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Methods for

Microbiological examination of food and animal feeding stuffs —

Part 0: General laboratory practices

ICS 07.100.30; 65.120; 67.040

Committees responsible for this British Standard

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Association of Clinical Pathologists
Association of Public Analysts
BLWA Ltd. (the Association of the Laboratory Supply Industry)
Leatherhead Food Research Association
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Dairy Industry Federation
Department of Agriculture Northern Ireland
Department of Health
Department of Trade and Industry (Laboratory of the Government Chemist)
Food and Drink Federation
Institute of Brewing
Meat and Livestock Commission
Ministry of Agriculture, Fisheries and Food
Natural Resources Institute
Public Health Laboratory Service
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Contents

National foreword

This Part of BS 5763 has been prepared by Technical Committee AW/9 and is identical with ISO 7218:1996 *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations,* including amendment 1:2001, published by the International Organization for Standardization (ISO) and in the preparation of which the United Kingdom played a full part. This edition supersedes BS 5763-0:1986 which is withdrawn and from which it differs in that the technical requirements have been generally updated in line with current microbiological practice.

Cross reference

The British Standards which implement international or European publications referred to in this document may be found in the BSI standards Catalogue under the section entitled "International Standards Correspondence Index", or by using the "Find" facility of the BSI Standards Electronic Catalogue.

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Summary of pages

This document comprises a front cover, an inside front cover, pages i and ii, pages 1 to 35 and a back cover.

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Introduction

When conducting microbiological examinations, it is especially important

— that only those microorganisms which are present in the samples are isolated or enumerated, and

— that the microorganisms do not contaminate the environment.

In order to achieve this, it is necessary to pay attention to personal hygiene and to use working techniques which ensure, as far as possible, exclusion of extraneous contamination (see clause **5**).

Since, in this International Standard, it is possible to give only a few examples of the precautions to be taken during microbiological examinations, a thorough knowledge of the microbiological techniques and of the microorganisms involved is essential. It is important that the analyses be conducted as accurately as possible, including calculation of the number of microorganisms and the variability of the result (part of this is given by the confidence limits; see clause **9**).

Ultimately, it is the responsibility of the head of the laboratory to judge whether the manipulations are safe and can be considered to be good laboratory practice.

A large number of manipulations can, for example, unintentionally lead to cross-contamination and the analyst should always verify the accuracy of the results given by his or her technique.

In order to conduct the examinations correctly, it is necessary to take certain precautions when constructing and equipping the laboratory (see clause **3**).

Certain precautions must be taken, not only for reasons of hygiene, but also to ensure good reproducibility of the results. It is not possible to specify all the precautions to be taken in all circumstances, but this International Standard at least provides the main measures to be taken when preparing, sterilizing and storing the media and the equipment (see clauses **6** and **7**).

If the guidance in this International Standard is followed, this will also contribute towards the protection of the health of the personnel. Additional information on this subject is to be found in the literature listed in Annex C.

1 Scope

This International Standard gives general instructions for carrying out microbiological examinations in accordance with specific standards.

The purpose of this International Standard is to help to ensure the validity of the examinations, to ascertain that the general techniques used for conducting these examinations are the same in all laboratories, to help achieve homogeneous results in different laboratories, and to contribute towards the protection of the health of the laboratory personnel by preventing risks of infection.

This International Standard may be used wholly or partly for the accreditation of a laboratory by national organizations.

2 Normative reference

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

ISO 6887:1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination*.

3 Premises

3.1 Test areas

The areas required for the specific operation of a microbiology laboratory are as follows:

— receipt, storage, preparation and processing of the samples;

— preparation and sterilization of culture media and equipment;

— performance of analyses: weighing, dilutions, inoculations, subculturing, incubation, preservation of the strains, etc.;

— decontamination and cleaning of equipment, and processing of the analysis waste.

3.2 Additional areas

The areas included in this category are, for example:

— entrances, corridors, stairways, goods lifts or lifts;

— administrative areas (e.g. secretarial, offices, documentation rooms, etc.);

— cloakrooms and toilets;

— archive rooms;

— stores.

3.3 Location of the premises

The environment within which the microbiological analyses are carried out shall not affect the reliability of the analyses.

Care shall be taken to locate the premises so as to avoid risk of cross-contamination. Application of the "no-way-back" principle may help to achieve this aim.

Care shall be taken to ensure protection against extreme conditions such as excess temperature, dust, humidity, steam, noise, vibration, exposure to direct sunlight, etc.

The surface area shall be sufficiently large to keep the work areas clean and orderly. For all test premises, a work station of approximately 20 m^2 is recommended for each analyst.

During the course of the tests, care shall be taken to limit access to the test areas to only those persons required to conduct the tests.

Separate rooms and/or separate areas and/or specific enclosures shall be provided for the following:

— receipt and storage of samples;

— preparation of samples, particularly in the case of raw materials (e.g. powdered products containing a high number of microorganisms);

— manipulation of pathogens (e.g. *Salmonella, Listeria monocytogenes)*;

— preparation and sterilization of culture media and equipment;

— cleaning of glassware and of other equipment, as well as the decontamination of equipment and contaminated culture media;

— checking the sterility of foodstuffs.

Separation of the following areas should also be considered:

— the areas used for the preparation of culture media, and the room used for sterilization of culture media and of the equipment; and

— the decontamination area and washing area.

Incubators, refrigerators and freezers can be placed in specific, specially adapted rooms.

3.4 Equipping the premises

3.4.1 The test premises shall be fitted out in the following ways in order to reduce the risks of contamination by dust and therefore by microorganisms:

— the walls, ceilings and floors should be smooth, easy to clean, and resistant to detergents and disinfectants used in laboratories;

— overhead pipes conveying fluids should not cross the premises unless they are hermetically enclosed;

— solar radiation protection systems shall be installed on the outside of the windows, except in special cases;

— windows and doors shall be able to be closed hermetically when conducting the tests in order to minimize draughts; furthermore, they shall be designed so as to avoid the formation of dust traps and thus facilitate their cleaning.

3.4.2 The ambient temperature and air quality (microorganism content, humidity, dust spreading rate, etc.) shall be compatible with carrying out the tests. A filter ventilation system for incoming air is recommended for this purpose.

When tests are to be conducted in a low-contamination atmosphere, the room shall be specially equipped with a clean air laminar-flow cabinet and/or a safety cabinet.

This equipment shall comply with the relevant regulations.

3.4.3 The laboratory bench tops and furniture shall be manufactured in smooth, impermeable material, which is easy to clean and disinfect. In order to prevent the accumulation of dust, the cupboards shall, if possible, reach up to the ceiling.

Laboratory furniture shall be designed so as to facilitate cleaning the floors (e.g. movable furniture).

Enclosed storage facilities shall be available for storing documents used when manipulating the samples, culture media, reagents, etc.

NOTE It is desirable that documents or books which are not frequently used be placed outside the test areas.

3.4.4 The premises shall be well lit with avoidance of interfering reflections. It is advisable to avoid as far as possible exposure of the work places and sensitive equipment (incubators in particular) to direct sunlight.

3.5 Maintenance and inspection

The floors, walls, ceilings, laboratory bench tops and furniture shall be subjected to regular maintenance and repair in order to avoid cracks where dirt might particularly accumulate and thus cause a source of contamination.

Regular cleaning and disinfection shall be carried out in order to keep the premises in a condition suitable for conducting tests.

The ventilation systems and their filters shall be regularly maintained and filters changed when necessary.

The microbiological quality of surfaces and air shall be monitored regularly.

Surface contamination may be estimated by directly applying to the surface a contact plate containing suitable neutralizing agents. The air quality may be examined by exposing for 15 min an open Petri dish containing a non-selective agar culture medium [e.g. plate count agar (PCA)].

NOTE Other methods can also be used in order to estimate surface and air contamination.

4 Installations and equipment

In general, all installations and equipment shall be kept clean and in proper working condition. Maintenance operations should be monitored. The monitoring instruments shall be regularly serviced.

4.1 Microbiological cabinets

4.1.1 *Description*

A cabinet is a dust-removed work station equipped with horizontal or vertical laminar air-flow. In microbiology, a safety cabinet is used to retain the microorganisms on filters.

Conventionally, the maximum tolerable number of particles per cubic metre with a size greater than 0,5 μm represents the dust-spreading class of a safety cabinet. For cabinets used in food microbiology, the number of particles shall not exceed 4 000 per cubic metre.

Cabinets are of two types:

a) clean-air cabinets, which are intended to protect the product from extraneous contamination, and to minimize contamination due to the operator;

b) safety cabinets, which are intended to protect the product from extraneous contamination, and also to protect the operator and the environment.

Safety cabinets should be used for all work involving pathogens.

4.1.2 *Maintenance and inspection*

The efficiency of a safety cabinet shall be checked on receipt and thereafter at regular intervals by a qualified person (a yearly inspection is recommended). In the case of cabinets with prefilters, the latter shall be changed regularly.

Cabinets should be cleaned and disinfected after use. Periodic verification of any microbial contamination should be carried out by a check of the working surface and walls of the cabinet.

A periodic verification of the proportion of microorganisms present shall be carried out using the usual equipment. For example, expose several open Petri dishes containing a non-selective agar culture medium (e.g. PCA) in each cabinet for 30 min. Other methods can also be used.

4.2 Balance

4.2.1 *Use*

A food microbiology laboratory shall be equipped with balances of the required range and accuracy for the different products to be weighed. In general, two degrees of accuracy are required: ±0,01 g and ±0,000 1 g.

These balances are mainly used for weighing the test portion of the sample to be analysed and the components of the culture media and reagents. They may also possibly be used for carrying out measurements of dilution fluid volumes by weight.

4.2.2 *Maintenance and inspection*

The balance shall be placed on a stable horizontal support and shall be protected from vibrations.

It shall be checked regularly by calibration with working standards (preferably each working day). At least once a year, its entire range shall be monitored by a qualified person.

The balance pan shall be cleaned, if necessary, after each use and at least once a day. The mechanism shall be cleaned and checked by a qualified service engineer at least once a year.

4.3 Homogenizer

4.3.1 *Description*

This equipment is used to prepare the initial suspension from the test sample of non-liquid products.

The following apparatus may be used:

— a peristaltic homogenizer with sterile plastic bags, possibly with a device for adjusting velocity and time; or

— a rotary homogenizer, the rotational speed of which is between 8 000 r/min and 45 000 r/min inclusive, with sterilizable glass or metal bowls equipped with covers.

In certain special cases, the homogenization can be carried out with sterilizable glass beads having an appropriate diameter (approximately 6 mm; see specific standards).

4.3.2 *Use*

The usual operating time of a peristaltic homogenizer is 1 min to 2 min. This type of apparatus shall not be used for certain foodstuffs, such as

— products which risk puncturing the bag (presence of sharp, hard or dry particles), or

— products which are difficult to homogenize because of their texture (e.g. salami-type sausage).

The rotary homogenizer shall operate for a duration such that the number of revolutions is between 15 000 and 20 000 inclusive. Even with the slowest homogenizer, this time shall not exceed 2,5 min.

Glass beads can be used for the preparation, by shaking, of the initial suspensions of certain viscous or thick products, in particular certain dairy products (see specific standards).

4.3.3 *Maintenance and inspection*

The different appliances shall be inspected and maintained in accordance with the manufacturers' instructions.

4.4 pH-meter

4.4.1 *Description*

A pH-meter is used to measure the potential difference, at a determined temperature, between a measuring electrode and a reference one, both electrodes being introduced into the product. It shall be capable of measuring to an accuracy of ±0,1 pH units and its minimum measuring threshold shall be 0,01 pH units. The pH-meter shall be equipped with either manual or automatic temperature equalization.

NOTE The measuring electrode and the reference electrode are usually grouped together in a combined electrode system.

4.4.2 *Use*

A pH-meter is used to measure the pH of each batch of culture media and reagents (**7.2**) to check if adjustment is needed. It may also be used to measure and/or adjust the pH of the test sample or of the initial suspension. Use of a pH-meter will be discussed in the standard specific to the product to be analysed, in which the conditions for the determination of the pH, for the adjustment of the pH, as well as the method of cleaning and of decontamination of the electrodes will be specified.

4.4.3 *Maintenance and inspection*

The pH-meter shall be calibrated, in accordance with the manufacturer's instructions, using at least two standard buffer solutions, at least daily before use. The standard solutions have pH values which are known to be within the second decimal at the measurement temperature (in general, pH 4,00 and pH 7,00 at 20 °C). They shall encompass the measured pH values.

The electrodes shall be checked and maintained in accordance with the manufacturer's instructions. It is necessary, in particular, to monitor regularly:

— the condition of the electrodes with respect to ageing and soiling;

— the response time and stability.

Prior to each use, check that the measuring bulb of the electrodes is completely immersed in distilled water or any other liquid, as recommended by the manufacturer; otherwise, immerse it 24 h prior to conducting any measurements.

Clean the electrodes after each use. In order to take into account the soiling and ageing of the electrodes, regularly clean them more thoroughly in accordance with the manufacturer's instructions.

Store the electrodes in accordance with the manufacturer's instructions.

4.5 Autoclave

4.5.1 *Description*

An autoclave is an appliance which enables a saturated steam temperature of at least 121 °C to be attained with a view to the destruction of microorganisms.

4.5.2 *Use*

During the same sterilization cycle, the autoclave shall not be used to sterilize clean equipment (and/or culture media) and also to decontaminate used equipment (and/or used culture media). It is preferable to use separate autoclaves for these two processes.

The autoclave shall be equipped with:

— at least one safety valve;

— a pressure gauge;

— a drain cock;

— a regulation device allowing the temperature to be maintained to within ±1 °C of the scheduled value;

— a thermometer or a recording thermocouple.

It should preferably also be equipped with a duration indicator or a programmer/timer.

With steam sterilization all air must be expelled prior to the pressure build-up. If the autoclave is not fitted with an automatic evacuation device, it is necessary to remove the air until a continuous jet of steam is emitted.

4.5.3 *Maintenance and inspection*

The autoclave shall be kept in perfect operating condition and shall be regularly inspected by the competent departments in accordance with the manufacturer's instructions.

All the monitoring instruments shall be kept in perfect working order and shall be verified regularly.

Descaling, if necessary, and draining operations shall be carried out regularly.

4.6 Incubator

4.6.1 *Description*

An incubator consists of a chamber which enables a temperature to be kept stable and evenly distributed to within ±1 °C, unless otherwise stated.

4.6.2 *Use*

Incubators shall be equipped with a regulation system which allows the temperature to be kept even and stable over their entire working volume.

If the ambient temperature is close to or higher than that of the incubator, it is necessary to fit a cooling system to the chamber.

Incubators walls should be protected from direct sunlight.

If possible, incubators should not be completely filled in one single operation because the culture media will take a long time to equilibrate to temperature, whatever type of incubator is used (forced-air convection or otherwise).

When loading incubators, attention should be paid to air circulation; under no circumstances shall Petri dishes or tubes be placed within 25 mm of the inside walls of the incubator. Stacks shall not be of more than six Petri dishes and shall be separated by at least 25 mm.

4.6.3 *Maintenance and inspection*

The homogeneous temperature within the working volume shall be checked using several thermometers or thermocouples.

The measurement accuracy should be four times better than the requested accuracy (e.g. for a requested accuracy of ±2 °C, the measurement accuracy should be ±0,5 °C).

The temperature stability shall be checked, for example, with one or more maximum and minimum thermometers.

The incubator temperature shall be checked at least every working day. For this purpose, each incubator shall incorporate at least one thermometer, whose bulb is immersed in glycerol contained in a sealed bottle. Other checking systems of equivalent performance can be used.

The inner and outer walls of the incubator shall be regularly cleaned and disinfected and, if appropriate, dust shall be removed from the ventilation system.

4.7 Refrigerator or cold-storage room

4.7.1 *Description*

These are chambers which allow cold storage to be guaranteed. The temperature, unless otherwise specified, shall be +3 °C ± 2 °C except for the conservation of analysis samples where the temperature shall be +2 °C ± 2 °C.

4.7.2 *Use*

Different chambers shall be available for the storage of:

— non-inoculated culture media and reagents;

— samples for analysis;

— microorganism strains and incubated media.

The refrigerators and cold-storage rooms shall be loaded in such a way that appropriate air circulation is maintained.

4.7.3 *Maintenance and inspection*

The temperature of each chamber shall be checked each working day using a thermometer or a permanently installed probe. (See **4.6.3** for the accuracy of the apparatus).

The following maintenance operations shall be carried out regularly:

— removal of dust from the blades or from the external heat-exchange plates;

— defrosting;

— cleaning and disinfection of the inside of the chambers.

4.8 Freezer

4.8.1 *Description*

A freezer has chambers which allow frozen storage to be guaranteed. The temperature, unless otherwise specified, shall be below –18 °C, preferably equal to –24 °C ± 2 °C.

4.8.2 *Use*

Different chambers shall be available for the storage of:

— some non-inoculated culture media and reagents;

— samples for analysis;

— microorganism strains.

The freezer shall be loaded in such a way that a sufficiently low temperature is maintained, in particular when unfrozen products are introduced.

4.8.3 *Maintenance and inspection*

The temperature of each chamber shall be checked each working day using a thermometer or a permanently installed probe. (See **4.6.3** for the accuracy of the apparatus.)

The following maintenance operations shall be carried out regularly:

— removal of dust from the blades and from the external heat-exchange plates;

— defrosting;

— cleaning and disinfection of the inside of the chambers.

4.9 Thermostatically controlled bath

4.9.1 *Description*

This is a bath which allows a specified temperature to be maintained. Unless otherwise stated, the accuracy shall be ±0,5 °C. The working temperatures are stipulated in each method of application.

4.9.2 *Use*

The main uses are the following:

— maintenance of sterile molten agar media at 47 °C ± 2 °C;

— incubation of inoculated culture media at a constant temperature;

— preparation of initial solutions at a controlled temperature (e.g. preparation of the initial suspensions of caseinates requires the latter to be maintained for 15 min in the bath at 37 °C);

— heat treatment of initial suspensions at a controlled temperature (e.g. counting of spores can require the destruction of vegetative cells).

For precise temperature control, the thermostatically controlled bath shall be equipped with a circulating water pump and an automatic heating-regulation system. The agitation of the liquid shall not cause dispersal of droplets.

4.9.3 *Maintenance and inspection*

Each bath shall be equipped with a thermometer or a thermocouple independent of the automatic regulation system.

The bath temperature shall be checked each time it is used, preferably daily.

The level of the liquid in the bath (water, ethylene glycol, etc.) shall be checked regularly.

In order to avoid any microbial proliferation, the liquid shall be changed frequently.

4.10 Sterilizing oven

4.10.1 *Description*

A sterilizing oven is a chamber which allows a temperature of 170 °C to 180 °C to be attained for the destruction of microorganisms by dry heat.

4.10.2 *Use*

Only metal or glass equipment is sterilized in the sterilizing oven. When the correct temperature is reached, the sterilizing operation shall last at least 1 h.

WARNING — Volumetric glassware shall not be sterilized in a sterilizing oven.

The temperature shall be evenly distributed within the chamber.

The oven shall be equipped with:

— a thermostat;

— a thermometer or a recording thermocouple.

It should preferably also be equipped with a duration indicator or a programmer/timer.

4.10.3 *Maintenance and inspection*

Check that the temperature is homogeneous over the working volume.

The oven shall be kept in perfect operating condition and the monitoring instruments shall be verified regularly.

Regular cleaning is recommended.

4.11 Microwave oven

4.11.1 *Description*

This is an apparatus which allows a product to be heated using microwaves.

4.11.2 *Use*

In the present state of the art, only one use is possible: melting of the agar culture media.

The apparatus used operates at atmospheric pressure. It shall be able to heat the culture media in a controlled manner via a microwave emission cycle. The distribution of the microwaves shall be homogeneous within the product so as to avoid zones of overheating. For a better heat distribution, apparatus equipped with a turntable should be used.

WARNING — Handle with care! Heating up agar culture media in a microwave oven can cause delay in boiling.

NOTE In the absence of sufficient assessment of the efficiency of microwaves for sterilizing culture media, this use cannot be yet included in this International Standard.

4.12 Optical microscope

4.12.1 *Use*

An optical microscope contains objectives with different magnifications. Using a high-magnification objective with oil immersion allows observation of the morphology of the micro-organism in its aqueous suspension or after staining. The microscope should preferably be fitted with a phase-contrast objective and substage condenser to improve the examination of live cultures.

NOTE Instruments (plate microscopes) with low-power magnification and ideally stereoscopic focusing are available for the examination of colonies of bacteria on or in agar culture media.

4.12.2 *Maintenance and inspection*

After use involving immersion, the lens shall be cleaned, in such a way as not to affect the optical quality, in order to remove the product used for immersion.

At least once a year, an authorized member of the personnel shall carry out a general cleaning operation, checking the mechanism and optics.

4.13 Gas burner or wire incinerator

A gas burner is used for creating and maintaining a protection zone around a hot spot. It is used for sterilizing metal wires and loops, by bringing them up to red heat.

The wire incinerator is preferable when sterilizing metal wires or loops after manipulating pathogenic bacteria.

4.14 Dispenser for culture media and reagents

4.14.1 *Description*

This is an instrument or apparatus used to distribute culture media and reagents into tubes, bottles or Petri dishes (e.g. measuring cylinder, manual syringe, automatic syringe, peristaltic pump or machine with automated delivery).

4.14.2 *Use*

In the case of the aseptic distribution of sterile culture media and reagents, all parts of the instrument or apparatus in contact with the product to be dispensed shall be sterile (see **6.2**).

The accuracy of the instrument or apparatus shall be appropriate to the accuracy of the volume to be dispensed. In particular, the accuracy required for the volumes of dilution fluid used for conducting the decimal dilutions is ±2 %.

4.14.3 *Maintenance and inspection*

These instruments shall be kept in perfect condition in accordance with the manufacturer's instructions.

The volumes delivered shall be monitored regularly.

4.15 Mechanical stirrer

This allows the homogeneous mixing of diverse liquid media (e.g. decimal dilutions and samples of liquid for testing) or a suspension of bacterial cells in a liquid.

Its principle is based on an eccentric rotational movement of the contents of test tubes (vortex).

4.16 Colony-counting device

This should preferably be equipped with a lighting system with a dark background, a magnifying glass having a magnification of at least ×1,5, and a mechanical or electronic digital counter. Any other automated counting systems of equivalent efficiency may be used (for example a laser counter).

4.17 Equipment for culture in a modified atmosphere

4.17.1 *Description*

This is a jar that can be hermetically sealed or any other appropriate apparatus which enables modified atmospheric conditions (e.g. anaerobiosis) to be maintained for the total incubation time of the culture medium. Other systems of equivalent performance, for example anaerobic chambers, may be used. Follow the manufacturer's instructions for installation and maintenance.

4.17.2 *Use*

The composition of the atmosphere obtained by a gas mixture (e.g. from a gas cylinder) or by any other appropriate means (such as commercially available gas packs) will be stipulated in the specific International Standard.

4.17.3 *Maintenance and inspection*

An indicator for monitoring the nature of the atmosphere shall be placed in each chamber during each use.

If a catalyst is fitted, this shall be regularly regenerated according to the manufacturer's instructions.

This equipment shall be regularly cleaned and disinfected.

4.18 Other equipment

Other equipment and apparatus are in everyday use, including the following: filtration apparatus, glass or plastic containers (test tubes, flasks, bottles), glass or plastic Petri dishes, (most commonly between 90 mm and 100 mm in diameter), glass or plastic pipettes (10 ml, 2 ml, 1 ml), sampling instruments, wires and loops (of nickel/chromium, platinum/iridium or disposable plastic, etc.).

5 Personnel

5.1 Competence

All personnel working in a microbiology laboratory shall have received adequate training to enable them to conduct properly the operations entrusted to them.

The personnel in charge of performing the tests shall have a good knowledge of and sufficient practical experience with microbiological techniques and the microorganisms sought. They shall be able to interpret the accuracy and precision required to yield acceptable results. For that purpose, they can, for example, take part in ring tests, use reference materials, or achieve self-assessment tests for enumeration of microorganisms (see, in particular, relevant IDF publications).

All personnel shall receive relevant updated information, as necessary, in hygiene and laboratory safety matters.

5.2 Hygiene

In the field of personal hygiene, the following precautions shall be taken in order to avoid contaminating the samples and culture media, but also in order to avoid risk of infection of personnel:

— wear laboratory clothing that is light-coloured, clean and in good condition, manufactured from a fabric which limits the risks of flammability; this clothing shall not be worn outside the work areas and, possibly, cloakrooms;

— wear protection for the hair and beard, if necessary;

— keep nails perfectly clean and well-groomed, and preferably short;

— wash hands thoroughly in lukewarm water, preferably delivered by a non-manual commanding tap, using liquid or powder soap or, possibly, disinfectant, delivered preferably by a dispenser maintained in a correct state of cleanliness, before and after microbiological examinations and immediately after visiting the toilets; for drying hands, use single-use paper or single-use cloth towels;

— when inoculating, avoid speaking, coughing, etc.;

— do not smoke, drink or eat in the test areas;

— special precautions shall be taken by persons having infections (whitlow) or illnesses where germs are likely to contaminate samples and may invalidate results;

— do not put food for personal consumption in the laboratory refrigerators.

6 Preparation of the equipment

6.1 Preparation

The equipment used in microbiology shall be prepared in such a manner as to guarantee its cleanliness and/or sterility up until the time of use.

The equipment used shall be washed prior to use, even if new.

Stopper tubes and cap bottles prior to sterilization by appropriate means (combed cotton, metal, plastic stoppers, etc.).

Stopper the pipettes with combed cotton or any other appropriate material.

If necessary, the equipment to be sterilized should be placed in special containers or wrapped in an appropriate material (special paper, aluminium, etc.). Equipment to be autoclaved empty (i.e. without media or other aqueous solutions) should allow free access of steam, otherwise sterilization will not be achieved.

6.2 Sterilization

6.2.1 ***Sterilization by dry heat***

Heat in a sterilizing oven (**4.10**) for at least 1 h at 170 °C to 180 °C.

6.2.2 ***Sterilization by moist heat***

Heat for at least 15 min at a minimum of 121 °C in an autoclave (**4.5**), preferably equipped with a vacuum drying device. Temperature indicators can be used in order to make certain that the temperature has been achieved (e.g. special papers).

6.3 Disposable apparatus

Disposable apparatus may be used in the same way as the re-usable glassware (Petri dishes, pipettes, bottles, tubes, etc.) if the specifications are similar.

It is then advisable to make certain, by asking the manufacturer, that the proposed equipment is indeed suitable for use in microbiology (in particular sterility) and that the material contains no substances that inhibit the growth of microorganisms.

Disposable apparatus shall be decontaminated prior to its disposal. Besides the methods described in **6.6**, incineration may be used. If there is an incinerator on the premises, decontamination and disposal may be achieved in a single operation.

6.4 Management of clean equipment

Clean equipment shall be protected against dust during storage, under conditions which maintain its cleanliness.

6.5 Management of sterile equipment

Prior to use, the equipment shall be stored under conditions which allow it to remain sterile. Single-use equipment shall be stored in accordance to the manufacturer's specifications, without any deterioration of the packaging; laboratory-prepared equipment shall be stored in clean containers.

When sterilizing equipment intended for microbiology, an expiry date (or a manufacturing date) shall be put on each packaging. Hermetically sealed equipment can be stored for up to 3 months prior to use. Packaged equipment that is not hermetically sealed shall be stored for a shorter period of time (e.g. 8 days).

6.6 Decontamination

After use (culture of microorganisms or contact with microorganisms), the equipment and its contents shall be decontaminated, prior to cleaning or disposal, whatever the microorganism involved. For example, the cotton wool plugs shall only be removed after pipettes have been decontaminated; tubes containing liquid media shall be decontaminated prior to being washed. Decontamination can be performed according to one of the following two techniques:

— sterilization in moist heat in an autoclave (**4.5**) for at least 30 min at a minimum of 121 °C of all equipment which has been in contact with microorganism cultures (incubated, solid or liquid culture media, contaminated reagents, equipment, etc.);

— decontamination by immersion in a disinfectant solution for small-sized, corrosion-resistant equipment (pipettes, for example).

Pasteur pipettes shall only be used once.

Plastic equipment may be directly incinerated, if permitted by the laws of the country.

6.7 Washing

Wash equipment only after it has been decontaminated.

Empty the containers of their contents.

Prior to washing, separate seals from stoppers or caps, if appropriate.

Carefully wash the stoppers or caps and the glassware in hot water with a commercial detergent solution. In the absence of any commercial product, a sodium carbonate solution at 0,125 % (*m*/*m*) can be used, followed by immersion in dilute acid (e.g. hydrochloric acid (HCl) at ρ = 0,1 mol/l).

Rinse all equipment in distilled water or water of equivalent quality.

Specialized equipment may be used in order to facilitate cleaning operations (e.g. pipette washers, dishwasher, ultrasonic troughs, etc.).

7 Preparation and sterilization of culture media and reagents

The accurate preparation of culture media is one of the fundamental steps in microbiological analysis and it shall be given special care.

7.1 Distilled water

The water used shall be distilled water or water of equivalent quality; i.e. free from substances likely to inhibit or to influence the growth of microorganisms under the test conditions. If the distilled water is prepared from chlorinated water, neutralize the chlorine prior to the distillation.

The distilled water shall be stored in containers manufactured from inert materials (e.g. neutral glass, polyethylene, etc.) which shall be shown to be free of any inhibitory substances prior to use.

NOTE In some cases, it is necessary to use freshly prepared water, without any dissolved carbon dioxide.

In order to be considered as being of good quality, the distilled water shall possess a resistivity of at least 300 000 Ωcm.

NOTE Due to passing through an ion exchanger, demineralized water often has a high microorganism content; therefore, it is advisable not to use this process without verifying that the microorganism content of the water is low. Consult the manufacturer for the best way to minimize microbial contamination. After filter sterilization, deionized water heavily contaminated with microbes may still contain substances inhibitory to the growth of some microorganisms.

7.2 Preparation of culture media

Two types of preparation exist:

— from basic ingredients, dehydrated or not; or

— from dehydrated complete media.

The bottles containing the dehydrated basic ingredients or the dehydrated complete media shall be kept, protected against light, in a dry place at the temperature stated by the manufacturer.

Do not use beyond the stated shelf-life.

As the dehydrated components and media are hygroscopic, it is essential to close the bottles quickly and carefully after sampling. A dehydrated medium which shows signs of caking or solidifying, indicative of water uptake, shall not be used.

7.2.1 *Rehydration*

Follow the recommendations of the International Standard in question and/or those of the manufacturer.

7.2.2 *Measurement of pH*

Measure the pH using a pH-meter (**4.4**) and adjust it, if necessary, so that after sterilization and cooling at 25 °C the medium is at the required pH ± 0,2 pH units, unless otherwise stated.

The adjustment is normally carried out using a solution of approximately 40 g/l (about 1 mol/l) of sodium hydroxide (NaOH) or approximately 36,5 g/l (about 1 mol/l) of hydrochloric acid (HCl).

7.2.3 *Dispensing*

Dispense the medium into appropriate containers, either manually or using an automatic apparatus (**4.14**).

Use a container having a volume one, two or three times the volume to be dispensed in order to prevent the medium from boiling over during autoclaving.

7.3 Sterilization

The sterilization of culture media and of reagents can be carried out using various techniques, including:

— sterilization by moist heat;

— sterilization by filtration.

However, certain media and reagents can be used without any special sterilization procedures (refer to the International Standard and/or the manufacturer's specifications).

After sterilization, the media shall be monitored, in particular with respect to pH, colour, sterility and bacteriological performance.

7.3.1 *Sterilization by moist heat*

This is performed in an independent autoclave (**4.5**) or in a medium preparator and dispenser. Generally, the autoclaving operation takes 15 min at 121 °C (245 kPa). For volumes greater than 1 litre, adapt the sterilization cycle as necessary. In all cases, follow the instructions given in the International Standard and/or those of the manufacturer.

Prior to autoclaving, a representative number of containers, in each part of the autoclave, shall be equipped with a test strip paper (commercially available) which indicates that the desired temperature has been attained.

7.3.2 *Sterilization by filtration*

This can be performed under vacuum or pressurized conditions.

Use membranes and filter elements with a pore diameter of 0,22 µm. They shall have been sterilized in the autoclave (**4.5**) prior to use. Refer to the manufacturer's instructions regarding the use of filter elements or membranes which have been purchased in a sterile condition.

Sterilize the different components of the filtration apparatus, assembled or not, in the autoclave (**4.5**) for 15 min at 121 °C. If necessary, aseptic assembly can be performed in a safety cabinet after autoclaving. Certain assembled appliances can be purchased in a sterile condition.

7.4 Storage

Each package of bottles, tubes and Petri dishes shall be labelled and shall bear the following details:

— name of the medium;

— date of preparation and/or expiry date.

7.4.1 *Laboratory-prepared culture media and reagents*

Culture media dispensed in tubes or bottles and reagents which are not used immediately shall be protected against light and desiccation (e.g. using rubber caps during storage or screw-on lids).

Unless specified in the International Standard in question, they shall be kept in the refrigerator (**4.7**) for a maximum period of 3 months, or between 18 °C and 23 °C for a maximum period of 1 month under conditions which prevent their composition from being modified.

Never use media which have become dehydrated.

Prior to use, it is desirable that the temperature of the culture media be equilibrated to that of the laboratory.

7.4.2 *Ready-to-use culture media and reagents*

It is necessary to comply with the manufacturer's instructions: expiry date, storage temperature and conditions, conditions for use (pH, etc.) and efficiency control.

7.5 Melting of agar culture media

Melt a culture medium by placing it in a boiling water bath or by any other process which gives identical results (e.g. a steam flow-through autoclave or microwave oven). Avoid over-heating and remove it as soon as it has melted. Keep the culture medium in a molten state in a thermostatically controlled bath at 47 °C ± 2 °C (**4.9**) until such time as it is to be used. Never use culture medium at a temperature higher than 50 °C. It is preferable not to keep a molten medium more than 8 h. No unused medium shall be resolidified for subsequent use.

In the case of particularly sensitive culture media, this melting duration shall be shortened, and this will be specified in the relevant International Standard.

7.6 De-aeration of culture media

If necessary, just prior to use, heat the culture media in boiling water or under a flow of steam for 15 min, with lids or caps loose; after heating, tighten the caps and cool down rapidly to the operating temperature.

7.7 Preparation of Petri dishes

Pour the molten agar culture medium into Petri dishes so as to obtain a thickness of at least 2 mm (e.g. for 90 mm diameter dishes, 12 ml of agar are normally required). Allow the agar to cool and solidify by placing the Petri dishes on a cool, horizontal surface.

Use the thus-prepared Petri dishes immediately or store them under conditions which prevent their composition from being modified, in the dark, in the refrigerator (**4.7**) for a maximum period of 1 week. This duration can be, in some cases, longer or shorter; it will be specified in the relevant standard. Label the dishes as described in **7.4**.

Use them after drying.

In general, for the surface inoculation of an agar culture medium, dry the dishes, preferably with the lids removed and with the agar surfaces facing downwards, in an oven set at a temperature between 25 °C and 50 °C, until the droplets have disappeared from the surface of the medium. Do not dry them any further. The agar plates can also be dried in a laminar-flow safety cabinet for 30 min with half-open lids, or overnight with the lids in place.

Ready-prepared agar plates are available commercially. Store and use them according to the manufacturer's instructions.

8 Laboratory samples

8.1 Sampling

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

Sampling is not part of these general rules for microbiological examinations. See the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

8.2 Transport

The transportation of the samples to the laboratory shall ensure that they are kept under conditions which prevent any alteration in the number of microorganisms present. Preference should be given to those means of transport which are the fastest.

Particular attention shall be paid to observing the following storage temperatures for the following products:

— stable products: ambient temperature;
— fresh and refrigerated products: between 0 °C and +4 °C;
— frozen or deep-frozen products: below –18 °C;
— pasteurized and similar products: between 0 °C and +4 °C;
— spoiled stable units: between 0 °C and +4 °C.

WARNING — Sensitive foodstuffs (e.g. innards, fresh fish) shall be stored at a temperature of between 0 °C and +2 °C.

Spoiled stable units shall be transported in closed packaging for protection against possible leakage.

8.3 Receipt and storage

The laboratory personnel shall check the condition of the samples on receipt. If their condition is unsatisfactory or if the samples are insufficient, the laboratory shall generally refuse the samples. In special circumstances the personnel can analyse them but shall report reservations about the validity of the results.

The samples admitted into the laboratory shall be documented in such a manner that their progress through to the time of drafting the test report can be monitored.

The following information shall be noted:

— date of receipt;

— characteristics of the sampling operation (sampling date, sampling conditions, etc.);

— requesting party's name and address;

— characteristics of the product.

Samples awaiting examination shall be stored under conditions which prevent any alteration in the number of microorganisms present.

Particular attention shall be paid to the storage temperature (see **8.2**) and to the examination deadlines for the following products:

— stable products: as early as possible and before the storage limit date;

— fresh and refrigerated products: within 24 h after receipt (if a longer storage period cannot be avoided, freeze the sample as soon as possible at a temperature below –18 °C and mention this in the test report because, in certain products, freezing modifies the composition of the flora);

— pasteurized or similar products: as early as possible and before the storage limit date;

— spoiled stable units: as soon as possible and less than 48 h.

8.4 Test portions

In order to avoid contamination of the environment and of the test portion, it is recommended to work in special premises or in a safety cabinet. Failing this, those products known to contain very few microorganisms (e.g. pasteurized products, cooked dishes) shall always be examined first, followed by one known to be more highly contaminated.

The protection of the environment against contamination is of particular importance during the weighing and sampling of the test portion of highly contaminated powdered products. These procedures shall be carried out in a safety cabinet.

Handle the samples in such a manner as to avoid any and all risk of contamination. To achieve this, take the following precautions:

— when not working in a safety cabinet, work in the vicinity of a flame;

— for a packaged product, clean the outside of the packing with 70 % ethanol where it will be opened; flame if possible;

— any instrument which is used for opening the packaging (tin-opener, scissors, etc.) shall be sterile;

— any instrument which is used for taking the sample (spoon, forceps, pipette, etc.) shall be sterile;

— carefully mark the reference of the laboratory sample on the containers, plastic bags, etc. (see **8.3**) containing the test sample.

8.5 Conservation and destruction of laboratory samples

Except for special cases, keep the laboratory samples until all results have been obtained, or longer if necessary, and pack them in sterile containers (e.g. plastic bags) and restore them to the storage temperature of the sample. Refrigerated fresh products should be frozen.

Before discarding, decontaminate any deteriorated or dangerous laboratory samples.

Discard undeteriorated laboratory samples directly.

9 Examination techniques and expression of results

9.1 Hygienic precautions during the examination

Precautions shall be taken in order to conduct the work as far as possible under aseptic conditions, for example:

— make sure that the work area is clean and that there are no draughts (doors and windows closed);

— before and after the work, decontaminate the work surface with an appropriate disinfectant;

— make sure, prior to starting, that everything required for carrying out the work is available;

— in the case of work conducted in a safety cabinet, use sterile gloves or decontaminate hands prior to starting work (e.g. with a mixture of polyalcohols) and avoid crossing forearms and hands;

— when not working in a safety cabinet, open the test tubes and bottles in the vicinity of a flame, holding them in the most inclined position possible;

— carry out the work as quickly as possible without making any unnecessary movements;

— if the total contents of a bag of disposable pipettes, Petri dishes, etc. is not used during the course of an examination, make sure that the container is properly closed after taking the appropriate number of units;

— sterilize loops and inoculation wires, etc. before and after use with a flame; in order to avoid splatter of substances and microorganisms, preferably use a wire incinerator (**4.13**); whenever possible, use single-use sterile loops and wires;

— place used pipettes, spatulas, etc. in specific receptacles containing an appropriate disinfectant (e.g. a sodium hypochlorite solution for the pipettes) prior to decontamination (**6.6**);

— place the Petri dishes, culture media and all other equipment which may contain microorganisms into specific containers prior to decontamination, which precedes the washing operation;

— place disposable apparatus in appropriate containers prior to decontamination or incineration (**6.6**);

— immediately mop up any contaminated or other spillage by means of cotton pads or any other appropriate material impregnated with 70 % (*V*/*V*) ethanol or any other disinfectant, then clean and disinfect the work surface prior to continuing.

The manipulation of products likely to contain pathogenic bacteria (*Salmonella, Listeria monocytogenes*, etc.) or toxins requires special precautions, which are described in specialized publications. The following are recommended:

— a safety cabinet for all manipulations required for conducting the analysis;

— a mechanical suction device; pipetting by mouth suction is prohibited.

Aerosols are a major cause of environmental contamination and of infection. Aerosols can be formed for example:

— when opening Petri dishes, tubes and bottles;

— when using shakers, syringes, centrifuges, etc.;

— when emptying pipettes by blowing;

— when sterilizing wet inoculation loops or needles;

— when opening ampoules containing freeze-dried cultures.

It is therefore necessary to avoid their formation.

9.2 Preparation of the initial suspension and dilutions

See ISO 6887.

In the case of the preparation of an initial suspension, the time which elapses between the end of the preparation and the moment the inoculum comes into contact with the culture medium shall not exceed 45 min, unless specifically mentioned in the relevant International Standard.

9.3 Counting using a solid medium

9.3.1 *Inoculation for poured plates*

Prepare the medium (by surfusion on a water bath at 47 °C), the Petri dishes, the dilution fluid and the dilutions to be examined (in accordance with **9.2**) in quantities and numbers corresponding to the inoculation plan specified in the relevant International Standard.

Dispense into the Petri dishes (labelled) the defined volumes of the dilutions to be examined. Pour into each dish the volume of medium specified in **7.7**. Immediately mix the molten medium and the inoculum carefully so as to obtain a homogeneous distribution of the microorganisms within the mass of the medium. Allow to cool and solidify by placing the Petri dishes on a cool horizontal surface (the solidification time of the agar shall not exceed 10 min).

If the presence of spreading colonies (e.g. *Proteus* spp.) in the product to be examined is expected, overlay the solidified plates with sterile non-nutritive agar or agar identical to the culture medium used in the analysis.

9.3.2 *Surface inoculation*

Deposit the inoculum in the centre of the labelled Petri dish onto the agar culture medium (prepared in accordance with **7.7**). Spread it uniformly and as quickly as possible on the surface of the medium using a sterile glass or plastic spreader until there is no longer any liquid visible on the agar surface.

Glass balls may be used to assist in this spreading.

In certain cases (stated in the relevant International Standard), the inoculum may be deposited on a membrane then spread as previously described.

9.3.3 *Incubation*

Unless otherwise stated, immediately invert the inoculated dishes and place them quickly in the incubator set at the appropriate temperature. If excessive dehydration occurs (e.g. at 55 °C or in the event of strong air circulation), wrap the dishes loosely in plastic bags prior to incubation or use any similar system of equivalent efficiency. During the incubation period, variations in the incubation temperature may be acceptable, for example during the usual operations of loading or unloading the incubator.

NOTE In certain cases, it may be useful to make provision for duplicate inoculated dishes which will be stored at +2 °C for comparison with incubated inoculated dishes when counting, in order to avoid confusing particles of the product being examined with colonies.

After incubation, the dishes shall, if possible, be examined immediately. Otherwise, they may be stored, unless otherwise specified, for up to a maximum of 24 h in the refrigerator (**4.7**).

9.3.4 *Counting of colonies*

Following the period of incubation stated in the specific standard, count the colonies (total colonies, typical colonies or presumed suspect colonies) for each dish containing less than 300 colonies (or any other number stated in the specific standard).

The different methods of calculation defined in **9.3.5** shall take account of dishes containing 0 colonies if these dishes have been retained.

When counting typical or presumed suspect colonies, the maximum number of all typical or atypical colonies present on a dish shall not exceed 300 (or any other number stated in the specific standard).

NOTE 1 In certain cases, it may be difficult to count the colonies (for example where spreading microorganisms are present). These cases are dealt with in the specific standards.

NOTE 2 When counting typical or presumed suspect colonies, the description of the colonies will possibly be given in the specific standard.

9.3.5 ***Expression of results***

9.3.5.1 *General*

9.3.5.1.1 In this subclause, the cases dealt with correspond to the following general cases:

— inoculation of two Petri dishes, 90 mm in diameter, per dilution;

— maximum number for the counting of total colonies is 300 per dish;

— maximum number of all typical and atypical colonies present on a dish when counting typical or presumed suspect colonies is 300 per dish;

— maximum number for the counting of typical or presumed suspect colonies is 150 per dish;

— number of presumed suspect colonies (**9.3.5.3**) inoculated for identification or confirmation, from each dish retained is 5;

— minimum number of colonies [total colonies, typical colonies or colonies complying with identification or confirmation criteria (**9.3.5.3**)] on at least one dish is 15.

These figures will be defined in the specific standards.

When dishes with a diameter different from 90 mm are used, the maximum number of colonies shall be increased proportionately to the surface area of the dishes.

9.3.5.1.2 The methods of calculation defined below take account of the cases which occur most frequently when tests are carried out in accordance with good laboratory practice. Rare special cases may occur (for example, significant discrepancy between the number of colonies in two dishes with the same dilution, or a very different ratio to that of the dilution factor between the dishes of two successive dilutions) and it is therefore necessary that results obtained from counting be examined, interpreted or possibly refused by a qualified microbiologist.

9.3.5.2 *Method of calculation: General case (counting of total colonies or typical colonies)*

For a result to be valid, it is generally considered necessary to count the colonies on at least one dish containing a minimum of 15 colonies [total colonies, typical colonies or colonies complying with identification or confirmation criteria (**9.3.5.3**)].

Calculate the number N of microorganisms present in the test sample as a weighted mean from two successive dilutions using the following equation:

$$N = \frac{\sum C}{V \times [n_1 + (0{,}1 \times n_2)] \times d} \qquad (1)$$

where

$\sum C$ is the sum of the colonies counted on all the dishes retained from two successive dilutions, and where at least one contains a minimum of 15 colonies;

V is the volume of inoculum applied to each dish, in millilitres;

n_1 is the number of dishes retained at the first dilution;

n_2 is the number of dishes retained at the second dilution;

d is the dilution factor corresponding to the first dilution retained [$d = 1$ when the undiluted liquid product (test sample) is used].

Round off the results calculated to two significant figures. In order to do this, if the third figure is less than 5 do not modify the preceding figure; if the third figure is greater than or equal to 5, increase the preceding figure by one unit.

Take as the result a number preferably between 1,0 and 9,9 multiplied by the appropriate power of 10, or a whole number with two significant figures.

Express the result as follows:

— number N of microorganisms per millilitre (liquid products) or per gram (other products).

EXAMPLE

Counting has produced the following results:

— at the first dilution (10^{-2}) retained: 168 and 215 colonies;

— at the second dilution (10^{-3}) retained: 14 and 25 colonies.

$$N = \frac{\sum C}{V \times [n_1 + (0{,}1 \times n_2)] \times d} = \frac{168 + 215 + 14 + 25}{1 \times [2 + (0{,}1 \times 2)] \times 10^{-2}} = \frac{422}{0{,}022} = 19\ 182$$

By rounding off the result as recommended above, the number of microorganisms is 19 000 or $1{,}9 \times 10^4$ per millilitre or per gram of product.

9.3.5.3 *Method of calculation: Case after identification or confirmation*

When the method used requires identification or confirmation, a given number, A (generally 5), of presumed suspect colonies is inoculated from each of the dishes retained for the counting of colonies. After identification or confirmation, calculate, for each of the dishes, the number, a, of colonies complying with identification or confirmation criteria, using the following equation:

$$a = \frac{b}{A} \times C \qquad (2)$$

where

b is the number of colonies complying with identification or confirmation criteria among the inoculated colonies, A;

C is the total number of presumed suspect colonies counted on the dish.

Round off the results calculated to the nearest whole number. In order to do this, if the first figure after the decimal point is less than 5, do not modify the preceding figure; if the first figure after the decimal point is greater than or equal to 5, increase the preceding figure by one unit.

Calculate the number N, N_E or N' of identified or confirmed microorganisms present in the test sample, by replacing ΣC by Σa using the formulae provided in **9.3.5.2**, **9.3.5.4.1** and **9.3.5.5.3**, respectively.

Round off the result as recommended in **9.3.5.2**.

Express the result as recommended in **9.3.5.2**, **9.3.5.4.1** and **9.3.5.5.3**, respectively.

EXAMPLE

Counting has produced the following results:

— at the first dilution (10^{-3}) retained: 66 and 80 colonies;

— at the second dilution (10^{-4}) retained: 4 and 7 colonies.

Testing of selected colonies was carried out:

— for 66 colonies, 8 colonies, 6 of which complied with the criteria; hence $a = 50$;

— for 80 colonies, 9 colonies, 6 of which complied with the criteria; hence $a = 53$;

— for 7 colonies, 5 colonies, 4 of which complied with the criteria; hence $a = 6$;

— for 4 colonies, all 4 of which have complied with the criteria; hence $a = 4$.

$$N = \frac{\sum a}{V \times [n_1 + (0{,}1 \times n_2)] \times d} = \frac{50 + 53 + 6 + 4}{1 \times [2 + (0{,}1 \times 2)] \times 10^{-3}} = \frac{113}{0{,}002\ 2} = 51\ 364$$

By rounding off the result as recommended in **9.3.5.2**, the number of microorganisms is 51 000 or $5{,}1 \times 10^4$ per millilitre or per gram of product.

9.3.5.4 *Method of calculation: Estimated counts*

9.3.5.4.1 *Case of two dishes (test sample or initial suspension or first dilution) containing less than 15 colonies*

If the two dishes from the test sample (liquid products), or from the initial suspension (other products), or from the first dilution inoculated or retained, contain less than 15 colonies (total colonies, typical colonies or colonies complying with identification or confirmation criteria), calculate the estimated number N_E of microorganisms present in the test sample as an arithmetical mean of the colonies counted on the two dishes using the following equation.

$$N_E = \frac{\sum C}{V \times n \times d} \qquad (3)$$

where

$\sum C$ is the sum of the colonies counted on the two dishes;

V is the volume of inoculum applied to each dish, in millilitres;

n is the number of dishes retained (in this case, $n = 2$);

d is the dilution factor of the initial suspension or of the first dilution inoculated or retained [$d = 1$ when the undiluted liquid product (test sample) is used].

Round off the result as recommended in **9.3.5.2**.

Express the result as follows:

— estimated number N_E of microorganisms per millilitre (liquid products) or per gram (other products).

EXAMPLE

Counting has produced the following results:

— at the first dilution (10^{-2}) retained, 12 and 13 colonies were counted.

$$N_E = \frac{12 + 13}{1 \times 2 \times 10^{-2}} = \frac{25}{0{,}02} = 1\ 250$$

By rounding off the result as recommended in **9.3.5.2**, the estimated number N_E of microorganisms is 1 300 or $1{,}3 \times 10^3$ per millilitre or per gram of product.

9.3.5.4.2 *Case of two dishes (test sample or initial suspension or first dilution) containing no colonies*

If the two dishes from the test sample (liquid products), or from the initial suspension (other products), or from the first dilution inoculated or retained, do not contain any colonies, express the results as follows:

— less than $1/d$ of microorganisms per millilitre (liquid products) or per gram (other products);

where d is the dilution factor of the initial suspension or of the first dilution inoculated or retained [$d = 1$ when the undiluted liquid product (test sample) is used].

9.3.5.4.3 *Special cases (counting of typical or presumed suspect colonies)*

9.3.5.4.3.1 If the number of all typical and atypical colonies for the two dishes containing a first dilution d_1 is greater than 300 (or any other number stated in the specific standard), with visible typical colonies or confirmed colonies and if, for the two dishes of the subsequent dilution d_2 containing less than 300 colonies (or any other number stated in the specific standard) no typical or confirmed colony can be counted, express the result as follows:

— less than $1/d_2$ and more than $1/d_1$ of microorganisms per millilitre (liquid products) or per gram (other products);

where d_1 and d_2 are the dilution factors corresponding to dilutions d_1 and d_2.

EXAMPLE

Counting has produced the following results:

— at the first dilution (10^{-2}) retained: more than 300 colonies on each of the two dishes, with typical or confirmed colonies present;

— at the second dilution (10^{-3}) retained: 33 and 35 colonies, with no typical or confirmed colonies present.

The result expressed in microorganisms is less than 1 000 and more than 100 per millilitre or per gram of product.

9.3.5.4.3.2 If the number of all typical and atypical colonies for the two dishes containing a first dilution d_1 is greater than 300 (or any other number stated in the specific standard), without visible typical colonies or confirmed colonies and if, for the two dishes of the subsequent dilution d_2 containing less than 300 colonies (or any other number stated in the specific standard) no typical or conformed colony can be counted, express the result as follows:

— less than $1/d_2$ of microorganisms per millilitre (liquid products) or per gram (other products);

where d_2 is the dilution factor corresponding to the dilution d_2.

EXAMPLE

Counting has produced the following results:

— at the first dilution (10^{-2}) retained: more than 300 colonies on each one of the two dishes, with no typical or confirmed colonies present;

— at the second dilution (10^{-3}) retained: 33 and 35 colonies, with no typical or confirmed colonies present.

The result expressed in microorganisms is less than 100 per millilitre or per gram of product.

9.3.5.5 *Method of calculation: Special cases*

9.3.5.5.1 When the number of colonies counted (total colonies, typical colonies or presumed suspect colonies) is greater than 300 (or any other number stated in the specific standard) for the two dishes containing a first dilution d_1, with a number of colonies (total colonies, typical colonies or colonies complying with identification or confirmation criteria) of less than 15 for the two dishes of the subsequent dilution d_2:

— if the number of colonies for each of the two dishes containing dilution d_1 is within the 324 to 300 interval (upper part of the confidence interval for a weighted mean equal to 300), use the calculation method for general cases (**9.3.5.2**);

— if the number of colonies for each of the two dishes containing the dilution d_1 is greater than 324 (upper limit of the confidence interval for a weighted mean equal to 300), only take account of the result of the count of dilution d_2 and then proceed with the estimated count (**9.3.5.4**), except when referring to a maximum number set at 300 for the counting of colonies, if the latter result is less than 10 (lower limit of the confidence interval for a weighted mean equal to 15) since the difference between the two dilutions is then unacceptable.

NOTE The figures corresponding to confidence intervals should be adapted to the maximum number stated for the counting of colonies.

EXAMPLE 1

Counting has produced the following results:

— at the first dilution (10^{-2}) retained: 310 and 322 colonies;

— at the second dilution (10^{-3}) retained: 8 and 12 colonies.

Use the method of calculation for general cases using the dishes from the two dilutions retained.

EXAMPLE 2

Counting has produced the following results:

— at the first dilution (10^{-2}) retained: more than 324 colonies on each one of the dishes;

— at the second dilution (10^{-3}) retained: 12 and 14 colonies.

Begin estimated counting on the basis of the colonies counted on the two dishes from the 10^{-3} dilution.

EXAMPLE 3

Counting (when a maximum number of 300 has been set for the counting of colonies) has produced the following results:

— at the first dilution (10^{-2}) retained: more than 324 colonies on each one of the two dishes;

— at the second dilution (10^{-3}) retained: 8 and 6 colonies.

The result of this counting is unacceptable.

EXAMPLE 4

Counting (when a maximum number of 150 has been set for the counting of colonies) has produced the following results:

— at the first dilution (10^{-2}) retained: more than 167 colonies on each one of the two dishes (upper limit of the confidence interval with a weighted mean equal to 150);

— at the second dilution (10^{-3}) retained: 8 and 6 colonies.

Begin estimated counting on the basis of colonies counted on the two dishes from the 10^{-3} dilution.

9.3.5.5.2 Where the counting of colonies (total colonies or typical colonies or presumed suspect colonies) for each one of the dishes for all inoculated dilutions produces a number greater than 300 (or any other number stated in the specific standard), express the result as follows:

— more than $300/d$ (case of total colonies or typical colonies) or more than $300 \times b/A \times 1/d$ (case of confirmed colonies) microorganisms per millilitre (liquid products) or per gram (other products);

where

d is the dilution factor of the last inoculated dilution;

b is the number of colonies complying with identification or confirmation criteria among the inoculated presumed suspect colonies, A.

9.3.5.5.3 Where only the two dishes containing the last inoculated dilution contain less than 300 (or any other number stated in the specific standard) colonies (total colonies, typical colonies or presumed suspect colonies), calculate the number N' of microorganisms present in the test sample as an arithmetical mean of the colonies counted on the two dishes, using the following equation:

$$N' = \frac{\sum C}{V \times n \times d} \qquad (4)$$

where

$\sum C$ is the sum of colonies counted on the two dishes, and where at least one contains a minimum of 15 colonies;

V is the volume of the inoculum applied to each dish, in millilitres;

n is the number of dishes retained (in this case, $n = 2$);

d is the dilution factor corresponding to the dilution retained.

Round off the result as recommended in **9.3.5.2**.

Express the result as follows:

— number N' of microorganisms per millilitre (liquid products) or per ram (other products).

EXAMPLE

Counting has produced the following results:

— at the last dilution (10^{-4}) inoculated: 120 and 130 colonies.

$$N' = \frac{120 + 130}{1 \times 2 \times 10^{-4}} = \frac{250}{0{,}000\ 2} = 1\ 250\ 000$$

By rounding off the result as recommended in **9.3.5.2** the number N' of microorganisms is 1 300 000 or $1{,}3 \times 10^6$ per millilitre or per gram of product.

9.3.6 ***Confidence limit***

In order to estimate the validity of the result and to avoid too strict an interpretation, it is necessary to determine the confidence interval which characterizes the statistical distribution of the microorganisms within the sample.

Other variations due to the technique itself intervene, particularly those connected with dilution errors, the extent of which varies from one laboratory to another.

9.3.6.1 *General case*

With a probability of 95 %, the confidence interval δ, which characterizes the microbial dispersion within the sample, is calculated using the equation:

$$\delta = \left[\frac{\sum C}{B} + \frac{1{,}92}{B} \pm \frac{1{,}96\sqrt{\sum C}}{B} \right] \frac{1}{d}$$

when

$$B = (n_1 + 0,1 n_2)$$

and where

$\sum C$ is the sum of the colonies counted on all the dishes retained;

n_1 is the number of dishes retained at the first dilution;

n_2 is the number of dishes retained at the second dilution;

V is the volume of inoculum applied to each dish, in millilitres;

d is the dilution factor corresponding to the first dilution retained.

EXAMPLE 1: Extreme cases (see Table 1)

Table 1 — Extreme cases

Number of colonies counted		Weighted mean	Confidence interval
Dilution n	Dilution $n + 1$		
300	30	300	278 to 324
300	30		– 7 % to + 8 %
15	1	14	10 to 20
15	1		– 29 % to + 43 %

EXAMPLE 2: Confidence interval for the example given in

With $N = 1{,}9 \times 10^4$ per gram for 422 colonies counted, the confidence interval δ is

$$\delta = \left[\frac{422}{2{,}2} + \frac{1{,}92}{2{,}2} \pm \frac{1{,}96\sqrt{422}}{2{,}2} \right] \times \frac{1}{10^{-2}}$$

$$\delta = (191{,}82 + 0{,}87 \pm 18{,}30) \times 10^2$$

The limits of the confidence interval are therefore

$$\delta_1 = 1{,}7 \times 10^4 \text{ and } \delta_2 = 2{,}1 \times 10^4$$

In this case, the limits of the confidence interval, expressed as a percentage and calculated on non-rounded data, are between – 9,1 % and + 10,0 % inclusive.

9.3.6.2 *Case after identification*

With a probability of 95 %, the confidence interval δ, which characterizes the microbial dispersion within the sample, is given by the equation stated in **9.3.6.1**, where $\sum C$ is replaced by $\sum a$.

EXAMPLE: For the case given in **9.3.2**, the number N of microorganisms found is $5{,}1 \times 10^4$ per millilitre for 113 colonies retained, therefore

$$\delta = \left[\frac{113}{2{,}2} + \frac{1{,}92}{2{,}2} \pm \frac{1{,}96\sqrt{113}}{2{,}2}\right] \times \frac{1}{10^{-3}}$$

$$\delta = (51{,}36 + 0{,}87 \pm 9{,}47) \times 10^3$$

The limits of the confidence interval are therefore

$$\delta_1 = 4{,}3 \times 10^4 \text{ and } \delta_2 = 6{,}2 \times 10^4$$

In this case, the limits of the confidence interval are between –16,7 % and +20,1 % inclusive.

9.3.6.3 *Estimated counts*

The confidence limits are given in Table A.1 and Table A.2 of Annex A.

9.4 Counting using a liquid medium: Most probable number technique

NOTE Two inoculation systems are possible. The most frequently used systems (called "symmetric" systems) have the same number of tubes for each dilution, the volume ratios between two dilutions being generally 1 to 10. These systems are especially used when it is not only a question of checking that a certain limit is not exceeded, but also of determining the number of microorganisms present. There also exist systems called "asymmetric" systems which comprise different numbers of tubes for different dilutions. Only the "symmetric" systems with decimal dilutions will be mentioned in this International Standard.

9.4.1 *Inoculation*

Depending on the desired accuracy of the results, inoculate an appropriate number of flasks or tubes with the same dilution (e.g. three, five or ten flasks or tubes). As a general rule, the techniques implemented require three flasks or tubes per dilution.

Introduce, by means of a pipette, the inoculum into the corresponding flasks or tubes.

Use a new sterile pipette for each dilution.

9.4.2 *Incubation*

Place the inoculated flasks and/or tubes in an incubator or, preferably, in a thermostatically controlled bath (**4.9**).

9.4.3 *Interpretation of results*

9.4.3.1 *Selection of dilutions*

NOTE The initial suspension and the test sample, if liquid, are considered as being "dilutions".

In the general case where three tubes or flasks per dilution have been inoculated, select, for each sample examined, three consecutive dilutions complying, as appropriate, with **9.4.3.1.1**, **9.4.3.1.2** or **9.4.3.1.3**.

9.4.3.1.1 *Case 1: At least one dilution reveals three positive tubes (see Table 2)*

Select the highest dilution (i.e. the one having the smallest concentration of sample) which reveals three positive tubes, as well as the next two highest dilutions (i.e. those the sample concentrations of which are equal to 1/10 and 1/100 of that of the first dilution selected). See example 1 and **9.4.3.1.2**.

If an insufficient number of dilutions has been prepared beyond the highest dilution revealing three positive tubes, select instead the three highest dilutions of the series (i.e. those having the smallest concentration of sample). See example 2.

Table 2 — Examples of the selection of positive results for calculating MPN values

Example	Number of positive tubes obtained from three incubated tubes for the following inoculated quantities of sample per tube[a]					MPN[b]	
	Liquid products	10 ml	1 ml	10^{-1} ml	10^{-2} ml	10^{-3} ml	**Liquid products**	**Other products**
	Other products	1 g	10^{-1} g	10^{-2} g	10^{-3} g	10^{-4} g	ml^{-1}	g^{-1}
1		3	**3**	**2**	**1**	0	$1,5 \times 10^{1}$	$1,5 \times 10^{2}$
2		3	**3**	**3**	**0**	—	$2,4 \times 10^{1}$	$2,4 \times 10^{2}$
3		2	2	**1**	**1**	**0**	7,4	$7,4 \times 10^{1}$
4		**3**	**3**	**0**	0	0	2,4	$2,4 \times 10^{1}$
5		**2**	**2**	**0**	1	0	$2,1 \times 10^{-1}$	2,1

[a] Bold: combination selected.
[b] Calculated from the MPN index for three tubes (Table B.1).

9.4.3.1.2 *Case 2: No dilution revealing three positive tubes*

Case 1 cannot be applied. Select the three highest dilutions of the series (i.e. those having the smallest concentration of sample) which contain at least one positive response. See example 3. See also **9.4.3.1.3**.

9.4.3.1.3 *Special cases*

In all cases where more than one of the three dilutions selected according to **9.4.3.1.1** and **9.4.3.1.2** do not reveal any positive tubes, select the lowest dilution which does not contain any positive tubes (i.e. the one having the highest concentration of sample) and the two smallest preceding dilutions (i.e. those of which the concentrations of sample are equal to 10 and 100 times that of the first dilution selected; see examples 4 and 5), except when postive tubes are only found at the level of the first dilution prepared from the sample. In the latter case, it is necessary to select the first three dilutions for calculating the MPN, even if this series contains two dilutions which do not reveal any positive tube.

9.4.3.2 *Calculation of the most probable number (MPN)*

9.4.3.2.1 Check, according to the number of samples examined by batch in Table B.1, whether the sequences of number of positive tubes, corresponding to the dilutions selected in accordance with **9.4.3.1**, are acceptable from the statistical standpoint. This acceptability depends both on the number of samples examined and on the decision to accept or refuse the results of categories 2 and 3 (see Table B.2).

Thus, for example, when only the results of category 1 are accepted, sequence 221 can only be taken into account if 10 samples of the relevant batch have been examined. On the other hand, when the less probable results of category 2 are also accepted, sequence 221 will also be taken into account in the case where only two, three or five samples have been examined. If sequence 221 is the result of a single test, it will not be acceptable under any circumstances.

9.4.3.2.2 For each sequence considered as being acceptable according to **9.4.3.2.1**, the MPN index will be obtained by means of Table B.1.

9.4.4 *Expression of results*

From the MPN index (see **9.4.3.2**) read from Table B.1, determine the most probable number of microorganisms in the reference volume, using the following equation:

$$C_s = M\frac{F}{V_0}V_s$$

where

C_s is the most probable number of microorganisms in the reference volume V_s;

M is the MPN index read from Table 2 for the basic dilution V_0;

F is the reciprocal of the dilution factor corresponding to the dilution, if any, of the sample taken as the basic dilution used in the table (generally $F = 10$, 100, etc.);

V_s is the reference volume chosen for expressing the microorganism concentration;

V_0 is the basic dilution.

In the case where the smallest dilution selected corresponds to the tubes prepared from a double concentration medium (10 ml of inoculation), divide the MPN index beforehand by 10.

If the MPN is smaller than 0,3 microorganisms per millilitre (liquid products) or per gram (other products) and if the procedure appropriate for a small number of microorganisms has been used, the result shall be expressed in the following manner: less than 1 microorganism in 1 ml (liquid products) or in 1 g of product (other products).

EXAMPLE

In the case of a solid product, the MPN index is 24; the first tube selected corresponds to the inoculation of 1 ml of the initial suspension ($F = 10$), C_s, is, per gram

$C_s = 24 \times 10 = 2{,}4 \times 10^2$

9.4.5 *Confidence limits*

The confidence limits are given in the MPN table (Table B.1).

It is well known that large variations in results can be observed with the MPN technique. Consequently, results obtained according to this method shall be used with care.

9.5 Detection method

A detection method is a method which determines the presence or absence of microorganisms defined in a given quantity of product.

9.5.1 *Principle*

Unless otherwise stated in the relevant International Standard, mix (liquid products) or homogenize (other products) a quantity p of the product be examined with $9 \times p$ ml or g of an elective or selective broth. After incubation, preferably in a thermostatically controlled bath (**4.9**), spread a loop of the culture obtained over the surface of a selective agar medium in such a manner as to obtain isolated colonies. A number (generally five) of the colonies obtained after incubation is then identified.

In certain cases, it is desirable to precede enrichment in the elective or selective broth by a pre-enrichment in a nutrient broth so as to resuscitate the stressed microorganisms. Likewise, it sometimes appears worthwhile using at the same time two or more elective or selective broths as well as two or more selective agar media.

9.5.2 *Interpretation of the result*

If the target microorganism has been detected, give the result in the form:

"present in the analysed p ml (liquid products) or p g (other products)".

If the target microorganism has not been detected, give the result in the form:

"absent in the analysed p ml (liquid products) or p g (other products)".

Under no circumstances generalize to other higher quantities of product.

9.6 Basic identification techniques

9.6.1 *Preparation of a pure culture*

9.6.1.1 *General*

Begin the preparation of a pure culture by the selection of a colony on or in an agar medium which has been inoculated with a dilution of the test sample or with a culture.

Then inoculate the selected colony on a non-selective agar culture medium. After incubation, select a well-isolated colony. Repeat the operation if necessary.

Use the plating-out techniques described in **9.6.1.2**. Different methods may prove to be necessary in specific cases. For anaerobic microorganisms, the procedure described in **9.6.1.2** can often be followed, taking care to leave the cultures in the presence of air for as short a time as possible.

9.6.1.2 *Plating out*

Take a small quantity from the surface of a well-isolated colony using the tip of a sterile loop. Then plate out, either directly with the cells present on the loop (**9.6.1.2.1**), or after having prepared a suspension of these cells (**9.6.1.2.2**).

9.6.1.2.1 *Direct method: Example*

Using the tip of the loop, inoculate, in close streaks, a portion of about one-third of the agar medium surface area. Sterilize and cool down the loop. From the edge of the inoculated area, make another series of streaks, less close together than in the first case, over half of the surface area not yet inoculated. Repeat the operation over the remaining surface area making more-spread-out streaks (see Figure 1).

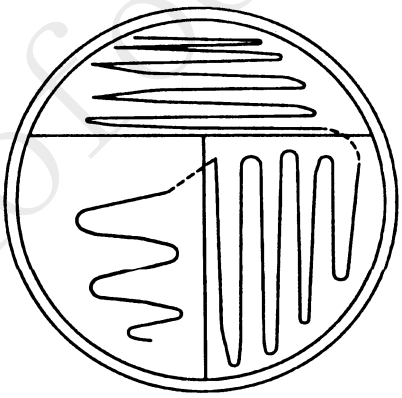

Figure 1 — Example of plating out: Direct method

9.6.1.2.2 *Method using dilution fluid*

Suspend the cells in 1 ml to 2 ml of the selected dilution fluid, rubbing the inoculated loop against the wall of the tube at the surface of the liquid, then mix well.

Sterilize and cool down the loop. Using the loop, take a small portion of the microbial suspension and proceed as stated in **9.6.1.2.1**.

9.6.1.3 *Incubation*

Unless otherwise stated, invert the inoculated Petri dishes and place them in the incubator for the chosen time at the chosen temperature.

9.6.1.4 *Selection*

After incubation, select a well-isolated colony from the dish, either for subsequent plating out, or for the tests to be performed.

If possible, the final tests should be carried out using cells stemming from one single colony. If there is insufficient cell material in one colony, it should first be subcultured in a liquid medium or on the agar medium slant, after which the subculture can be used for the tests to be performed.

9.6.2 ***Gram's stain (Modified Hucker technique)***

This staining of bacterial cells allows description of the morphology of the bacteria and classification of them into two groups as a function of whether or not they are capable of retaining the violet stain of Crystal violet under the test conditions. This division results mainly from differences in the structure of the cell walls of the two groups and it is correlated with other major differences between the two groups. There are a number of ways to conduct a Gram's stain, but all follow the sequences given below.

9.6.2.1 *Solutions*

Commercially available solutions may be used. In this case, follow the manufacturer's recommendations.

9.6.2.1.1 *Crystal violet solution*

9.6.2.1.1.1 *Composition*

Crystal violet	2,0 g
Ethanol (95 %)	20 ml
Ammonium oxalate ($C_2H_8N_2O_4$)	0,8 g
Distilled water	80 ml

9.6.2.1.1.2 *Preparation*

Dissolve the Crystal violet in the ethanol and the ammonium oxalate in the distilled water. Mix the two solutions and allow the mixture to stand for 24 h prior to use.

9.6.2.1.2 *Iodine solution*

9.6.2.1.2.1 *Composition*

Iodine	1,0 g
Potassium iodide (KI)	2,0 g
Distilled water	100 ml

9.6.2.1.2.2 *Preparation*

Dissolve the potassium iodide in 10 ml of distilled water; add the iodine in fractions. After dissolution, make up to 100 ml in a volumetric flask.

9.6.2.1.3 *Safranine solution*

9.6.2.1.3.1 *Composition*

Safranine O	0,25 g
Ethanol (95 %)	10 ml
Distilled water	100 ml

9.6.2.1.3.2 *Preparation*

Dissolve the safranine in the ethanol then mix with the distilled water.

9.6.2.2 *Staining technique*

After fixing the bacterial film on the microscope slide prepared from a culture 18 h to 24 h old, or when the broth is turbid, cover the film with the Crystal violet solution (**9.6.2.1.1**). Allow to react for 1 min.

Gently rinse the inclined slide with water for a few seconds.

Cover the slide with the iodine solution (**9.6.2.1.2**). Allow to react for 1 min.

Gently rinse the inclined slide with water for a few seconds.

Pour gently and continuously a film of ethanol (95 %) onto the inclined slide over a period of not more than 30 s until no more of the violet colour is emitted.

Gently rinse the inclined slide with water in order to eliminate the ethanol.

Cover the slide with the solution of safranine (**9.6.2.1.3**) for 10 s.

Gently rinse the inclined slide with water.

Dry the slide.

9.6.2.3 *Interpretation*

Examine the slide under the high-power oil objective of the microscope (**4.12**). Those bacterial cells which appear blue or violet are termed Gram-positive (Gram +); those which are coloured dark pink to red are termed Gram-negative (Gram –).

For a pure culture of certain bacterial types, both Gram-positive and Gram-negative cells can be obtained in a same microscope field.

NOTE Densely packed cells may give an uncharacteristic response.

9.6.3 ***Test for catalase***

The detection of this enzyme, which decomposes hydrogen peroxide (H_2O_2) into water and oxygen, can be carried out using a broth culture, an agar culture or one single colony on an agar medium.

In all cases, unless otherwise stated in the relevant International Standard, the culture medium shall not contain any blood, unless the latter has been heated (cooked blood medium).

NOTE 1 Certain lactic bacteria possess a "pseudo-catalase" which does not contain any haem group when they are cultured in the absence of glucose or in the presence of a small glucose concentration (0,1 %).

NOTE 2 In the case of anaerobic bacteria, wait 30 s in the open air prior to adding the hydrogen peroxide.

9.6.3.1 *From a broth culture*

Add to 1 ml of the culture 0,5 ml of a 10 volume [3 % (*m*/*m*)] hydrogen peroxide solution. Observe the occurrence of oxygen bubbles (catalase positive) or absence (catalase negative).

9.6.3.2 *From an agar medium culture*

Cover the culture with 1 ml to 2 ml of a 10 volume hydrogen peroxide solution. Observe immediately and after 5 min whether or not oxygen bubbles have formed.

9.6.3.3 *From a colony*

Place separately two drops of a 10 volume hydrogen peroxide solution on a microscope slide.

Pick off a colony with a sterile glass or plastic rod (especially not a metallic wire) and gently emulsify it in one of the two drops. Observe immediately and over several minutes (at least 1 min) whether or not oxygen bubbles have formed. In the event of doubt, cover each of the drops with a microscope slide and compare the occurrence of bubbles under both slides.

The observations can be conducted macroscopically or using a low-magnification microscope.

9.6.4 ***Test for oxidase***

The detection of oxidase is carried out by the change in colouring of a compound at the time of oxidation under the action of this enzyme.

9.6.4.1 *Reagent*

9.6.4.1.1 *Composition*

N, *N*, *N*′, *N*′-Tetramethyl-*p*-phenylenediamine dihydrochloride ($C_{10}H_{16}N_2 \cdot 2HCl$)	1,0 g
Distilled water	100 ml

9.6.4.1.2 *Preparation*

Dissolve the reagent in the cold water. The reagent shall be prepared immediately prior to use.

Commercially available disks or sticks may be used. In this case, follow the manufacturer's recommendations.

9.6.4.2 *Technique*

Moisten a piece of filter paper with the reagent. Take a sample of the bacterial culture obtained from an agar medium using a platinum wire or a glass or plastic rod (a nickel/chrome wire gives false positives) and deposit it on the moistened filter paper.

9.6.4.3 *Interpretation of the result*

In the case of the presence of oxidase, a violet to purple colour appears within a period of between 5 s and 10 s. If the colour has not changed after 10 s, the test is considered as being negative.

9.6.5 ***Use of biochemical galleries for identification***

Currently available biochemical galleries may be used for identification. However, all commercialized galleries do not present the same level of reliability. Their performance shall therefore be assessed before use, especially if they have not been validated by an independent system.

Annex A (normative)
Confidence interval limits for estimated counts

The values given in Table A.1 and Table A.2 are based on reference [10].

Table A.1 — Counting from one Petri dish

Number of microorganisms[a]	Confidence limit at 95 % level		Percent error for the limit[b]	
	Lower	Upper	Lower	Upper
1	<1	6	–97	+457
2	<1	7	–88	+261
3	<1	9	–79	+192
4	1	10	–73	+156
5	2	12	–68	+133
6	2	13	–63	+118
7	3	14	–60	+106
8	3	16	–57	+97
9	4	17	–54	+90
10	5	18	–52	+84
11	6	20	–50	+79
12	6	21	–48	+75
13	7	22	–47	+71
14	8	24	–45	+68
15	8	25	–44	+65

[a] Equal to the number of colonies.
[b] Compared to the microorganism count (1st column).

Table A.2 — Counting from two Petri dishes

Number of colonies[a]	Number of microorganisms	Confidence limit at 95 % level		Percent of error for the limit[b]	
		Lower	Upper	Lower	Upper
1	1	<1	3	–97	+457
2	1	<1	4	–88	+261
3	2	<1	4	–79	+192
4	2	1	5	–73	+156
5	2	1	6	–68	+133
6	3	1	6	–63	+118
7	4	2	7	–60	+106
8	4	2	8	–57	+97
9	4	2	9	–54	+90
10	5	2	9	–52	+84
11	6	3	10	–50	+79
12	6	3	10	–48	+75
13	6	3	11	–47	+71
14	7	4	12	–45	+68
15	8	4	12	–44	+65
16	8	5	13	–43	+62
17	8	5	14	–42	+60
18	9	5	14	–41	+58
19	10	6	15	–40	+56
20	10	6	15	–39	+54
21	10	6	16	–38	+53
22	11	7	17	–37	+51
23	12	7	17	–36	+50
24	12	8	18	–36	+49
25	12	8	18	–35	+48
26	13	8	19	–35	+47
27	14	9	20	–34	+46
28	14	9	20	–34	+45
29	14	9	21	–33	+44
30	15	10	21	–32	+43

[a] Counted on two Petri dishes.
[b] Compared to the microorganism count (column 2).

Annex B (normative)
MPN tables

Table B.1 — MPN table for 3 × 1 g (ml), 3 × 0,1 g (ml) and 3 × 0,01 g (ml)

Number of positive results			MPN	Category when the number of tests is					Confidence limits			
				1	2	3	5	10	> 95 %	> 95 %	> 99 %	> 99 %
0	0	0	< 0,30						0,00	0,94	0,00	1,40
0	0	0	0,30	3	2	2	2	1	0,01	0,95	0,00	1,40
0	1	0	0,30	2	1	1	1	1	0,01	1,00	0,00	1,60
0	1	1	0,61	0	3	3	3	3	0,12	1,70	0,05	2,50
0	2	0	0,62	3	2	2	2	1	0,12	1,70	0,05	2,50
0	3	0	0,94	0	0	0	0	3	0,35	3,50	0,18	4,60
1	0	0	0,36	1	1	1	1	1	0,02	1,70	0,01	2,50
1	0	1	0,72	2	2	1	1	1	0,12	1,70	0,05	2,50
1	0	2	1,1	0	0	0	3	3	0,4	3,5	0,2	4,6
1	1	0	0,74	1	1	1	1	1	0,13	2,00	0,06	2,70
1	1	1	1,1	3	3	2	2	2	0,4	3,5	0,2	4,6
1	2	0	1,1	2	2	1	1	1	0,4	3,5	0,2	4,6
1	2	1	1,5	3	3	3	3	2	0,5	3,8	0,2	5,2
1	3	0	1,6	3	3	3	3	2	0,5	3,8	0,2	5,2
2	0	0	0,92	1	1	1	1	1	0,15	3,50	0,07	4,60
2	0	1	1,4	2	1	1	1	1	0,4	3,5	0,2	4,6
2	0	2	2,0	0	3	3	3	3	0,5	3,8	0,2	5,2
2	1	0	1,5	1	1	1	1	1	0,4	3,8	0,2	5,2
2	1	1	2,0	2	2	1	1	1	0,5	3,8	0,2	5,2
2	1	1	2,0	2	2	1	1	1	0,5	3,8	0,2	5,2
2	1	2	2,7	0	3	3	3	3	0,9	9,4	0,5	14,2
2	2	0	2,1	1	1	1	1	1	0,5	4,0	0,2	5,6
2	2	1	2,8	3	2	2	2	1	0,9	9,4	0,5	14,2
2	2	2	3,5	0	0	0	0	3	0,9	9,4	0,5	14,2
2	3	0	2,9	3	2	2	2	1	0,9	9,4	0,5	14,2
2	3	1	3,6	0	3	3	3	3	0,9	9,4	0,5	14,2
3	0	0	2,3	1	1	1	1	1	0,5	9,4	0,3	14,2
3	0	1	3,8	1	1	1	1	1	0,9	10,4	0,5	15,7
3	0	2	6,4	3	3	2	2	2	1,6	18,1	1,0	25,0
3	1	0	4,3	1	1	1	1	1	0,9	18,1	0,5	25,0
3	1	1	7,5	1	1	1	1	1	1,7	19,9	1,1	27,0
3	1	2	12	3	2	2	2	1	3	36	2	44
3	1	3	16	0	0	0	3	3	3	38	2	52
3	2	0	9,3	1	1	1	1	1	1,8	36,0	1,2	43,0
3	2	1	15	1	1	1	1	1	3	38	2	52
3	2	2	21	2	1	1	1	1	3	40	2	56
3	2	3	29	3	3	3	2	2	9	99	5	152
3	3	0	24	1	1	1	1	1	4	99	3	152
3	3	1	46	1	1	1	1	1	9	198	5	283
3	3	2	110	1	1	1	1	1	20	400	10	570
3	3	3	> 110									

NOTE The confidence limits given in Table B.1 are only intended to provide some idea about the influence of statistical variations on the results. There will always be other sources of variation, which may sometimes even be significant.

Table B.2 — Explanation of the results

Category	Definition
1	When the number of microorganisms in the product is equal to the MPN found, the result is one of those which has the greatest chance of being obtained. At most there is only a 5 % chance of obtaining a result that is less probable than the least probable in this category.
2	When the number of microorganisms in the product is equal to the MPN found, the result is one of those that have less chance of being obtained than even the least probable one in category 1, but at the most there is only a 1 % chance of obtaining a result that is less probable than the least probable one in this category.
3	When the number of microorganisms in the product is equal to the MPN found, the result is one of those that have less chance of being obtained than even the least probable one in category 2, but at the most there is only a 0,1 % chance of obtaining a result that is less probable than the least probable one in this category.
0	When the number of microorganisms in the product is equal to the MPN found, the result is one of those that have less chance of being obtained than even the least probable one in category 3. There is only a chance of 0,1 % of obtaining a result in this category without anything being wrong.

Before starting testing, it shall be decided which category will be acceptable, i.e. only 1, 1 and 2, or even 1, 2 and 3 (see Table B.1 and Table B.2). If the decision, to be taken on the basis of the results, is very important, only the results of category 1 or at most those of categories 1 and 2 should be accepted. The results of category 0 should be considered with the greatest caution.

Annex C (informative)
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