosen depend on the slaughtering practice and equipment used. For poultry, neck skin or, if notpresent, breast skin is usually sampled.

**B-6.3 Game -** For larger game, the sampling sites can be similar to those for large mammals. In general, for each species, samples are to be collected from sites most likely to be contaminated.

**B-7 SAMPLING TECHNIQUES**

**B-7.1 General**

**B-7.1.1** For a given sampling situation, the same sampling technique shall be used each time, to ensure that results are comparable. In general, three different methods can be used, the excision (destructive) method, the swabbing (non-destructive) method or the rinsing method. As the surface of the carcass is being sampled, the results are expressed in terms of colony forming units (CFU) per cm2. When using the rinsing technique, the results are usually expressed as CFU per carcass. When sampling skin from poultry carcasses, the results are expressed as CFU/g.

**B-7.1.2** A number of samples from one carcass, or from several carcasses at the same sampling site, can be combined to make one pooled sample that is taken as a whole for analysis in the laboratory. Alternatively, a number of samples from one carcass can be combined to one composite sample, from which a test portion is taken for analysis (*see* IS 10232).

**B-7.2 Excision Methods**

In general, two different methods are used, the cork borer and the template method. Both sample the surface of the meat (as the inner part of the meat is normally sterile). The cork borer usually samples smaller areas than the template method, but is easier to use when examining frozen meat.

**B-7.2.1** *Cork Borer Method*

**B-7.2.1.1** *Reagent*-Ethanol, 70 percent volume fraction

**B-7.2.1.2** *Equipment*

* Sterile scalpels;
* Sterile forceps;
* Sterile cork borers, with a cutting area of at least 5 cm2 (diameter about 2.5 cm);
* Sterile scissors;
* Portable gas blow torch or portable Bunsen burner (optional);
* Tissues or cotton wool;
* Sterile plastic bags, for a peristaltic type or sonic homogenizer of appropriate size for thearea being sampled;
* Diluent.

**B-7.2.1.3** *Collection of Samples* **-** At the relevant sites on the carcass, circular incisions are made in the surface with a sterile cork borer. The cork borer is removed and discs of skin or tissue (approximately 2 mm thick) are thencut loose from the surface end with a sterile scalpel or scissors and forceps and put into a labelled sterile plastic bag.

**B-7.2.1.4** *Cleaning and Sterilization of Equipment -* Each sample shall be taken using clean and sterile equipment. Sterilization can be achieved by autoclaving suitably wrapped equipment. Equipment may be reused during sampling, provided it is carefully cleaned and disinfected between sampling. This may be done as follows:

1. clean with tissues or cotton wool dipped in 70 percent ethanol;
2. dip in 70 percent ethanol in a bottle;
3. burn the ethanol off if the use of a naked flame is hazardous, then, simply allow the ethanol to evaporate);
4. allow to cool.

**B-7.2.1.5** Due to the time needed to carry out the cleaning, it is useful to have several sets of pre-sterilized equipment (For example, cork borer, scalpels, and forceps) available. It is essential that these tools are not contaminated again before use. As an alternative, use sterile disposable instruments.

NOTE: If samples are combined into a pooled or composite sample, it is not necessary to clean and disinfect between taking these samples.

**B-7.2.2** *Template Excision Method*

This method is identical to the cork borer method, except for the use of templates and scalpel or knife instead of a cork borer. Templates are usually made from metal or plastic, and are used to define the sampling area, e.g. 10 cm2, 50 cm2, or 100 cm2.

**B-7.3 Skin Sampling**

**B-7.3.1** *Neck Skin* **-** Neck skins are often removed from poultry carcasses as they pass on the production line, so they haveto be cut off rapidly, but may be trimmed later and weighed (individually or as a composite sample).

Take a pair of sterile scissors. Open a sterile plastic bag without touching the sterile interior of the bag. Grip the bag at the bottom seam and fold it back over the hand so that it inside out. Avoiding carcasses with very short neck skins, grip the neck skin of a carcass firmly through the bag and cut it off as rapidly as possible. This is usually done using scissors. Measure the sample size by weighing (one neck skin weighs about 10 g). If necessary, several samples shall be combined to give the desired sample size, e.g. 25 g or 50 g.

**B-7.3.2** *Breast Skin* **-** It is quick and easy to remove the skin from poultry breasts. Take the carcass to be sampled and put it on a flat surface, avoiding any contact with the parts of the skin to be sampled. Remove as much of the breast skin as possible using and forceps and weigh it. The results are then expressed with respect to the mass (as CFU per gram or as presence/absence in 25 g).

**B-7.4 Swabbing Methods**

Swabbing is a non-destructive method especially used for sampling larger areas. The technique includes the use of sticks with absorbent material, swabs, tampons, sponges, and cloths, mainly depending on the circumstances and area to be examined.

**B-7.4.1** *Wet and Dry Stick Swab Method*

**B-7.4.1.1** *Reagent* **-** Sterile diluent, dispensed in 10ml amounts in tubes or bottles, Equipment, Sterile cotton wool swabs with wooden shafts. The size depends on the area to be swabbed (e.g. small swabs for 10 cm2 on small game carcasses, large swabs for 50 cm2 on beef carcasses), Sterile square templates.

**B-7.4.1.2** *Collection of Samples***-** Moisten a swab in 10 ml diluent at a selected carcass-sampling site, press thetemplate hard on to the surface. Rub the swab over the whole area using pressure, movingin at least two directions, e.g. firstly horizontally and then vertically, at least 10 times in each direction. Place the swab into the diluent used to wet the swab, breaking off the wooden shaft against the inside of the bottle. Then, with a dry swab, sample the same area again, using the same technique to absorb any liquid remaining from the wet swab as above, and place this swab into the same container of diluent. The templates may be cleaned, disinfected, and reused as described under.

**B-7.4.2** *Sponge, Tampon Swab, and Small Cloth Method* **-** Sponges and tampon swabs are used particularly for the sampling of larger areas. Sampling of larger non-template areas can increase the chance of detecting pathogens that are present at low levels on the carcasses. More than one site may be sampled using the same swab.

**B-7.4.2.1** *Reagent* - *Sterile diluent*

**B-7.4.2.2** *Equipment*

* Sterile specimen sponge (free of inhibitory substances) of 25 cm3 to 50 cm3 in a sterile plastic bag; Large cellulose sponges may also be suitable;
* Sterile tampon swabs: these swabs typically comprise large composite fabric (cloth) swabs with multiple layers of gauze or cotton wool encased in gauze;
* Sanitary towels or tampons (free of inhibitory substances);
* Sterile small cloth (free of inhibitory substances),
* Plastic bags, for a peristaltic type or sonic type homogenizer;
* Sterile template, with hollow internal area of not less than 100 cm2;
* Sterile gloves.

**B-7.4.2.3** *Collection of Samples* **-** At the sampling point, open the plastic bag containing the sterile sponge, sterile tampon swab or sterile small cloth and add sufficient diluent from a defined volume of diluent (For example, 25 ml or 100 ml) to wet the sponge or the tampon swab without excess fluid being evident. Massage the sponge or the tampon swab from outside the bag to moisten it thoroughly. Place the template over the test area. Either use the bag as a glove by turning it inside out and grasping the sponge or tampon through the bag, as for taking neck skins or use a fresh pair of sterile gloves to wipe the sponge or tampon swab over the test surface.

Sample by rubbing in at least two directions, for example, firstly horizontally and then vertically, at least 10 times in each direction. After swabbing, place the sponge or the tampon swab back in its plastic bag and add the remainder of the diluent. The template may be reused.

**B-7.5 Carcass Rinsing Method**

**B-7.5.1** *Reagent* **-** Sterile diluent (300 ml, 400 ml, or 600 ml volumes)

**B-7.5.2** *Equipment* - Large stomacher type plastic bags

**B-7.5.3** *Collection of Samples (For Example, Poultry)*

**B-7.5.3.1** Carcasses are normally taken off the moving production line. Open a large stomacher type bag without touching the sterile interior. Enclose a carcass with the bag while it is still on the line, and,using both hands while holding the legs of the carcass through the bag, lift the carcass off the line (i.e.detach its legs from the shackles). Try to avoid taking carcasses with significant volumes of water stilldraining off them. If such carcasses are taken, remove them, under aseptic conditions, to a separate,disinfected set of shackles and allow the water to drain off before enclosing it in a bag.

**B-7.5.3.2** Rest the bottom of the bag containing the carcass on a flat surface. Holding the top of the bag slightly open, add sterile diluent usually 400 ml (chickens) or 600 ml (turkeys), to the bag, pouring the solution into the carcass cavity and over the exterior of the carcass. Expel most of the air from the bag and then close the top of the bag with, e.g. a tie wrap. Holding the bag securely, rinse the carcass inside and out, using a rocking motion, for approximately 1 min. Do this by holding the carcass through the bottom of the bag with one hand and the closed top of the bag with the other hand. Holding the carcass securely in this way, move it through an arc, shifting the weight of the carcass from one hand to the other to ensure that all surfaces (interior and exterior) of the carcass are rinsed. Rest the bag with the carcass on a flat surface and, while supporting the carcass, open the bag. Remove the carcass from the bag, firstly letting any excess fluid drain back into the bag. Take care not to touch the interior of the bag. Secure the top of the bag so that the rinse fluid will not spill out or become contaminated. Alternatively, transfer the rinse fluid under aseptic conditions from the bag to a sterile container (For example, the bottle that contained the sterile diluent). This may be done without taking the carcass from the bag.

**B-7.5.4** *Storage and Transport of Samples*

**B-7.5.4.1** Transport the samples in an insulated cool box with frozen freezer blocks, a crushed melting ice cool box, or a (portable) refrigerator. Do not allow the samples to freeze or to be exposed to the frozen blocks, if used. Keep the temperature between 1 °C to 8 °C.

**B-7.5.4.2** Examine the samples as soon as possible after receipt, or store them at 3 °C ± 2 °C for a maximum of 24 h (*see* IS 16122).

**B-7.6** **Microbiological Examination of Samples**

Test shall be performed in accordance with the relevant standard methods. The quantitative results from samples obtained by excision or swabbing shall be expressed as colony forming units (CFU) per square centimetre and not CFU per gram or per millilitre, with the exception of results from poultry skin, which can be expressed as CFU per gram. For carcass rinse samples, the results of plate counts shall be expressed as CFU per ml carcass rinse stating the volume of the rinse liquid, or as CFU per carcass. In the case of enrichment procedures, report the target microorganism as detected or not detected in the area/test portion analyzed or per carcass tested.

**B-7.7 Sampling Sites**

The sampling sites to be chosen depend on the slaughterhouse practices for different animals. The purpose is to examine the sites with the highest prevalence of contamination (see Table B.1). Figure B.1, Figure B.2, and Figure B.3 show examples of the sampling sites on the surface of the carcass of pigs, cattle, and sheep respectively. For other large animals, e.g. horses, deer, and similar animals, sampling sites will be similar to those for cattle. However, this should be verified. The slaughter techniques used will affect the sites most highly contaminated.

**Table B.1**

**Sites Most Often Identified as More Highly Contaminated**

|  |  |  |
| --- | --- | --- |
| Pigsa | Cattlea | Sheepa |
| 1 Pelvic channel internal | 1 Pelvic channel internal | 1 Knee external aspect |
| 2 Pelvic channel internal | 2 Pelvic channel internal | 2 Pelvic channel internal |
| 3 Abdominal | 3 Hock external aspect | 3 Abdominal external |
| 4 Xiphoid external | 4 Hock internal aspect | 4 Anterior sternum external |
| 5 Xiphoid internal | 5 Internal thigh | 5 Foreleg, elbow, external aspect |
| 6 Pillar of diaphragm | 6 Sternum external | 6 Neck, prescapular nregion external |
| 7 Submaxillary external | 7 Sternum internal |  |
| 8 Submaxillary internal | 8 Xiphoid external |  |
| 9 Fore foot external aspect | 9 Xiphoid internal |  |
| 10 Fore foot internal aspect | 10 Foreleg internal aspect |  |
|  | 11 Atlanto-occipital internal aspect |  |
|  | 12 Atlanto-occipital external aspect |  |
| a The numbers 1 to 12 indicate the sampling sites in Figure B.1 to Figure B.3 | | |



Figure B.1 – Pig: examples of sampling sites (left = lateral, right = medial)



Figure B.2 – Cattle: examples of sampling sites (left = lateral, right = medial)



Figure B.3 – Sheep: examples of sampling sites (left = lateral, right = medial)

**ANNEX C**

( *Clause* 6.15.5.3 )

**Classification of Major Taxa of Fish**

|  |  |  |
| --- | --- | --- |
| **Taxonomical Division** | | **Examples** |
| Phylum — Chordata | Class — Myxini | Hagfish, Nuta-unagi, Meokjango, Yu sheng |
| Class — Petromyzontida | Lamprey |
| Class — Chondrichthyes | Whitefish, Makorepe, ghost shark |
| Class — Elasmobranchii | Sharks, flake, sora, rays, skates |
| Class — Actinopterygii | Fin fish |
| Phylum — Arthropoda, subphylum — Crustacea | | Crayfish, yabby, marron, scampi, clawed lobster, spiny lobster, langoustines, shrimp, prawns, crabs, |
| Phylum — Mollusca | Class — Cephalopoda | Octopus, squid, cuttlefish, nautilius. |
| Class — Bivalvia | Oysters, mussels, scallops, clams, cockles |
| Class — Gastropoda | Abalone (paua), conch, periwinkles, whelks, limpets, sea slugs, snails |
| Phylum — Chordata, subphylum — Tunicata | | Sea squirts, sea pork, sea tulips, sea violet, piure |
| Phylum — Echinodermata | Class — Holothurian | Sea cucumber, trepan, sea slug |
| Class — Echinoidea | Sea urchins (hota, ututuk, kina, uni) star fish |

**BIBLIOGRAPHY**

* + - 1. IS 11546 : 2012/ ISO 707 : 2008 Milk and milk products - Guidance on sampling ( second revision )
      2. IS 15989 : 2023/ ISO 17604 : 2015 Microbiology of food chain carcass sampling for microbiological analysis ( first revision )
      3. IS 15990 : 2023/ ISO 6887-2 Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination: Part 2 Specific rules for the preparation of meat and meat products ( *first revision* )
      4. IS 16980 : 2018/ ISO 6887-6 : 2013 Microbiology of food and animal feed - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Specific rules for the preparation of samples taken at the primary production stage
      5. IS 17447 : 2020/ ISO 6887-4 : 2017 Microbiology of the food chain - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Specific rules for the preparation of miscellaneous products
      6. IS 17448 : 2020 / ISO 6887-3 : 2017 Microbiology of the food chain - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Specific rules for the preparation of fish and fishery products
      7. ISO/TS 17728:2015 Microbiology of the food chain - Sampling techniques for microbiological analysis of food and feed samples
      8. IS 17779 : 2021/ ISO 6887-5 : 2020 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination: Part 5 Specific rules for the preparation of milk and milk products
      9. American Public Health Association (APHA), Compendium Methods Microbiological Examination Foods, 5th Edition
      10. Bacteriological Analytical Manual (BAM), Chapter 1, Updated April, 2022– In: *Food Sampling/Preparation of Sample Homogenate*
      11. CODEX General Guidelines on Sampling - CAC/GL 50-2004
      12. FAO/WHO, Microbiological risk assessment series, 24- In: *Statistical Aspects of Microbiological Criteria Related to Foods*
      13. International Commission on Microbiological Specifications for Foods (ICMSF) – In: *Microorganisms in Foods 7*, *Microbiological Testing in Food Safety Management*
      14. Food Safety and Standards Authority of India (FSSAI) – General guidelines for sampling for microbiological analysis, October, 2022. [*https://www.fssai.gov.in/upload/uploadfiles/files/Sampling\_Guidelines\_Microbiology\_07\_10\_2022.pdf*](https://www.fssai.gov.in/upload/uploadfiles/files/Sampling_Guidelines_Microbiology_07_10_2022.pdf)

**ANNEX D**

(*Foreword*)

**COMMITTEE COMPOSITION**

FOOD MICROBIOLOGY SECTIONAL COMMITTEE, FAD 31

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| *Organization* |  | *Representative(s)* |
| ICAR - lndian Veterinary Research Institute, Bareilly |  | DR KIRAN N. BHILEGAONKAR, **(*Chairperson*)** |
| 3 M India Ltd., Bangalore |  | SHRI KULVEEN SINGH BALI |
|  | SMT KAVITHA KULKARNI (*Alternate*) |
| All India Food Processors' Association (AIFPA), New Delhi |  | DR K. L. GABA |
|  | SHRI VIJAY GAUR (*Alternate*) |
| Central Research Institute, Kasauli |  | DR YASHWANT KUMAR |
|  | DR SUBHADIP MAHAPATRA (*Alternate*) |
| ICAR-Central Institute of Fisheries Technology, Kochi |  | DR SATYEN KUMAR PANDA |
|  | DR B. MADHUSUDANA RAO (*Alternate*) |
| CSIR-Central Food Technological Research Institute, Mysore |  | DR ALOK SRIVASTAVA |
|  | DR ASHA MARTIN (*Alternate*) |
| CSIR-Institute of Microbial technology, Chandigarh |  | DR SURESH KORPOLE |
|  | DR P. ANIL KUMAR (*Alternate*) |
| Confederation of Indian Food Trade & Industry, New Delhi |  | SHRI KRISHNA KUMAR JOSHI |
|  | SMT PRIYANKA SHARMA (*Alternate*) |
| Confederation of Indian Industry (CII), New Delhi |  | SHRI SAM THOMAS |
|  | DR. KHURSHID ALAM KHAN (*Alternate*) |
| Defence Food Research Laboratory (DFRL), Mysore |  | DR JOSEPH KINGSTON |
|  | DR BALAKRISHNA (*Alternate*) |
| Export Inspection Council (EIC) of India, New Delhi |  | DR WASHI ASGHAR |
|  | SHRI ANGSHUMAN SAHA (*Alternate*) |
| Hi Media Laboratories Private Ltd., Mumbai |  | DR RAHUL G. WARKE |
|  | DR GIRISH B. MAHAJAN (*Alternate*) |
| ICAR-Indian Veterinary Research Institute, Bareilly |  | DR TRIVENI DUTT |
|  | DR D. K. SINGH (*Alternate*) |
| Marine Products Export Development Authority, Cochin |  | DR SREENATH P. G |
|  | SHRI VINOD V. (*Alternate*) |
| Merck Life Science Private Limited, Mumbai |  | SMT SUJATA SAINDANE |
|  | SHRI SACHIN MALI (*Alternate*) |
| National Accreditation Board for Testing and Calibration of Laboratories, Gurugram |  | DR SUNITA RAWAT |
|  | SHRI VINAY TYAGI (*Alternate*) |
| National Dairy Development Board, Anand |  | SHRI SD JAISINGHANI |
|  | DR JITENDER SINGH (*Alternate I*) |
| National Dairy Research Institute, Karnal |  | DR RAVINDER KUMAR MALIK |
|  | DR NARESH KUMAR GOEL (*Alternate*) |
| National Institute of Cholera & Enteric Disease, Kolkata |  | DR SHANTA DUTTA |
|  | DR ASISH KUMAR MUKHOPADHYAY (*Alternate*) |
| National Institute of Food Technology Entrepreneurship and Management, Haryana |  | DR NEETU KUMRA TANEJA (*Alternate*) |
| ICMR-National Institute of Nutrition, Hyderabad |  | DR R. NAVEEN KUMAR (*Alternate*) |
| BIS Directorate General |  | SHRIMATI SUNEETI TOTEJA, SCIENTIST ‘E’ AND HEAD (FOOD AND AGRICULTURE)  (REPRESENTING DIRECTOR GENERAL (*Ex-Officio*) |

*Member Secretary*

SHRIMATI VARSHA GUPTA

Scientist ‘D’/JOINT DIRECTOR,

(FOOD AND AGRICULTURE), BIS

Panel Responsible for Review and Revision of IS 5404 : 1984 Sampling, Transport, Storage and

Sample Preparation of Food Samples for Microbiological Analysis, FAD 31 : Panel 1

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| *Organization* |  | *Representative(s)* |
| ICAR-Central Institute of Fisheries Technology, Kochi |  | DR S. K. PANDA (*Convenor*) |
| Export Inspection Council of India, New Delhi |  | SHRI ANGSHUMAN SAHA |
| National Dairy Development Board, Anand |  | DR JITENDER SINGH |
| ICMR-National Institute of Nutrition, Hyderabad |  | DR R. NAVEEN KUMAR |