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Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations

AMENDMENT 1

Microbiologie des aliments — Exigences générales et recommandations

AMENDEMENT 1

Reference number
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Foreword

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

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received, www.iso.org/patents.

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The committee responsible for this document is ISO//TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

This corrected version of ISO 7218:2007/Amd.1:2013 incorporates corrected values in Tables C.5, C.6 and C.7, i.e.

— columns four, five, six, seven and eight of Table C.5,

— columns four, five, six, seven, eight and nine of Table C.6, and

— columns four and nine of Table C.7.

Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations

AMENDMENT 1

Page 1, Clause 2

Delete ISO 8261. This has been superseded by ISO 6887-5 [already included in "ISO 6887 (all parts)"].

Delete the entries numbered "ISO 835 (all parts)", "ISO 8655-1", "ISO/TS 11133 (all parts)", and "ISO 16140" and insert the following.

ISO 835, *Laboratory glassware — Graduated pipettes*

ISO 8655 (all parts), *Piston-operated volumetric apparatus*

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

ISO 16140-2, *Microbiology of food and animal feed — Method validation — Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method*

Pages 6 to 30, Clauses 5 and 6

Delete the existing text and insert the following.

5 Apparatus and equipment

5.1 General

In accordance with good laboratory practice, all apparatus and equipment should be kept clean and in good working condition. Before use, equipment should be verified as fit for the intended purpose and its performance monitored during use, where appropriate.

Where necessary, equipment and monitoring devices should be calibrated to traceable national standards, and recalibration and any necessary intermediate checks performed, and procedures and results documented.

Equipment should be regularly checked and maintained to ensure safety and fitness for use. Equipment should be monitored according to the working conditions and the accuracy demanded for the results.

The frequency of calibration and verification checks of each item of equipment is, in most cases, not specified in this International Standard, since it shall be determined by each laboratory, depending on the type of equipment and on the laboratory's level of activity, and in accordance with the manufacturer's instructions. In a limited number of cases, a frequency has been specified since it was considered to be essential.

Apparatus and equipment shall be constructed and installed to facilitate operation and to allow for ease of maintenance, cleaning, decontamination and calibration.

Any measurement uncertainties given in this clause relate to the apparatus and equipment concerned and not to the whole method of analysis.

Throughout this clause, requirements for accuracy of measuring equipment are given. These are based on the practical tolerance required to demonstrate suitable control of equipment in routine use. The accuracy stated is related to the metrological uncertainty of the device (see ISO/IEC Guide 99).

For temperature control equipment, check the stability and homogeneity of the temperature before initial use and after any repair or modification which might have an effect on the temperature control.

5.2 Protective cabinets

5.2.1 Description

A protective cabinet is a work station with horizontal or vertical laminar airflow to remove dust and other particles, such as microbes, from the air.

The maximum tolerable number of particles per cubic metre with a size greater than or equal to 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 for use in food microbiology laboratories are of four types.

a) Class I biosafety cabinets are open-fronted exhaust-protective cabinets that are intended to protect the operator and the environment but will not protect the product from extraneous contamination. Potentially infected aerosols will be contained within the cabinet and trapped by impaction on the filter. The filtered air is normally discharged to the atmosphere; if this is not done, the air shall pass through two high-efficiency particulate air (HEPA) filters mounted in series. They are not recommended for work with risk category 3 pathogens because of the difficulties in maintaining and ensuring appropriate operator protection.

b) Class II biosafety cabinets protect the product, the operator and the environment. They recirculate some filtered air, exhaust some to the atmosphere and take in replacement air through the working aperture, thereby providing operator protection. They are suitable for work with risk category 2 and 3 pathogens.

c) Horizontal laminar outflow cabinets protect the work from contamination, but blow any aerosols generated into the operator's face. Therefore they are not suitable for handling inoculated cultures or preparation of tissue culture.

d) Vertical laminar airflow cabinets protect the product by the use of vertical laminar flow of HEPA-filtered air. They also protect the operator by the use of internally recirculated air. They are particularly suitable for providing an aseptic environment for handling sterile products and for protecting the operator when handling powders.

Use protective cabinets for all work involving the handling of pathogens and contaminated powders, if required by national regulations.

The use of a gas burner or wire incinerator is not recommended in protective cabinets. If it is necessary, the gas burner should have a small flame so that the airflow is not disturbed. The use of disposable equipment (loops, pipettes, etc.) is a suitable alternative.

5.2.2 Use

Use protective cabinets that are appropriate for the intended application and environmental conditions in the laboratory.

Cabinets should be kept as free of equipment as possible.

Where practicable, place everything needed inside the cabinet before starting work to minimize the number of arm movements into and out of the working aperture. Position equipment and materials so as to minimize disturbance to the airflow at the working aperture.

Operators should be adequately trained in the correct use of cabinets to ensure their safety and the integrity of the product or culture.

5.2.3 Cleaning and disinfection

Clean and disinfect the working area after use with appropriate and non-corrosive disinfectant in accordance with the manufacturer's instructions. Regularly examine wire grids protecting prefilters, if they exist, and wipe clean with a disinfectant-soaked cloth.

For laminar flow cabinets, the filter face should be vacuum cleaned regularly, taking care not to damage the filter medium.

Safety cabinets should be fumigated before filter changing or servicing.

After cleaning of the cabinets, ultraviolet (UV) lamps may be used for disinfection. UV lamps should be regularly cleaned and replaced in accordance with the manufacturer's instructions. If they are used, they should be cleaned regularly to remove any dust and dirt that may block the germicidal effectiveness of the light. Ultraviolet light intensity should be checked when the cabinet is recertified to ensure that light emission is according to the manufacturer's instructions.

See Reference [17].

5.2.4 Maintenance and inspection

The efficiency of a protective cabinet shall be checked by a qualified or certified person on receipt and thereafter at regular intervals as recommended by the manufacturer, as well as after any repair or modification. The efficiency should be checked after relocation.

Periodic verification of freedom from any microbial contamination should be carried out by a check of the working surface and walls of the cabinet.

A periodic verification of the number of airborne microorganisms present should be carried out during operation of the filters using the usual equipment. For example, expose several open Petri dishes containing a non-selective agar culture medium (e.g. Plate Count Agar) in each cabinet for 30 min. Other methods may be used.

5.3 Balances and gravimetric diluters

5.3.1 Use and measurement uncertainty

Balances are mainly used for weighing the test portion of the sample to be examined and the components of the culture media and reagents. In addition, they may be used for carrying out measurements of dilution fluid volumes by mass.

Gravimetric diluters are electronic instruments consisting of a balance and programmable liquid dispenser and are used during the preparation of initial sample suspensions; they function by adding diluent to a subsample at a set ratio. The subsample is then weighed to the tolerance specified in the application, and the diluter set to dispense sufficient diluent for the ratio required (e.g. 9 to 1 for decimal dilutions). See ISO 6887-1.

A food microbiology laboratory shall be equipped with balances of the required range and measurement uncertainty for the different products to be weighed.

Unless otherwise stated, the resolution of the balance should achieve a tolerance of 1 % but shall be sufficient to achieve a maximum tolerance of 5 % of the mass.

EXAMPLE To weigh 10 g, the balance should be capable of being read to 0,1 g.

To weigh 1 g, the balance should be capable of being read to 0,01 g.

Place the equipment on a stable horizontal surface, adjusted as necessary to ensure that it is level and protected from vibration and draughts.

5.3.2 Cleaning and disinfection

Equipment should be cleaned and disinfected after use or following spillage during weighing with an appropriate and non-corrosive disinfectant.

5.3.3 Performance verification and calibration

5.3.3.1 Calibration

Calibration shall be checked across the entire range by a qualified person at a frequency dependent on use.

5.3.3.2 Verification

The performance of the balance system shall be regularly verified during use and after cleaning with check weights in the range of use by a qualified person.

NOTE Check weights may also be verified immediately after calibration of the balance.

5.4 Homogenizers, blenders and mixers

5.4.1 Description

This equipment is used to prepare the initial suspension from the test sample.

The following apparatus may be used:

— a peristaltic blender with sterile bags, possibly with a device for adjusting speed and time; or

NOTE The Stomacher® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

— a rotary homogenizer (blender), the notional speed of which is between 8 000 r/min and 45 000 r/min inclusive, with sterilizable bowls equipped with covers; or

— a vibrational mixer with sterile bags; or

NOTE The Pulsifier® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

— another homogenizing system with equivalent efficiency.

In certain cases, manual mixing may be carried out using sterile glass beads having an appropriate diameter (approximately 6 mm; see ISO 6887-2 to ISO 6887-5).

5.4.2 Use

The usual operating time of a peristaltic homogenizer is 1 min to 3 min (see ISO 6887-2 to 6887-5 for specific foods).

Do not use this type of apparatus for certain foodstuffs, such as:

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

— 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 total number of revolutions is between 15 000 r/min and 20 000 r/min inclusive. Even with the slowest homogenizer, this time shall not exceed 2,5 min.

The vibrational mixer may be used for most foodstuffs, including hard or dry products. The usual operating time is 0,5 min to 1 min. If microorganisms are likely to be encountered deep inside cohesive structures, the sample should be cut into small pieces prior to processing.

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).

5.4.3 Cleaning and disinfection

Clean and disinfect peristaltic homogenizers and vibrational mixers regularly and after any bag spillage or leakage.

For rotary homogenizers, clean and sterilize the glass or metal bowl after each use.

5.4.4 Maintenance

Inspect and maintain equipment in accordance with the manufacturer's instructions.

5.5 pH meter

5.5.1 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 being read to the nearest 0,01 pH unit, enabling measurements to be made with a tolerance of ±0,1 pH unit. The pH meter shall be equipped with either manual or automatic temperature compensation.

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

5.5.2 Use

A pH meter is used to measure the pH value of culture media and reagents to check if adjustment is needed during preparation and as a quality check after sterilization.

It may also be used to measure the pH value of samples and sample suspensions. The use of a pH meter is discussed in the standard specific to the product to be analysed, in which the conditions for the determination of the pH value and for adjustment of the pH value are specified.

Adjust the pH meter as indicated in the manufacturer's manual to measure the pH value at a standardized temperature, e.g. 25 °C. Read the pH value after stabilization has been reached. Record the value to two decimal places.

NOTE The reading may be considered stable when the pH value measured over a period of 5 s varies by not more than 0,02 pH units. Using electrodes in good condition, equilibrium is normally achieved within 30 s.

5.5.3 Calibration and verification

5.5.3.1 Calibration

Calibrate the pH meter in accordance with the manufacturer's instructions, using at least two, and preferably three, standard buffer solutions at least daily before use. Define the maximum permissible tolerances for these readings, which shall be more stringent than the tolerance permitted in general use.

The standard solutions should be traceable and shall have pH values specified to two decimal places at the measurement temperature (in general, pH 7,00 and pH 4,00 and/or pH 9,00 at 25 °C, in accordance with the manufacturer's instructions). The standards used shall encompass the pH value to be measured.

5.5.3.2 Verification

After calibration of the pH meter with the two traceable standard buffer solutions, a third buffer standard solution should be used to perform a check reading (in "read" mode) to demonstrate the functionality of the pH meter.

If readings fall outside the maximum permissible limits adjust the pH meter in accordance with the manufacturer's instructions. This adjustment shall be followed by further calibration and check.

5.5.4 Maintenance

Check and maintain the electrodes 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; and

— the response time and stability.

Rinse the electrodes with distilled or deionized water 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.

5.6 Autoclave

5.6.1 Description

An autoclave enables a saturated steam temperature to be attained in the chamber.

The autoclave should be equipped with

— at least one safety valve;

— a drain cock;

— a regulation device allowing the temperature in the chamber to be maintained to within ±3 °C of the target temperature (to take into account the measurement uncertainty associated with the measuring thermocouple); and

— a temperature probe or a recording thermocouple.

It should also be equipped with a timer and temperature recorder.

5.6.2 Use

With steam sterilization, all air is 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.

For the sterilization of culture media, the saturated steam in the chamber shall be at a temperature of at least 121 °C ± 3 °C or temperature specified by manufacturers or production instructions or specified in the test method.

For the destruction of cultured microorganisms and decontamination of used culture media, the saturated steam in the chamber shall be at a temperature of at least 121 °C ± 3 °C.

During the same sterilization cycle, do not use the autoclave to sterilize clean equipment (and/or culture media) and at the same time to decontaminate used equipment (and/or used culture media).

It is preferable to use separate autoclaves for these two processes. After autoclaving, all materials and equipment should be allowed to cool within the autoclave before removal.

For safety reasons, do not remove the contents until the temperature has dropped below approximately 80 °C.

5.6.3 Maintenance

Clean the chamber, drain filter and door seals regularly. Check the door seals for integrity. Carry out draining operations and descaling, if necessary, at regular intervals. Follow the manufacturer's recommendations.

5.6.4 Verification

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

Keep the monitoring instruments in good working order and verify them by calibration and regular checks.

Initial validation should include performance studies for each operating cycle and each load configuration used in practice. This process should be repeated after significant repair or modification. Sufficient temperature sensors should be positioned within the load to demonstrate adequate heat penetration at all locations. Validation and revalidation should consider the suitability of heat-up and cool-down times as well as the sterilization temperature.

For each load, as a minimum, a process indicator should be included at the centre of the load to verify the heating process where a traceable record of process efficiency is not available.

5.7 Media preparator

5.7.1 Description

A media preparator is principally designed for the sterilization of large volumes of media (>1 l). It consists of a heating vessel, water jacket and continuous stirring device. The equipment shall also be fitted with a temperature gauge, pressure gauge, timer and safety valve.

In addition, the unit should have a safety lock to prevent opening until a temperature of <80 °C is reached.

5.7.2 Use

Follow the manufacturer's instructions at all times.

The entire production process takes place within the apparatus. After addition of all the ingredients, they are dissolved by stirring and heating. This is followed by sterilization.

5.7.3 Maintenance

Wash the preparator and rinse thoroughly with purified water between each media batch.

5.7.4 Verification

The preparator shall be kept in good working condition and inspected regularly by competent qualified personnel in accordance with the manufacturer's instructions.

Keep the monitoring instruments in good working order and verify them by calibration and regular checks.

Initial validation should include performance studies for each operating cycle and each load size used in practice. This process should be repeated after significant repair or modification. Two temperature probes, one adjacent to the control probe and another remote from it, may be used to demonstrate uniform heating.

The temperature and duration of each cycle should be checked.

5.8 Incubator

5.8.1 Description

An incubator consists of an insulated chamber which enables the temperature to be kept stable and uniformly distributed to within the maximum permissible temperature tolerance specified in the test method.

5.8.2 Use

Incubators shall be equipped with a regulation system that allows the temperature or other parameters to be kept even and stable over their entire working volume. Define the working volume to ensure that this is achieved.

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.

The walls of incubators should be protected from sunlight.

If possible, incubators should not be completely filled in a single operation because the culture media will take a long time to come to temperature equilibrium, whatever type of incubator is used (forced-air convection or otherwise). Refrain from leaving the incubator door open for long periods.

When loading incubators, attention should be paid to air circulation (see 10.2.5).

5.8.3 Cleaning and sanitization

Clean and sanitize regularly the inner and outer walls of the incubator and, if appropriate, remove dust from the ventilation system.

5.8.4 Verification

Check the temperature stability and the homogeneity of the temperature distribution at the working temperature(s) throughout the working volume of the incubator through simultaneous use of a number of thermometers or thermocouples of known accuracy and appropriate temperature range.

Use the information to define the acceptable operating range of the incubator and the optimum position of the thermometer or recording thermocouple used to monitor working temperatures.

For example, to achieve a target temperature of (37 ± 1) °C when the profiling data shows a range of 36,8 °C to 37,3 °C across the incubator, then the operating range should be reduced to 36,2 °C to 37,7 °C in order to ensure all parts of the incubator achieve the target temperature of 37 °C.

This process should be repeated after each significant repair or modification.

The temperature of operation should be checked with one or more maximum and minimum thermometers or recording thermocouples, for example.

Check the incubator temperature at least every working day. For this purpose, each incubator shall incorporate at least one working measurement device, which can be immersed in glycerol (or other appropriate heat sink). Other checking systems of equivalent performance may be used.

5.9 Refrigerator, cold-storage room

5.9.1 Description

These are chambers which allow maintenance of cold storage. For the conservation of food samples for analysis, the temperature shall be (3 ± 2) °C (maximum permissible tolerances), except for particular applications. For other uses, the temperature, unless otherwise specified, shall be (5 ± 3) °C.

5.9.2 Use

In order to avoid cross-contamination, use different chambers, or at least different containers, to achieve physical separation, for the storage of

— uninoculated culture media and reagents;

— test samples; and

— microorganism cultures and incubated media.

Load refrigerators, chillers and cold-storage rooms in such a way that appropriate air circulation is maintained and the potential for cross-contamination is minimized.

5.9.3 Verification

Check the temperature of each chamber each working day using a thermometer or a permanently installed probe. The accuracy required of the temperature-monitoring device is dependent on the purpose for which the unit is used.

5.9.4 Maintenance and cleaning

Carry out the following maintenance operations at regular intervals to ensure proper operation:

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

— defrosting;

— cleaning and sanitization of the inside of the chambers.

5.10 Freezer and deep freezer

5.10.1 Description

A freezer is a chamber which allows frozen storage to be guaranteed. The temperature, unless otherwise specified, shall be below −15 °C, preferably below −18 °C for food samples.

A deep freezer is a chamber which allows deep-frozen storage to be guaranteed. The temperature, unless otherwise specified, shall be below –70 °C.

5.10.2 Use

5.10.2.1 Freezer

Different chambers, or at least different containers, shall be available to achieve physical separation for the storage of

— uninoculated reagents;

— samples for analysis; and

— microorganism cultures.

Load the freezer in such a way that a sufficiently low temperature is maintained, in particular when unfrozen products are introduced.

5.10.2.2 Deep freezer

The principle use is storage of microorganisms, reference and/or working cultures, and reagents.

Load the freezer in such a way that a sufficiently low temperature is maintained and cross-contamination between microorganisms and reagents is prevented.

5.10.3 Verification

Check the temperature of each chamber regularly using a suitable temperature-monitoring device.

5.10.4 Maintenance

Carry out regularly the following maintenance operations:

— removal of dust from the motor blades and from the external heat-exchange plates (if accessible);

— defrosting;

— cleaning and sanitization of the inside of the chambers.

5.11 Thermostatically controlled bath

5.11.1 Description

A thermostatically controlled bath, filled with a liquid (water, ethylene glycol, etc.), with or without a fitted lid or other device to limit evaporation, is required to maintain a specified temperature. Temperature control is often more precise than an air incubator, enabling maximum permissible tolerance of ±0,5 °C or better to be achieved. The working temperatures and required maximum permissible tolerance are stipulated in each individual application or standard method. A cooling system is necessary to maintain a temperature near or below ambient temperature.

5.11.2 Use

The main uses are as follows:

— incubation at a constant temperature of inoculated culture media;

- maintenance of sterile molten agar media during media preparation;
- tempering of sterile molten agar media for use in specific standard methods;
- preparation of initial sample suspensions or solutions at a controlled temperature;
- heat treatment of initial sample suspensions at a controlled temperature (e.g. pasteurization).

Where precise temperature control is required, the bath shall be equipped with a circulating-water pump and an automatic temperature-regulation system. Any agitation of the liquid shall not cause droplet dispersal.

Lidded baths are preferable for precise or high-temperature usage. Sloping lids that allow condensate to drain should be used.

For incubation of inoculated media, maintain the liquid level so that the top of the test medium is at least 2 cm below the liquid level in the bath throughout the incubation.

Other containers should be placed within baths such that the level of their contents is below that of the liquid.

The depth of immersion shall preclude entry of water through the closure.

Devices to maintain stability of the containers may be required, for example racks.

All containers should be dried after removal from the bath and before further use.

5.11.3 Verification

Check the stability and homogeneity of the temperature throughout the bath before initial use and after any repair or modification having an effect on the temperature control.

Monitor each bath with a thermometer, thermocouple or temperature-recording device of suitable minimum measurement uncertainty (see 5.28.2), and independent of the automatic temperature-regulation system.

NOTE A digital display can also be used, provided that its accuracy and resolution are verified.

Monitor the temperature of the bath during each use and at least daily for periods of extended incubation.

5.11.4 Maintenance

Baths should be filled with liquid as recommended by the manufacturer. For incubation of cultures, distilled or deionized water should preferably be used.

Check regularly the level of the liquid to ensure the correct functioning of the bath and satisfactory immersion of items in the bath. The liquid level shall always cover the heating elements.

Baths should be emptied, cleaned, sanitized and refilled regularly and at a frequency depending on usage, or after a spillage occurs.

5.12 Steamers, including boiling-water baths

5.12.1 Description

Steamers and boiling-water baths consist of a heating element surrounded by water in a vessel with a close-fitting lid. In a steamer, this creates steam at atmospheric pressure; in a boiling-water bath this heats the water to a temperature at or close to the boiling point, with or without the production of steam.

5.12.2 Use

The main uses are as follows:

— melting of agar media;

— preparation of heat-labile media;

— reduction of contamination of small items of equipment between use.

A safe and adequate level of water shall be present in the vessel to ensure that the heating elements are covered at all times.

An autoclave with a free-steaming facility may also be used.

5.12.3 Maintenance

Keep steamers and boiling water baths clean.

If necessary, regular descaling should be performed at a frequency dependent on local water hardness.

5.13 Sterilizing oven

5.13.1 Description

A sterilizing oven is a chamber that is capable of maintaining a temperature of 160 °C to 180 °C for the destruction of microorganisms by dry heat.

5.13.2 Use

Only robust equipment such as glass and metalware shall be sterilized in the sterilizing oven; do not use it for plastic and rubber items.

Before sterilization, clean all glassware and metalware to be sterilized in the oven.

If volumetric glassware is sterilized in the sterilizing oven, verify regularly the accuracy of marked volumes.

The temperature shall be uniform throughout the chamber. The oven shall be equipped with a thermostat and a thermometer or temperature-recording device of suitable accuracy.

It should be equipped with a duration indicator, programmer or timer.

Once the operating temperature is reached, the sterilizing procedure shall last for at least 1 h at 170 °C ± 10°C or an equivalent time/temperature combination.

After sterilization, to prevent cracking, glassware should be allowed to cool in the oven before removal.

5.13.3 Verification

Check the stability and homogeneity of the temperature throughout the oven before initial use and after any repair or modification which might have an effect on the temperature control.

The oven shall be fitted with a calibrated thermometer, thermocouple or temperature-recording device of suitable accuracy which is independent of the automatic temperature-regulation system. The monitoring device shall have a resolution of 1 °C or better at the oven temperature used.

The temperature of the oven should be monitored and recorded during each use.

5.13.4 Maintenance

Clean internal surfaces when required.

5.14 Microwave oven

5.14.1 Description

A microwave oven is a device that allows heating of items by microwave energy at atmospheric pressure.

5.14.2 Use

Use the equipment currently available only to heat liquids or melt agar culture media.

WARNING — Do not heat media containing heat-sensitive components in a microwave unless it has been verified that this way of heating has no effect on medium performance. No assessment has yet been made of the efficiency of microwaves for sterilizing culture media and microwave ovens shall not be used for this purpose.

The oven shall be capable of heating liquids and culture media in a controlled manner via a microwave emission cycle. The distribution of microwaves shall be homogeneous to avoid zones of overheating. Ovens fitted with a turntable or a stirrer for the microwaves give better heat distribution.

Do not use metal equipment, including metal closures. Loosen bottle caps or stoppers before heating.

Heating for longer periods at lower power ratings can give better heat distribution.

WARNING — Handle heated items with care. Contents can become super-heated and boil out or bottles can explode.

When melting agar media, a low power setting (e.g. defrost cycle) and a water heat sink (e.g. 50 ml to 100 ml of water in a microwaveable beaker) are recommended to aid control of the heating process.

A standing time of at least 5 min is recommended after the heating process before removal from the microwave oven.

5.14.3 Verification

Suitable heating times and power settings shall be established at initial commissioning for the different volumes of liquids and culture media routinely handled, to ensure optimum performance and avoid overheating of sensitive products.

5.14.4 Maintenance

Clean the oven immediately any spillage occurs, as well as at regular intervals dependent on usage.

Oven door seals should be inspected for integrity and the oven checked for radiation leakage at regular intervals with a probe or equivalent device according to the manufacturer's instructions.

5.15 Glass washer

5.15.1 Description

Laboratory glass washers are electronically controlled machines for washing general laboratory glassware, which can be programmed for different washing cycles and rinses (e.g. distilled or deionized water or acid).

Devices for washing glass pipettes are special glass washers designed to clean the narrow bores of pipettes.

5.15.2 Use

Many types of glass washer are available, and these shall generally be installed and used following the manufacturer's instructions.

5.15.3 Verification

Check the effectiveness of cleaning by visual inspection and, in critical applications, carry out tests to ensure that glassware is free from inhibitory substances.

Alkaline or acidic residues may be checked for by using a universal pH indicator solution; a pH within the range 6,5 to 7,3 should be achieved.

5.15.4 Maintenance

Progamme regular maintenance as specified by the manufacturer at a suitable frequency.

More frequent servicing may be required for heavily used equipment or in hard water areas.

5.16 Optical microscope

5.16.1 Description

There are several different types of microscope: monocular, binocular, with a visual display unit, a camera or fluorescence equipment, etc., and with an internal or external light source. For bacteriological examinations, objectives with magnifications from ×10 (dry lens) to about ×100 (oil immersion with spring-loaded turret) are used to obtain an overall magnification of ×100 to ×1 000. Phase contrast and dark field microscopy are also invaluable for examination of "wet preparations".

5.16.2 Use

Set up the optics of the microscope in accordance with the manufacturer's instructions. The optical axis of the light from the high-intensity light bulb shall pass through the centre of the substage condenser, the slide and the object lens to the eyepiece so that spherical and chromatic aberrations do not occur.

5.16.3 Maintenance

Follow the manufacturer's instructions concerning storage, cleaning and servicing. Prevent condensation occurring where humidity is high as this may lead to deterioration of lens quality.

Each day or after use, remove oil from the immersion lenses and related parts using lens tissue. Use a solvent recommended by the manufacturer. Regularly remove grease caused by eyelashes from the eyepiece lens.

The optical systems can be easily damaged, and servicing, preferably by the manufacturer, is therefore desirable.

5.17 Gas burner or wire incinerator

5.17.1 Description

Gas (Bunsen) burners produce a narrow naked flame from either mains or bottled gas. Varying the amount of air mixed with the gas controls the degree of heat produced.

A gas or electronic burner is mainly used for sterilizing metal loops and straight wires by bringing them to red heat and for flame-sterilizing other small durable items of equipment.

5.17.2 Use

The wire incinerator is used for sterilizing metal loops and straight wires and is preferred when handling pathogenic bacteria as it prevents splatter and avoids risk of cross-contamination.

Gas burners can produce much heat and air turbulence in the laboratory.

Aseptic techniques can be achieved without a gas burner by using disposable materials.

In protective cabinets, the use of gas burners should be avoided, because they may interfere unacceptably with the laminar airflow. In this case, use of sterile disposable equipment is recommended.

5.17.3 Maintenance

Regularly clean and disinfect burners and covers on wire incinerators, particularly if any microbial culture has been spilled on the devices.

5.18 Dispenser for culture media and reagents

5.18.1 Description

A dispenser is an instrument or device used to distribute culture media and reagents into tubes, bottles or Petri dishes. Such devices range from simple measuring cylinders, pipettes or manual syringes, through automatic syringes and peristaltic pumps to programmable electronically controlled devices with variable automated delivery.

5.18.2 Use

Clean equipment used for dispensing culture media and reagents shall be free of inhibitory substances. Use separate tubing for selective media to minimize leaching/carryover of such substances.

If aseptic distribution of sterile culture media and reagents is required, all parts of the dispensing equipment in contact with the product shall be sterile.

5.18.3 Verification

The volume delivered by the dispenser for culture media shall have a tolerance not exceeding ±5 % of the designated volume.

The maximum permissible tolerance for dispensing measured volumes of decimal dilution fluid shall be ± 2 %.

Check volumes dispensed before initial use, then regularly in accordance with a documented schedule, and always after any adjustments affecting the volume dispensed. Details can be found in ISO 835 and ISO 8655.

5.18.4 Cleaning and maintenance

Clean the outer surface of the dispenser after each use. Wash and rinse thoroughly all parts of the dispenser that come in contact with the product and sterilize them if required for use in dispensing sterile liquid. Do not use disinfectants on surfaces that come into contact with the product to be dispensed as they may impart inhibitory properties.

All automated dispensers shall be kept in good condition by regular servicing in accordance with the manufacturer's instructions.

5.19 Vortex mixer

5.19.1 Description

This instrument facilitates the homogeneous mixing of liquid media (e.g. decimal dilutions and samples of liquid for testing) or suspensions of bacterial cells in a liquid.

Mixing is achieved by an eccentric rotational movement of the contents of the tube or container (producing a vortex).

5.19.2 Use

Press the base of the tube or container containing the liquid to be mixed against the mixer head. The speed of mixing is controlled by varying the speed of the motor or the angle of contact with the mixer head.

The operator should ensure that spillage does not occur during mixing by adjusting the speed as necessary and by holding the tube approximately one-third of its length below the top in order to be able to control the tube better and hence avoid the liquid rising too high in the tube.

Appropriate precautions should be taken to minimize the release of aerosols when opening vortexed containers.

5.19.3 Verification

Adequate mixing is evidenced by the appearance of a vortex throughout the depth of the liquid during the mixing operation.

5.19.4 Maintenance

Keep equipment clean. If spillage occurs, decontaminate the equipment using an appropriate laboratory disinfectant.

5.20 Colony-counting device

5.20.1 Description

Manual colony-counting devices use a pressure-actuated counting device and usually give an audible indication of each count and a digital readout of the overall count. They may be simple pen-like devices or may consist of an illuminated stage with a calibrated grid for the plate and a magnifying screen to aid colony detection. Automated electronic colony counters, incorporating image analysers, operate by a combination of hardware and software systems incorporating the use of a camera and a monitor.

5.20.2 Use

Follow the manufacturer's instructions. Adjust the sensitivity of an automated counter to ensure that all target colonies are counted. Automated electronic colony counters also require separate programming when used with different types of agar and matrices, and for surface counts and pour plate counts to ensure adequate discrimination of target colonies.

5.20.3 Verification

Checks should be made manually on a regular basis to ensure that accurate counts are obtained using a colony counter.

In addition, automated colony counters should be checked every day of use with a calibration plate containing a known number of countable particles or colonies.

5.20.4 Maintenance

Keep equipment clean and free of dust; avoid scratching of surfaces that are an essential element of the counting process. Programme regular maintenance of electronic counters incorporating image analysers as specified by the manufacturer, at a suitable frequency.

5.21 Equipment for culture in a modified atmosphere

5.21.1 Description

This may be a jar that can be hermetically sealed or any other appropriate equipment which enables modified atmosphere conditions (e.g. for anaerobiosis) to be maintained for the total incubation time of the culture medium. Other systems of equivalent performance, such as anaerobic cabinets, may be used.

Follow the manufacturer's instructions for installation and maintenance.

5.21.2 Use

The composition of the atmosphere required can be achieved by means of the addition of a gas mixture (e.g. from a gas cylinder) after evacuation of air from the jar, by displacement of the atmosphere in a cabinet or by any other appropriate means (such as commercially available gas packs).

In general, anaerobic incubation requires an atmosphere of less than 1 % volume fraction oxygen, 9 % volume fraction to 13 % volume fraction carbon dioxide; microaerobic (capnaerobic) incubation requires an atmosphere of 5 % volume fraction to 7 % volume fraction oxygen and approximately 10 % volume fraction carbon dioxide.

Conditions may need modification depending on the requirements of the specific microorganism.

5.21.3 Verification

Place a biological or chemical indicator for monitoring the nature of the atmosphere in each chamber during each use. Growth of the control strain or a change in colour of the chemical indicator verifies that appropriate incubation conditions have been achieved.

5.21.4 Maintenance

If a catalyst is fitted, regularly regenerate it in accordance with the manufacturer's instructions. If valves are fitted, clean and lubricate them to ensure proper functioning and replace as necessary.

Regularly clean and sanitize the equipment.

5.22 Centrifuge

5.22.1 Description

Centrifuges are mechanical or electronically operated devices that use centrifugal force to separate suspended particles, including microorganisms, from fluids.

5.22.2 Use

In some applications, concentration of target microorganisms is achieved by centrifuging liquid samples to provide a deposit, which can be resuspended in liquid and subjected to further examination.

Take necessary precautions to prevent aerosol generation and cross-contamination, by correct operation of the equipment and the use of sealed and sterile centrifuge tubes or pots.

5.22.3 Verification

Where the speed of centrifuging is critical to or specified in the application, the speed indicator or settings against a calibrated and independent tachometer should be checked regularly and after significant repairs or modifications.

5.22.4 Maintenance

Clean and disinfect centrifuges regularly and after any spillage involving microbial cultures or potentially contaminated samples.

Centrifuges should be serviced regularly.

5.23 Hotplate and heating mantle

5.23.1 Description

Hotplates and heating mantles are thermostatically controlled heating devices. Some hotplates and heating mantles incorporate magnetic stirring systems.

5.23.2 Use

Hotplates and heating mantles equipped with magnetic stirring systems are used for heating relatively large volumes of liquid such as media.

Do not use hotplates and heating mantles without stirring systems for preparation of media.

5.23.3 Maintenance

Clean up any spillages as soon as the unit is cool.

5.24 Spiral plater

5.24.1 Description

A spiral plater is a dispenser that distributes a predetermined volume of liquid over the surface of a rotating agar plate. The dispensing arm moves from the centre of the plate towards the outside edge in an Archimedean spiral. The volume dispensed is decreased as the dispensing stylus moves from the centre to the edge of the plate, so that an inverse relationship exists between the volume deposited and the radius of the spiral. The volume of sample dispensed on any particular segment is known and is constant. For loading and dispensing of liquids, a vacuum source, a motorized plunger if the instrument uses a disposable micro-syringe or an equivalent system is required.

5.24.2 Use

The equipment is used to dispense a liquid sample, sample homogenate or dilution on to an appropriate agar plate in order to determine a colony count. After incubation, colonies develop along the lines where the liquid was deposited. The number of colonies in a known area is counted using a counting grid supplied with the equipment and the count calculated.

The dispensing system shall be sanitized and then rinsed with sterile distilled or deionized water, before and after use, and between each sample. Disinfection may be achieved by flushing for example with a solution containing 0,5 % to 1 % mass fraction of free chlorine.

Equipment that operate with a different principle (for example disposable micro-syringes with plunger) shall be used according to the manufacturer's instructions.

The surface of agar plates to be used with the spiral plater shall be level and free of air bubbles.

Plates shall be free from excess surface moisture to ensure formation of discrete colonies.

Blockages can be prevented by allowing any particles to settle before loading the sample suspension and using a portion of the supernatant liquid. Blender bags with in-built filters may also be used.

5.24.3 Verification

Ensure that Petri dish is centred on the turntable.

The dispensing pattern shall be verified daily by dispensing washable ink. The spiral plater pattern shall be most dense near the centre of the plate where deposition begins and become steadily less dense to the point of stylus lift-off. The spiral shall be complete with no breaks. The clear portion of the plate shall be centred and approximately 2,0 cm in diameter. The position of the stylus at the start and the end of the inoculation shall be verified at the same time using the marks in the turntable.

If the pattern is not correct, the stylus should be cut according to the manufacturer's instructions. To ensure that the stylus tip is at the correct angle to the agar surface a check should be carried out by using a vacuum to hold a cover slip against the face of the stylus. The cover slip should be parallel to the surface of the plate.

The sterility of the spiral plater shall be verified by plating sterile water before each series of samples is examined.

A gravimetric check of the volume dispensed shall be performed regularly using distilled water. The maximum permissible tolerance for the expected mass of the volume dispensed shall be ±5 %.

5.24.4 Maintenance

Any spillages should be removed immediately and the equipment cleaned on a regular basis.

The equipment should be serviced and verified according to use.

5.25 Stills, deionizers and reverse-osmosis units

5.25.1 Description

These devices are used to produce distilled or deionized/demineralized water of the required quality (see ISO 11133) for preparation of microbiological culture media or reagents and for other laboratory applications.

5.25.2 Use

Install, commission and use equipment in accordance with the manufacturer's instructions, with due regard to the location of laboratory water, waste and electrical services.

5.25.3 Verification

Water shall be checked regularly or when used after storage for satisfactory conductivity and shall be no more than 50 µS/cm (equivalent to a resistivity ≥20 000 Ω.cm) for preparation of media and reagents.

If water is stored before use or produced through an ion exchanger, suitable checks for microbial contamination should be conducted in accordance with ISO 11133.

5.25.4 Maintenance

Stills should be cleaned and descaled with acid, e.g. citric acid at a mass fraction of 5 to 10 %, at a frequency dependent on the input water hardness and rinsed thoroughly with fresh water to remove residual acid. Deionizers and reverse-osmosis units should be maintained in accordance with the manufacturer's instructions.

5.26 Timers and timing devices

5.26.1 Description

Timers and integral timing devices are instruments that enable correct time periods to be used for many laboratory applications where the duration is specified and critical.

5.26.2 Use

Analog and digital handheld or bench timers used to monitor the duration of laboratory operations (e.g. application of stains to microbial films, homogenization of samples) shall be in good operating condition and capable of achieving the accuracy required.

Operate integral timers on laboratory equipment (e.g. autoclaves, centrifuges, homogenizers) in accordance with the manufacturer's instructions. These timers shall be capable of achieving the accuracy required depending on the criticality of application.

5.26.3 Verification

Check all timers used in laboratory operations where the duration is critical to the result against the national time signal regularly and after significant repairs.

5.26.4 Maintenance

Regularly clean and check timers for correct functioning.

Integral timing devices should be checked as part of the maintenance procedure for the instrument.

5.27 Pipettes and pipettors

5.27.1 Description

Pipettes are glass or disposable plastic devices used to deliver volumes of liquid or viscous materials; graduated pipettes deliver measured volumes with an accuracy which is dependent on the specification.

Automatic (mechanical) pipettors fitted with plastic tips are devices that dispense fixed or adjustable volumes of liquids, by manually or electrically operated piston action.

5.27.2 Use

Discard pipettes that are damaged or broken.

Sterile Pasteur or graduated pipettes and pipettor tips should be fitted with a non-absorbent cotton wool plug to prevent contamination when used to manipulate microbial cultures.

Bulbs used on Pasteur or graduated pipettes and the tips for pipettors shall be of the correct size to prevent leakage and ensure efficient operation.

Further details can be found in ISO 7712.[51]

5.27.3 Verification

Check graduated pipettes to confirm delivery of correct volumes if the manufacturer does not declare their accuracy (trueness and precision).

The calibration of pipettes or pipettors is described in ISO 835 and ISO 8655, especially in ISO 8655-2.

Test new pipettors before use, and at regular intervals depending on the frequency and nature of use, to confirm that they meet the maximum permissible tolerance which should be within 2 % for dispensing decimal dilution volumes and inocula (to improve precision and to reduce the uncertainty of the final test result, a maximum permissible tolerance of ±2 % is preferable) or 5 % for other applications. Perform intermediate gravimetric checks using distilled or deionized water to ensure that volumes dispensed remain within the maximum permissible tolerance.

5.27.4 Maintenance

Decontaminate and clean/sterilize non-disposable pipettes and automatic pipettors as appropriate after each use.

If the barrels or pistons of automatic pipettors become contaminated in use, disassemble them for decontamination and cleaning. After re-assembly, recalibrate them. Where it is not possible for this to be done in the laboratory, return the pipettors to the manufacturer for re-assembly and recalibration.

5.28 Thermometers and temperature-monitoring devices, including automatic recorders

5.28.1 Description

Thermometers are devices of the mercury-in-glass or alcohol-in-glass type that are used to monitor temperatures across the range of laboratory activities.

Other temperature-monitoring devices include platinum resistance thermometers and instruments that use thermocouples to measure temperature and provide a visual, hardcopy or electronic record of temperature variation with time.

Reference thermometers and other temperature-monitoring devices shall be calibrated to national or international standards. They shall be used for reference purposes only and shall not be used for routine monitoring.

Working thermometers and other temperature-recording devices shall be calibrated in a way that allows traceability to national or international standards.

NOTE Devices of adequate accuracy that conform to an appropriate international or national specification may also be used as working thermometers after verification of their performance.

5.28.2 Use

Thermometers and other temperature-monitoring devices shall be of suitable resolution to measure the temperature within the specified maximum permissible tolerance, depending on the criticality of the application.

The resolution of the temperature-monitoring device should be at least four times smaller than the range of the required maximum permissible tolerance. For example, for a maximum permissible tolerance of ±1 °C, the resolution should be at least 0,2 °C; for a maximum permissible tolerance of ±0,5 °C, the resolution should be at least 0,1 °C.

The measurement uncertainty of the reference thermometer calibration should also be taken into account when determining the operating temperature.

Thermometers or thermocouples placed in air incubators should be secured in suitable containers filled with glycerol, liquid paraffin or polypropylene glycol to buffer against heat loss when the door is opened and provide a stable reading. Use partial-immersion thermometers with only the bulb immersed, or use equivalent means to ensure stability.

Thermometers placed in water baths should be immersed in the water in accordance with individual specifications, e.g. partial-immersion thermometers should be immersed to the depth specified for that thermometer, usually 76 mm or 100 mm.

Do not use thermometers if the mercury or alcohol column is broken.

Mercury-in-glass thermometers are fragile and, if there is a risk of breakage, they should be placed inside protective cases that do not interfere with temperature measurements.

WARNING — Mercury is hazardous to health. Remove spillages in accordance with national regulations.

5.28.3 Verification

Reference thermometers shall be calibrated across the entire range against traceable national or international standards before initial use and at least every 5 years. Intermediate single point (e.g. ice point) calibration shall be performed to verify performance.

Reference thermocouples shall be fully calibrated against traceable national or international standards before initial use, checked regularly and with a frequency defined by the laboratory, in accordance with manufacturer's instructions. Intermediate checks shall be made against a reference thermometer to verify performance.

Other temperature-monitoring devices (such as radio wave receivers) shall be calibrated against traceable national or international standards in accordance with the manufacturer's instructions.

Working thermometers and thermocouples should be checked at the ice point and/or against a reference thermometer in the working temperature range.

5.28.4 Maintenance

Maintain thermometers and thermocouples in a clean and sound condition.

Maintain other temperature-monitoring devices in accordance with the manufacturer's instructions.

5.29 Immunomagnetic separator

5.29.1 Description

This equipment is used to separate and concentrate target microorganisms in liquid cultures by means of paramagnetic beads coated with an appropriate antibody.

Manual separators consist of a rotary mixer capable of 12 r/min to 20 r/min and a particle concentrator with a removable magnetic bar.

Automated separators use comb-like arrays of magnetic rods and tube racks. The magnetic particles are moved from tube to tube and permit the entire separation procedure, including washing stages, to be performed automatically in an enclosed environment.

5.29.2 Use and verification

Follow the manufacturer's instructions for use and those given in specific standards (e.g. for *E. coli* O157).

For manual systems, check the speed of rotation of the mixer.

For manual and automated systems, verify that the system is able to isolate low levels of the target microorganism before putting it into routine use.

It is important to appreciate the potential for cross-contamination during manual separation procedures and to take appropriate steps to avoid this happening.

5.29.3 Maintenance

Inspect and maintain equipment in accordance with the manufacturer's instructions.

5.30 Filtration system

The filtration system used shall be as described in ISO 8199.

5.31 Other equipment and software

Other equipment and its associated software shall be capable of achieving the accuracy required and shall comply with specifications relevant to the tests concerned. Calibration programmes if possible, and checks shall be established for key quantities or values where these properties have a significant effect on the result. Before routine use, calibrate if possible and check the equipment to establish that it meets the laboratory's requirements and complies with the relevant standard specifications. Any reconfigurations or modifications made by the laboratory to the software shall be verified to ensure that the modified software gives the correct result.

6 Preparation of glassware and other laboratory materials

6.1 Preparation

Glassware and other laboratory materials used in microbiology shall be of suitable design, used properly and prepared in such a manner as to guarantee its cleanliness and/or sterility up until the time of use. Laboratory grade water in accordance with ISO 3696[50] shall be used for rinsing reusable glassware after washing. Alkaline or acidic residues may be checked for by using a universal pH indicator solution; a pH within the range 6,5 to 7,3 should be achieved.

Glassware should be designed to prevent or limit contact between the operator and infectious material.

Tubes and bottles should be stoppered by appropriate means. If necessary, glassware to be sterilized (e.g. for the pipettes) should be placed in special containers or wrapped in an appropriate material (special paper, aluminium foil, etc.). Glassware to be autoclaved empty should allow free access of steam, otherwise sterilization will not be achieved.

6.2 Sterilization or decontamination

6.2.1 General

The temperature and duration of sterilization/decontamination should be recorded. Sterilization indicators may be used to distinguish between sterilized and unsterilized materials.

6.2.2 Sterilization by dry heat

Heat glassware, etc., in a sterilizing oven for at least 1 h at 170 °C ± 10 °C or equivalent.

6.2.3 Sterilization by moist heat (steam)

Moist steam under pressure is the most effective method of sterilization of laboratory glassware and materials. The temperature of the autoclave chamber shall remain at 121 °C ± 3 °C for at least 15 min (see 5.6).

6.2.4 Decontamination with chemical compounds

Use chemical compounds (e.g. chlorine-based products, alcohols, quaternary ammonium compounds) at appropriate concentrations and for an appropriate contact time.

Ensure that chemical residues do not affect the recovery of microorganisms.

6.3 Disposable equipment and materials

Disposable equipment and materials may be used instead of re-usable equipment and materials (glassware, Petri dishes, pipettes, bottles, tubes, loops, spreaders, etc.) if the specifications are similar.

It is advisable to verify that such equipment is suitable for use in microbiology (in particular as regards its sterility) and that the material contains no substances that inhibit the growth of microorganisms (see ISO 9998).

6.4 Storage of clean glassware and materials

Protect clean glassware and materials against dust during storage, in conditions which will maintain its cleanliness.

6.5 Management of sterile glassware and materials

Store glassware and materials under conditions that ensure that they remain sterile. Store single use equipment in accordance with the manufacturer's instructions, without any deterioration of the packaging. Store laboratory-prepared equipment in clean conditions.

When sterilizing equipment is intended for microbiology, put an expiry date (or date of manufacture) on each package.

6.6 Decontamination and disinfection

6.6.1 Decontamination of disposable equipment

Decontaminate disposable equipment before disposal in the laboratory or have them removed by a specialized contractor.

Besides the methods described in this clause, incineration may be used. If there is an incinerator on the premises, decontamination and disposal may be carried out in a single operation.

6.6.2 Decontamination of glassware and materials prior to use

In general, decontamination by sterilization of equipment should be done by moist heat (see 6.2.3) or dry heat (see 6.2.2).

In certain situations (e.g. field sampling), chemical decontamination may be appropriate. After such treatment, ensure that chemical residues do not affect the recovery of microorganisms.

6.6.3 Decontamination of glassware and materials after use

Materials for decontamination and disposal should be placed in containers, e.g. autoclavable plastic bags. Autoclaving is the preferred method for all decontamination processes (at least 30 min at 121 °C ± 3 °C). The autoclave should be loaded in a way that favours heat penetration into the load, (e.g. without overpacking) and taking care to loosen caps/lids and open bags.

Alternative methods, other than autoclaving, may be used if allowed by national regulations.

Autoclave all equipment which has been in contact with microbiological cultures (solid or liquid culture media), including re-usable containers prior to being washed.

During examination, decontamination by immersion in freshly prepared use-dilution disinfectant may be used for small-sized and corrosion-resistant equipment (e.g. pipettes).

Use Pasteur pipettes only once. Further details can be found in ISO 7712.[51]

Most disinfectants (see Annex A) have some toxic effects. Wear gloves and eye protection when handling concentrated disinfectant.

Materials contaminated with risk category 3 microorganisms and their containers shall be autoclaved before they are incinerated.

6.7 Waste management

Correct disposal of contaminated materials does not directly affect the quality of sample testing, unless it gives rise to potential for cross-contamination, but it is a matter of good laboratory practice.

Waste management should conform to national environmental or health and safety regulations.

A system for the identification and separation of contaminated materials and their containers should be established for

— non-contaminated waste (e.g. uncultured food samples) that can be disposed of with general waste;

— sharps, scalpels, needles, knives, broken glass;

— contaminated material for autoclaving and recycling; and

— contaminated material for autoclaving and disposal, or for disposal only if the material is to be incinerated (see, however, the special requirements for risk category 3 microorganisms below).

6.8 Washing

Wash re-usable equipment only after it has been decontaminated. After washing, rinse all equipment with deionized water.

Specialized equipment may be used to facilitate cleaning operations (e.g. a pipette washer, dishwasher, ultrasonic trough).

After washing, rinse all equipment with deionized water and ensure that residues do not affect the recovery of microorganisms. Additional rinses or checks may be needed to establish that equipment is suitably free from residues.

Page 30, Clause 7

Delete "ISO/TS 11133-1 and ISO/TS 11133-2", insert "ISO 11133".

Page 31, 8.2, paragraph 7

Delete "and ISO 8261".

Page 32, 8.4, paragraph 2, entry 3

Delete "ISO 6887-4 or ISO 8261", insert "ISO 6887-5".

Page 32, 8.5.1; page 34, 9.2.1

Delete ", or ISO 8261,".

Page 34, 9.2.2.2, paragraph 2; page 54, 14.2

Delete "ISO 16140", insert "ISO 16140-2".

Pages 34–49, Clause 10

Delete the existing text and insert the following. Underlined text has been modified.

10 Enumeration

10.1 General

When assessing the microbiological quality and/or safety of food and feeding stuffs, it is often not enough to know only which microorganisms are present. In most cases, the quantitative aspect is equally important, which brings about the need to enumerate microorganisms. This may be achieved in various ways: through direct examination (microscopy), by inoculating solid or liquid media, with flow cytometry, by real time polymerase chain reaction, etc. However, this International Standard will only cover enumeration using solid and liquid media.

Enumeration on solid media is based on the capacity of many microorganisms to produce colonies in or on agar media that can be recognized as such with the naked eye or with the aid of a simple magnifying glass.

If the matrix contains a high level of interfering particles,these should first be separated by settlement or use of filter bags.

If the level of bacteria is expected to be very low (less than 10 colonies in or on a plate at the lowest dilution), enumeration using liquid media is recommended (e.g. MPN) to improve statistical reliability of the results (see Annexes B and C). Concentration by filtration, immunoseparation or centrifugation may also be used.

10.2 Enumeration using a solid medium

10.2.1 General

Petri dishes should be labelled with the sample number, dilution, date and any other desired information.

Dilutions should be selected to ensure that plates containing the appropriate number of colonies are obtained (see 10.3.1) and to overcome any possible inhibitory properties.

Use a separate sterile pipette for transfers from each dilution, except if working from the highest dilution to the lowest dilution.

NOTE This subclause is written for the general case of 10^{-1} dilutions, but other forms of dilutions are allowed (e.g. dilute 1→5 or 1→2).

10.2.2 Number of Petri dishes per dilution

For enumeration techniques in food microbiology, one plate per dilution shall be used with at least two successive dilutions. Two plates per dilution may also be used to improve reliability.

If only one dilution is used, then two plates of this dilution shall be used according to Annex D to improve reliability of the results.

For laboratories that do not operate under quality assurance principles, two plates per dilution shall be used according to Annex D to improve reliability of the results.

10.2.3 Pour plate techniques

Withdraw the defined volumes of the dilution to be examined, touching the tip of the pipette against the side of the tube to remove excess liquid adhering to the outside. Lift the sterile Petri dish lid just high enough to insert the pipette, then dispense the contents.

After removing tempered agar medium from the water bath, blot the bottle dry with a clean towel to prevent water from contaminating the plates. Avoid spilling the medium on the outside of the container or on the inside of the plate lid when pouring. To avoid contamination of the media, hold the bottle in a near horizontal position. Also avoid setting down the bottle between pouring steps. Pour molten agar medium at 44 °C to 47 °C into each Petri dish (generally 18 ml to 20 ml of agar in 90 mm Petri dishes and 45 ml to 50 ml in 140 mm Petri dishes, to obtain at least 3 mm thickness) within 15 min of inoculation (to avoid aggregation of colonies). Avoid pouring the molten medium directly on to the inoculum. Immediately mix the molten medium and the inoculum carefully so as to obtain a homogeneous distribution of the microorganisms within the medium, e.g. by gently moving the dish backwards and forwards, from side to side and in a circular direction. Allow to cool and solidify by placing the Petri dish 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.) is expected in the product to be examined, overlay the solidified plates with sterile non-nutritive agar or agar identical to the culture medium used in the test (generally 5 ml of agar in 90 mm Petri dishes), in order to prevent or minimize spreading.

10.2.4 Surface inoculation

10.2.4.1 General

Methods of plating designed to produce only surface colonies on agar plates have certain advantages over the pour plate method. The morphology of surface colonies is easily observed, improving the analyst's ability to distinguish between different types of colony. Microorganisms are not exposed to the heat of the melted agar medium, so higher counts may be obtained.

Use pre-poured plates, of at least 3 mm thickness of the agar medium, that are level and free from air bubbles and surface moisture.

To facilitate uniform spreading, the surface of solidified agar should be dried in accordance with ISO 11133 or as specified in the relevant International Standard so that the inoculum is absorbed within 15 min.

10.2.4.2 Spreading-spatula method

Using a sterile pipette, transfer the inoculum (usually 0,1 ml or 0,5 ml) of the liquid test sample or of the initial suspension in the case of other samples to the agar plate (90 mm or 140 mm in diameter, respectively). Repeat this step for the next decimal dilution (the colonies to be counted will then be present in a dilution step of 10^{-1} in the case of liquid sample material and 10^{-2} in the case of other sample material) and, if necessary, repeat for further decimal dilutions.

The limit of enumeration can be lowered by a factor of 10 by inoculating 1,0 ml of the test sample if liquid, or 1,0 ml of the initial suspension for other products, either on the surface of one large agar plate (140 mm) or on the surface of three small agar plates (90 mm). In both cases, if only one dilution is used, prepare duplicates by using two large plates or six small ones.

Using a spreading spatula made of glass, plastic or steel (for example made from a glass rod and shaped like a hockey stick about 3,5 mm in diameter and 20 cm long, bent at right angles at about 3 cm from one end and flattened at the ends by heating), spread the inoculum as quickly as possible evenly over the agar surface without touching the side walls of the Petri dish. Allow the inoculum to absorb with the lids in place for about 15 min at room temperature.

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

10.2.4.3 Spiral-plate method

10.2.4.3.1 General

The spiral-plate method for determining the level of microorganisms has been tested in interlaboratory trials with milk and milk products and other foods.

The equipment used — the spiral plater — is described in 5.24.

10.2.4.3.2 Preparation of agar plate

An automatic dispenser with a sterile delivery system is recommended for the preparation of agar plates, to help ensure that the plates are level of consistent thickness, free from bubbles, and sterile.

Pour the same quantity of agar into all the plates so that the same height of agar will be presented to the spiral plater stylus tip in order to maintain the correct contact angle.

Dry the plates (see ISO 11133) before use and ensure no water droplets are visible on the surface of the medium.

10.2.4.3.3 Plating procedure and counting

Decontaminate the stylus tip and tubing by drawing first sodium hypochlorite solution (see 5.24.4) and then sterile water through the system before drawing the liquid sample into the stylus. Alternatively replace the disposable micro-syringe in equipment where these are fitted.

Place a pre-poured agar plate in a Petri dish on the turntable and lower the stylus. The sample is differentially dispersed as the stylus tip rides on the surface of the rotating agar plate. Remove the inoculated plate and return the stylus to its starting position. Decontaminate the stylus or replace the micro-syringe and load for the inoculation of another plate.

Allow the inoculum to absorb, with the lid in place, for no longer than 15 min at ambient temperature, then incubate (see 10.2.5).

After incubation, put the spiral-plate counting grid centrally in place. Use the counting rule of 20 for determining counts. Choose any wedge and begin counting colonies from the outer edge of the first segment towards the centre until 20 colonies have been counted. Complete by counting the remaining colonies in the segment containing the twentieth colony. Count a corresponding area on the opposite side of the plate and divide the number of colonies counted on both sides by the sample volume deposited in these two areas. The volumes of sample associated with each portion of the counting grid are given in the operating manual that accompanies each spiral plater.

10.2.5 Incubation

Unless otherwise stated in specific standards, invert dishes once they have been inoculated, 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, minor variations in the incubation temperature may be unavoidable and acceptable, for example during the usual operations of loading or unloading the incubator, but it is important that these periods are kept to a minimum. The duration of these variations should be monitored to ensure that they do not have a significant effect on the result.

It may sometimes be useful to laboratory operations to refrigerate inoculated dishes before incubation for no more than 24 h. If this is done, the laboratory shall ensure that this practice does not affect the resulting counts.

Generally, Petri dishes should be stacked no more than six high for aerobic incubation and should be separated from each other and from the incubator walls by at least 25 mm. However, higher stacks with less spacing may be acceptable in incubators fitted with air circulation systems; in this case, the temperature distribution should be verified.

After incubation, the dishes should normally be examined immediately. They may, however, be stored, unless otherwise specified in specific standards, for up to 48 h in a refrigerator. Refrigerated storage is only acceptable if it has been shown to have no effect on the numbers, appearance or the subsequent confirmation of the colonies. With certain media containing indicator dyes, refrigerated plates should be allowed to equilibrate at room temperature before examining, to ensure that the correct colour is regained.

10.3 Calculation and expression of results obtained with solid media

10.3.1 Counting of colonies

Following the period of incubation stated in the specific standard, count the colonies (total colonies, typical colonies or presumptive colonies) for each dish containing up to and including 300 colonies (or any other number stated in the specific standard).

When counting typical or presumptive colonies, the description of the colonies shall be as given in the specific standard.

In certain cases, it may be difficult to count the colonies (e.g. where spreading microorganisms are present). Consider spreading colonies as single colonies. If less than one-quarter of the plate is overgrown by spreading, count the colonies on the unaffected part and calculate by extrapolation the theoretical number of colonies for the entire plate. If more than one-quarter is overgrown, discard the count. Consider a chain of colonies as one colony forming unit.

NOTE In certain cases, it can be useful to make provision for storing inoculated dishes at (3 ± 2) °C for use in comparison with incubated inoculated dishes when counting, in order to avoid confusing particles of the product being examined with colonies. A binocular magnifying glass can also be used to distinguish particles of product from the colonies.

Various methods of calculation are given in 10.3.2, Annex B and Annex D taking account of special cases and giving information on the confidence intervals

In the various methods of calculation, account shall be taken of dishes containing no colonies, where these dishes have been retained, because when calculating a weighted mean these are significant.

When a spiral plater has been used, the colony counting is as described in 10.2.4.3.3.

10.3.2 Expression of results

10.3.2.1 General

10.3.2.1.1 The cases dealt with in this subclause are general cases:

— inoculation of one 90 mm-diameter Petri dish per dilution, and at least two successive dilutions are performed;

— maximum countable number for the total colonies present: 300 per dish;

— maximum total number of colonies (typical and atypical) present on a dish when counting typical or presumptive colonies: preferably 300 per dish;

— maximum countable number for typical or presumptive colonies: 150 per dish;

— number of presumptive colonies inoculated for identification or confirmation (see 10.3.2.3) in each dish retained: in general 5.

These figures are defined in the specific standards. For microorganisms producing large colonies the maximum numbers of colonies in a plate are specified in the specific standard.

When dishes with a diameter different from 90 mm are used, the maximum number of colonies shall be increased or decreased in proportion to the surface area of the dishes (or membranes).

EXAMPLE

— For poured plates with a diameter of 55 mm, the maximum countable number of colonies shall be 110 (equivalent to a total count of 300 cfu on a 90 mm Petri dish).

— For plate with a diameter of 140 mm, the maximum countable number of colonies shall be 730 (equivalent to a total count of 300 cfu on a 90 mm Petri dish).

10.3.2.1.2 The methods of calculation given below are for the cases which occur most frequently when tests are carried out in accordance with good laboratory practice. Special cases may occasionally occur (for example, the ratio of the dilution factors used for two successive dilutions may be very different), and it is therefore necessary for the counting results obtained to be examined and interpreted by a qualified microbiologist and, if necessary, rejected.

10.3.2.2 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 at least 10 colonies [total colonies, typical colonies or colonies complying with identification criteria (see 10.3.2.3)].

Calculate the number N of microorganisms present in the test sample as a weighted mean from two successive dilutions using Formula (1):

$$N = \frac{\sum C}{V \times 1{,}1 \times d} \qquad (1)$$

where

ΣC is the sum of the colonies counted on the two dishes retained from two successive dilutions, at least one of which contains a minimum of 10 colonies;

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

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

If more than one dilution is used, the ratio between the colony count of dilution d_2 and the colony count of dilution d_1 is expected to be 10 %. The upper and lower limits should be specified by the laboratory for the colony count of dilution d_2. These limits may be the ones proposed in ISO 14461-2,[42] for example. When these limits are not followed, the results should be interpreted with caution.

EXAMPLE If colony count of dilution d_1 is 250, the colony count of dilution d_2 should not be less than 13 (5,2 %) and not be more than 39 (15,6 %). See ISO 14461-2.[42]

Round off the calculated result to two significant figures. When doing 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.

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

Report the result as the number N of microorganisms per millilitre (liquid products) or per gram (other products).

EXAMPLE Counting has produced the following results:

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

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

$$N = \frac{\sum C}{V \times 1{,}1 \times d} = \frac{168 + 14}{1 \times 1{,}1 \times 10^{-2}} = \frac{182}{0{,}011} = 16\,545$$

Rounding off the result as specified above, the number of microorganisms is 17 000 or $1{,}7 \times 10^4$ per millilitre or per gram of product.

10.3.2.3 Method of calculation: after identification

When the method used requires identification, a given number A (generally 5) of presumptive colonies is identified from each of the dishes retained for the colony counting. After identification, calculate, for each of the dishes, the number a of colonies complying with identification criteria, using Formula (2):

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

where

b is the number of colonies complying with identification criteria among the identified colonies A;

C is the total number of presumptive colonies counted on the dish.

Round off the calculated result to the nearest whole number. When doing 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 in the formula given in 10.3.2.2 or by replacing C by a in the formula given in 10.3.2.4.1 and 10.3.2.5.3. Round off the result as specified in 10.3.2.2.

Express the result as specified in 10.3.2.2, 10.3.2.4.1 and 10.3.2.5.3, respectively.

EXAMPLE Counting has produced the following results:

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

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

Testing of selected colonies was carried out:

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

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

$$N = \frac{\sum a}{V \times 1{,}1 \times d} = \frac{50 + 4}{1 \times 1{,}1 \times 10^{-3}} = \frac{54}{1{,}1 \times 10^{-3}} = 49\,090$$

Rounding off the result as specified in 10.3.2.2, the number of microorganisms is 49 000 or $4{,}9 \times 10^4$ per millilitre or per gram of product.

10.3.2.4 Method of calculation: low counts

10.3.2.4.1 Case when one dish (test sample or initial suspension or first dilution) contains less than 10 colonies

Counts from 10 up to the upper limit of each method are in the optimum precision range. Precision decreases rapidly as the number of colonies decreases below 10. However, depending on the purpose of the test, a lower limit of determination can be defined as given below for counts lower than 10.

According to ISO/TR 13843,[40] the definition of limit of determination is: "Lowest average particle concentration x per analytical portion where the expected relative standard uncertainty, equals a specified value (RSD)". The term "coefficient of variation" is now preferred to RSD. The coefficient of variation, C_V, is calculated by dividing the estimate of the standard deviation s for a population from a sample by the mean $\overline{x}$ for that sample. Thus, $C_V = s / \overline{x}$.

In the case of a Poisson distribution, x is calculated by Formula (3):

$$x = \frac{1}{C_V^2} \tag{3}$$

If C_V is set at 50 % as the limit of acceptable relative precision (which seems to be reasonable in microbiology), the lower limit of determination will be at the number of colonies given by:

$$x = \frac{1}{0{,}50^2} = 4$$

Thus, results based on counts less than four should be treated as mere detection of the presence of the microorganism.

Summarizing:

If the plate contains less than 10 colonies, but at least four, calculate the result using the formula:

$$N_E = \frac{C}{V \times d}$$

and report it as the estimated number N_E of microorganisms per millilitre (liquid products) or per gram (other products).

NOTE The term "estimated number" means a less precise estimate of the true value.

If the total is from 3 to 1, the precision of the result is too low and the result shall be reported as:

"Microorganisms are present but less than 4/Vd per gram or per ml".

10.3.2.4.2 Case when the dish (test sample or initial suspension or first dilution) contains no colonies

If the dish containing the test sample (liquid products) or the initial suspension (other products) or the first dilution inoculated or retained does not contain any colonies, report the result as follows:

"less than 1/Vd microorganisms per millilitre" (liquid products) or

"less than 1/Vd microorganisms per gram" (other products)

where

d is the dilution factor of the initial suspension or of the first dilution inoculated or retained ($d = 10^0 = 1$ where the directly inoculated test sample of liquid product is retained);

V is the volume of the inoculum used in each dish, in millilitres.

10.3.2.4.3 Special cases

10.3.2.4.3.1 General

These cases concern the counting of typical or presumptive colonies.

10.3.2.4.3.2 Case 1

If the number of typical and atypical colonies for the dish 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, the dish containing the subsequent dilution d_2 contains up to and including 300 colonies (or any other number stated in the specific standard), and no typical or confirmed colony is visible, report the result as follows:

"less than $1/V_2d_2$ and more than $1/V_1d_1$ microorganisms per millilitre" (liquid products) or

"less than $1/V_2d_2$ and more than $1/V_1d_1$ microorganisms per gram" (other products)

where

d_1 and d_2 are the dilution factors corresponding to the dilution d_1 and d_2;

V_1 is the volume of the inoculum used in dish of the first dilution d_1, in millilitres;

V_2 is the volume of the inoculum used in dish of the subsequent dilution d_2, in millilitres.

EXAMPLE Counting has produced the following results:

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

— at the second dilution retained (10^{-3}): 33 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.

10.3.2.4.3.3 Case 2

If the number of typical and atypical colonies for the dish 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 the dish containing the subsequent dilution d_2 contains up to and including 300 colonies (or any other number stated in the specific standard) and no typical or confirmed colonies are visible, report the result as follows:

"less than $1/V_2d_2$ microorganisms per millilitre" (liquid products); or

"less than $1/V_2d_2$ microorganisms per gram" (other products)

where

d_2 is the dilution factor corresponding to the dilution d_2;

V_2 is the volume of the inoculum used in dish of the subsequent dilution d_2, in millilitres.

EXAMPLE Counting has produced the following results:

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

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

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

10.3.2.5 Method of calculation: special cases

NOTE 1 Some examples do not comply with the rules in 10.3.2.2 which specified the upper and lower limits of colony count of the dilution d_2 compared to the results of dilution d_1. If repeat analysis is not possible or appropriate, results can be calculated and expressed as indicated in clause 10.3.2.2. but the precision is less than in the normal situation and this should be indicated in the test report.

NOTE 2 All the explanations and examples are made with the case when one Petri dish per dilution is used. For the case when two dishes per dilution is used see Annex D.

NOTE 3 The lower limits of colony count of the dilution d_2 proposed in the examples come from ISO 14461-2.[42]

NOTE 4 The figures of the confidence interval should be adapted to the maximal number specified for the colony count.

10.3.2.5.1 If the number of colonies counted (total colonies, typical colonies or presumptive colonies) is greater than the maximum countable number (300 or any other number stated in the specific standard) for the dish containing a first dilution d_1, with a number of colonies (total colonies, typical colonies or colonies complying with identification criteria) of less than 10 (low counts limit) for the dish containing the subsequent dilution d_2, express the results as follows:

NOTE These items of this subclause are given as example

a) If the number of colonies for the dish containing the dilution d_1 is within the interval [maximum countable number plus 1, upper limit of the confidence interval for the maximum countable number] (e.g. 301-334) (Annex B) and the colony count of the dilution d_2 is not less than the lower limit specified in 10.3.2.2, use the calculation method for general cases (see 10.3.2.2).

EXAMPLE 1 Counting (when a maximum number of 300 has been set for the counting of colonies) has produced the following results:

— at the first dilution retained (10^{-2}): 310 colonies (lower than 334, upper limit of the confidence interval for 300);

— at the second dilution retained (10^{-3}): 8 colonies. The lower limit for the colony count at the dilution (10^{-3}) is 18 colonies, specified from 310 colonies of the dilution (10^{-2}).

The result of this colony count is unacceptable.

EXAMPLE 2 Counting (when a maximum number of 150 has been set for the counting of colonies) gives the following results:

— at the first dilution retained (10^{-2}): 160 colonies (lower than 174, upper limit of the confidence interval for 150);

— at the second dilution retained (10^{-3}): 8 colonies. The lower limit for the colony count at the dilution (10^{-3}) is 7 colonies, specified from 160 colonies of the dilution 10^{-2}.

Use the method of calculation for general cases (10.3.2.2) using the dishes for the two dilutions retained.

b) if the number of colonies for the dish containing the dilution d_1 is greater than the upper limit of the confidence interval for the maximum countable number (for example 334) and the colony count of the dilution d_2 is not less than the lower limit specified in 10.3.2.2 and calculated from the upper limit of the confidence interval for the maximum countable number, only take account of the result of the count of dilution d_2 and calculate an estimated count (see 10.3.2.4.1).

EXAMPLE 1 Counting (when a maximum number of 300 has been set for the counting of colonies) has produced the following results:

— at the first dilution retained (10^{-2}): more than 334 colonies in the dish;

— at the second dilution retained (10^{-3}): 9 colonies. The lower limit for the colony count at the dilution (10^{-3}) is 20 colonies, specified from 334 colonies of the dilution 10^{-2}.

The result of this colony count is unacceptable.

EXAMPLE 2 Counting (when a maximum number of 150 has been set for the counting of colonies) has produced the following results:

— at the first dilution retained (10^{-2}): more than 174 colonies in the dish;

— at the second dilution retained (10^{-3}): 9 colonies. The lower limit for the colony count at the dilution (10^{-3}) is eight colonies, specified from 174 colonies of the dilution 10^{-2}.

Report an estimated count on the basis of the colonies counted in the dish for the 10^{-3} dilution (see 10.3.2.4.1).

EXAMPLE 3 Counting (when a maximum number of 150 has been set for the counting of colonies) has produced the following results:

— at the first dilution retained (10^{-2}): more than 174 colonies in the dish;

— at the second dilution retained (10^{-3}): 5 colonies. The lower limit for the colony count at the dilution (10^{-3}) is eight colonies, specified from 174 colonies of the dilution 10^{-2}.

The result of this count is unacceptable.

10.3.2.5.2 Where the counting of colonies (total colonies, typical colonies or presumptive 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), report the result as follows:

"more than $300/Vd$" (in the case of total colonies or typical colonies); or

"more than $(300/Vd) \times (b/A)$ (in the case of confirmed colonies), expressed in microorganisms per millilitre

(liquid products) or microorganisms per gram (other products)

where

d is the dilution of the last inoculated dilution;

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

b is the number of colonies complying with identification criteria among the inoculated colonies A.

10.3.2.5.3 Where only the dish containing the last inoculated dilution is countable and contains at least 10 colonies and up to and including 300 (or any other number stated in the specific standard) colonies (total colonies, typical colonies or presumptive colonies), calculate the number N' of microorganisms present using Formula (4):

$$N' = \frac{c}{Vd} \quad (4)$$

where

c is the number of colonies counted in the dish;

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

d is the dilution corresponding to the dilution retained.

Round off the result as specified in 10.3.2.2.

Report the result as the number N' of microorganisms per millilitre (liquid products) or per gram (other products).

EXAMPLE Counting has produced the following results:

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

Thus

$$N' = \frac{120}{1 \times 10^{-4}} = 1\,200\,000$$

Rounding off the result as specified in 10.3.2.2, the number N' of microorganisms is 1 200 000, or $1{,}2 \times 10^6$ per millilitre or per gram.

10.3.2.6 Measurement of uncertainty

See ISO/TS 19036 for quantitative determinations.

10.4 Enumeration of yeasts and moulds

10.4.1 General

Yeasts and moulds should usually be enumerated either by a pour-plate technique which allows easier enumeration or by a surface spread-plate technique which provides maximum exposure of the cells to atmospheric oxygen and avoids heat stress from molten agar. Pre-poured agar plates should be dried before being inoculated (see ISO 11133).

Some yeasts and moulds can be infectious or can elicit allergic responses, sometimes even in healthy individuals. Thus it is important to be reasonably cautious when working with them. Ideally, plates should be kept in incubators, not in an open room. Plate lids should be removed as infrequently as possible, normally only for essential purposes, e.g. the preparation of a slide for microscopic examination. Flamed needles should be cooled before making transfers, to avoid dispersal of conidia and other cells. Work benches and incubators should be disinfected routinely.

Petri dishes should be incubated in an upright position and not disturbed until the plates are ready to be counted, as movement can result in the release of mould conidia or spores and the subsequent development of satellite colonies, giving an overestimate of the population.

10.4.2 Counting of colonies for yeasts and moulds

Plates with 10 to 150 colonies are usually counted. If the mycoflora consists primarily of moulds, select dishes containing counts in the lower population range; if the mycoflora consists primarily of yeasts, dishes containing counts up to the upper limit may be selected for counting.

If the identity of the colonies is in doubt, examine wet mounts or stains of cells from at least five colonies per sample to confirm that bacteria are not present.

10.5 Enumeration using a liquid medium

10.5.1 Principle

Test portions are inoculated into a liquid medium that is designed to support the growth of a particular microorganism or a group of microorganisms and often inhibits proliferation of non-target microorganisms.

To determine whether growth of the target microorganisms has occurred, various criteria can be used, e.g. visual detection of turbidity, gas production, colour changes, subsequent isolation of the microorganisms on a selective agar medium along with incubation atmosphere and temperature. The composition of the growth medium and the criteria for discriminating between a positive and a negative result are defined in the corresponding standards.

Using this approach, only a qualitative value can be attributed to each test portion, i.e. the result is either positive or negative. To obtain an estimate of the quantity of microorganisms that is present, it is necessary to examine several test portions, initial suspension and dilutions and use statistical procedures to determine the most probable number (MPN).

10.5.2 Inoculation

10.5.2.1 General

If a selective growth medium is used, the addition of the test portion should not reduce its selective properties, thereby allowing the growth of non-target microorganisms. In most standards, information about the compatibility of a specific matrix and the liquid medium is described in the scope, but care should be taken with matrices like spices, cocoa, bouillon, etc. as they may contain growth-inhibiting substances which require the addition of neutralizing compounds, the use of higher dilution factors, centrifugation, filtration or immuno-magnetic separation to separate the target microorganisms from the matrix, even though this is not always specifically defined in the corresponding standards. Incompatibility can also be due to the biological composition of the matrix: heavily contaminated environmental samples, fermented products or products with probiotic bacteria obviously represent a bigger challenge for the analytical microbiologist than samples which contain only very few microorganisms. For these problematic matrices, spiking experiments using representative microorganisms should be performed to verify that the method is indeed compatible with the matrix.

If MPN is used for the analysis of solid samples (including powders), only one test portion shall be taken for preparing the initial suspension. From this initial suspension, aliquots should be used for examination.

10.5.2.2 Procedure

Unless stated otherwise in the corresponding standards, test portion volumes of less than, or equal to, 1 ml are normally added to five to 10 times the volume of single strength media. Test portions between 1 ml and 100 ml are normally added to equal volumes of double-strength media.

For volumes greater than 100 ml, more concentrated media may be used. For special purposes, sterile dehydrated media may be dissolved in the cold (or pre-warmed to 30 °C) sample to be analysed.

Unless stated otherwise, the time lapse between preparing the initial suspension of a sample and inoculation of the last tube, multiwell plate or bottle should be less than 30 min.

10.5.3 Choice of inoculation system

The essence of the MPN method is the dilution of a sample to such a degree that inocula will sometimes but not always contain viable target microorganisms. The "outcome", i.e. the number of inocula producing growth at each dilution, will give an estimate of the initial concentration of bacteria in the sample. In order to obtain estimates over a broad range of possible concentrations, microbiologists use serial dilutions, incubating several tubes (or plates, etc.) at each dilution. The MPN of microorganisms present in the original sample, and the precision of the estimate, can be calculated by statistical procedures on the basis of the numbers of positive and negative tubes observed after incubation.

Make a choice from the various MPN configurations available according to

— the expected number of microorganisms in the sample under investigation;

— regulatory requirements;

— the precision needed; and

— any other practical considerations.

The measurement uncertainty depends on the number of positive test portions observed in a similar way as the measurement uncertainty of a colony count depends on the number of colonies on a plate. Measurement uncertainty changes as a function of the square root of the number of tubes used, since precision increases with increasing numbers of replicate tests; but it is necessary to quadruple the number of tubes to halve the measurement uncertainty. When systems have only a few replicate tubes, the relative measurement uncertainty will be high.

Depending on their size, test portions can be inoculated into tubes or bottles containing the required amount of liquid medium. For small test portions, multiwell plates can also be used.

10.5.3.1 Single dilution system

When the expected concentration of microorganisms is small or expected to vary only moderately, the most appropriate inoculation system is a single series of equal test portions. Where the expected ratio between the maximum and minimum number of microorganisms is less than about 25, 10 parallel test portions is the smallest number expected to provide useful information; with 50 parallel tubes, a ratio of 200 is the limit. If the actual concentration is near the extreme of the possible MPN values, then the chance of all growth or all non-growth tubes probably is too high. Examples of single dilution MPNs are given in Tables C.1 to C.4.

10.5.3.2 Multiple-dilution system

When the concentration of microorganisms in the sample is unknown, or if great variation is anticipated, it may be necessary to inoculate series of tubes from several dilutions. Inoculate a sufficient number of dilutions to ensure a system with both positive and negative results. The number of dilutions to be used depends also on the calculation method used for estimating the MPN value. If MPN tables are to be used, then the configurations of the systems are restricted to those available in tables. With computer programs, the numbers of dilutions and parallel tubes are not restricted, which is important in regard to use of new commercial MPN test kits.

10.5.3.3 Symmetric dilution system

The most commonly applied symmetric MPN system uses three or five parallel tubes per dilution. The precision obtained with these systems with small numbers of tubes per dilution is very low. Results from a three-tube design are hardly more than indications of the order of magnitude of the concentration. If more precision is required, it is recommended that five or more parallel tubes be chosen. Examples of a three tube MPN, a five tube MPN and a 10 tube MPN are given in Tables C.5, C.6 and C.7, respectively.

10.5.3.4 Non-symmetric dilution system

Non-symmetric systems have different numbers of tubes at different dilution levels and should be used only to estimate numbers of microorganisms within a well-defined range (see for example ISO 8199). Occasionally a tube may be lost or broken leading to a non-symmetrical system. However, some commercial test kits are based on non-symmetrical systems. It is recommended that data from such systems be evaluated using an appropriate computer software program (see 10.5.6.3).

10.5.4 Incubation

Incubate the inoculated tubes, flasks or bottles in an incubator or in a water bath. Place multiwell plates in an incubator.

Choose the duration and the temperature of incubation after reference to a specific standard method, as they depend on the microorganism or group of microorganisms sought.

For some microorganisms, a two stage incubation procedure and/or a confirmation step may be necessary. Refer to the specific standards for details, but note that this may add a complication to the derivation of MPN values (see Reference [52]).

10.5.5 Interpretation of results

The criteria that distinguish positive from negative results vary with each microorganism or group of microorganisms and are defined in the corresponding standards. Using these criteria, count and record the number of positive results obtained with all the test portions derived from one sample.

10.5.6 Determination of MPN values

There are three different possibilities for determining the MPN value: calculation using mathematical formulae, consultation of MPN tables, or utilization of specific computer programs. Provided that they are based on the same statistical considerations, they are equally valid. These three approaches are detailed below.

10.5.6.1 Mathematical formulae

10.5.6.1.1 Approximate formula for all cases

The approximate MPN value (M) for any number of dilutions and parallel tubes can be derived by application of Formula (5):

$$M / \mathrm{g} = \frac{Z_\mathrm{p}}{\sqrt{m_\mathrm{s} m_\mathrm{t}}} \tag{5}$$

where

Z_p is the number of positive tubes;

m_s is the total mass, in grams, of sample in all tubes with negative reactions;

m_t is the total mass, in grams, of sample in all tubes.

EXAMPLE (from Reference [53])

Suppose you test 10 portions each of 0,1, 0,01 and 0,001 g of sample and find 10, 4 and 2 positive results then, for the fractional responses only (i.e. 4 & 2,) the total number of positives is $Z_\mathrm{p} = 6$. The total mass tested is $m_\mathrm{t} = 10 \times 0{,}01 + 10 \times 0{,}001 = 0{,}11$ g; and the total mass in negative tubes is $m_\mathrm{s} = 6 \times 0{,}01 + 8 \times 0{,}001 = 0{,}068$.

$$M / \mathrm{g} = \frac{6}{\sqrt{0{,}068 \times 0{,}11}} = \frac{6}{\sqrt{0{,}007\,5}} = \frac{6}{0{,}086\,5} = 69{,}4$$

This result compares well with the tabulated value for 10 – 4 – 2, which is an MPN per gram of 70.

10.5.6.1.2 "Exact" solution for one series of tubes

The MPN value (M) for a single series of tubes is derived from Formula (6):

$$M / \mathrm{g} = -\frac{1}{m} \ln \left[\frac{S}{N} \right] \tag{6}$$

where

m is the mass, in grams, of sample in each tube of the series;

ln is the natural logarithm;

N is the number of tubes in the series;

S is the number of tubes with a negative reaction.

10.5.6.1.3 Precision estimates for single dilution assays

The 95 % confidence bounds of the MPN estimate can be calculated approximately from the derived MPN and the standard deviation of $\log_{10} M$ using the procedure of Reference [55]), as described in Reference [53] — see also Reference [52]:

NOTE According to ISO 80000-2:2009,[57] Clause 12, the symbol for decimal logarithms is "lg". However, in this International Standard, the symbol "$\log_{10}$", widely used in the community of food microbiology laboratories, is preferred.

The standard error of the MPN ($s_{\log_{10} M}$) is calculated using Formula (7):

$$s_{\log_{10} M} = \frac{1 - \exp(-Mm)}{2{,}303 \times Mm \sqrt{G \exp(-Mm)}} \tag{7}$$

where

M is the MPN;

m is the mass of inoculum per tube;

G is the number of tubes showing growth and e = exponential factor.

The normal approximation to the confidence bounds is given by $M \pm 1{,}96 \times s_{\log_{10} M}$.

EXAMPLE

Suppose that 0,1 g of sample (m) was inoculated into each of n = 20 tubes and that you have G = 4 positive results (hence there are 16 negative results). The MPN value (M) per gram is given by

$$M = -\frac{1}{m} \ln \left(\frac{S}{N} \right) = -\frac{1}{0{,}1} \times \ln \left(\frac{16}{20} \right) = -10 \times \ln(0{,}8) = 2{,}23$$

and the standard error $s_{\log_{10} M}$ by

$$s_{\log_{10} M} = \frac{1-\exp(-Mm)}{2{,}303 \times Mm\sqrt{G\exp(-Mm)}}$$

$$= \frac{1-\exp(-0{,}223)}{2{,}303 \times 0{,}223\sqrt{4 \times \exp(-0{,}223)}}$$

$$= \frac{(1-0{,}80)}{0{,}513\,6 \times \sqrt{3{,}2}} = \frac{0{,}20}{0{,}918\,7} = 0{,}22$$

Now, $\log_{10} M$ = 0,35 and the normal approximation to the confidence bounds is given by $M \pm 1{,}96 \times s_{\log_{10} M}$.

The lower bound of $\log_{10} M$ is 0,35 – (1,96 × 0,22) = 0,35 – 0,43 = –0,08.

The upper bound of $\log_{10} M$ is 0,35 + 0,43 = 0,78.

So the lower bound of M is antilog_{10}(–0,08) = 0,82 and the upper bound of M is antilog_{10} (0,78) = 6,1.

However, it is not necessary to make these calculations manually since they can be determined using the MPN calculator (10.5.6.3).

10.5.6.1.4 Precision estimates for symmetrical multiple-dilution assays

The decimal logarithm of the standard deviation of a symmetrical multiple-dilution MPN system can be obtained from Cochran's approximate Formula (8) (Reference [28]):

$$s = 0{,}58\sqrt{\frac{\log_{10} f}{N}} \quad (8)$$

where

s is the standard deviation of the decimal logarithm of the MPN;

f is the dilution factor between consecutive dilutions (usually 10);

N is the number of tubes per dilution.

The upper and lower 95 % confidence bounds can be approximated, respectively, by multiplying and dividing the MPN estimate by the antilogarithm of $2s$. This procedure tends to exaggerate the upper confidence limit. A more precise estimate can be obtained using the MPN calculator (10.5.6.3).

10.5.6.2 MPN tables

10.5.6.2.1 Tables for single dilution systems

Tables C.1 to C.4 give the MPN values and the 95 % confidence bounds per test portion for 10, 15, 20 and 25 parallel tubes (assuming that each tube is inoculated with the same volume of a single dilution).

To express the outcome per sample reference mass (or volume for liquid samples), multiply the MPN and the 95 % limit values by the ratio of the reference mass to the test portion mass. Do not multiply the logarithmic standard uncertainty. The reference mass in food microbiology is usually 1 g. The test portion mass corresponds to the amount of sample (in grams) that is present in the volume used to inoculate the tubes, e.g. 0.1 g if 1 ml of the 10^{-1} homogenate has been used.

EXAMPLE (Reference [30])

A total of 20 tubes of double-strength broth were inoculated with 5 ml aliquots of a sample diluted 10-fold (0,1 g/ml). After incubation, 16 of the tubes showed visible growth. What was the MPN of bacteria (as organisms per gram) in the sample?

Table B.3 gives 1,61 as the MPN of organisms per tube, which is enclosed within lower and upper 95 % bounds of 0,93 and 2,77.

Each tube received a test portion of 5 ml, which corresponds to 0,5 g of sample. Therefore, the MPN of microorganisms (M) in 1 g of sample is given by:

$$M = \frac{1{,}61}{0{,}5} \Big/ \text{g} = 3{,}22/\text{g}$$

with 95 % confidence bounds ranging from, for the lower 2,5 % limit:

$$\frac{0{,}93}{0{,}5} \Big/ \text{g} = 1{,}9/\text{g}$$

for the upper 97,5 % limit:

$$\frac{2{,}77}{0{,}5} \Big/ \text{g} = 5{,}5/\text{g}$$

10.5.6.2.2 Tables for multiple-dilution systems: three successive dilutions

With symmetrical systems, it is common practice to use three successive dilutions with three (Table C.5), five (Table C.6) or 10 replicates (Table C.7). Record the number of positive results for each set of tubes and, from the MPN table for the inoculation system used, read the MPN of microorganisms present in the reference volume of the sample.

These revised tables also provide the decimal logarithm of the MPN, its standard deviation, $s_{(\log_{10} M)}$, the lower and upper confidence limits of the approximate 95 % confidence interval together with a 'rarity value and a 'rarity category'. The 'rarity value' (based on work by Blodgett, References [52]–[54]) provides a simpler approach to assessment of the likelihood that an observed result will be obtained in a test.

Some combinations of positive tubes are more likely to occur than others; for example, a combination of positive results 0 – 0 – 3 is much less likely to occur than the combination 3 – 2 – 1. To quantify this probability, the rarity index has been calculated as the ratio of two likelihood values:

$$r = \frac{L(\hat{\mu})}{L_0(\hat{\mu})}$$

where

$L(\hat{\mu})$ is the likelihood of the observed result, x_1, x_2 ... x_k, of the serial dilution test;

$L_0(\hat{\mu})$ is the likelihood if the result were most likely under a concentration μ equal to the estimate $\hat{\mu}$ of the concentration μ.

Full details of the procedure for calculation of the likelihood functions are given in Reference [56].

The rarity index is a value between 0 and 1. It is 1 if the result of the serial dilution test is most likely a concentration equal to the estimated MPN. If it is in the neighbourhood of 0 the result of the serial dilution test is very unlikely for a concentration equal to the estimated MPN. Following the approach of Reference [27] we use three categories of rarity:

Category 1: the MPN value would be very likely to occur if its rarity value falls within the range 0,05 to 1,00, $0,05 \le n \le 1,00$; i.e. such a result would be likely to occur by chance on 95 % of occasions.

Category 2: the MPN value would be expected to occur only rarely if its rarity value falls within the range 0,01 to 0,05, $0,01 \le n \le 0,05$; such a result would be likely to occur by chance with a frequency less than 5 %.

Category 3: the MPN value would be expected to occur extremely rarely if its rarity value falls within the range 0 to 0,01, $0 \le n \le 0,01$; i.e. such a result would be expected to occur by chance less than once in 100 tests.

The tables show only those combinations of results that fall within categories 1 and 2.

In any circumstance when more than three dilutions are made, it is essential that all measured data values be used. It is not scientifically correct to "select" any combination of values on the premise that these values are more "correct" than other combinations. The results from all possible combinations of positive tubes should be recorded and the MPN calculator (10.5.6.3) used to derive MPN values.

10.5.6.3 Computer programs

Programs used to determine MPN values are required to identify improbable tube combinations since such combinations may reflect a problem with the performance of the microbiological test (see 10.5.6.2.2 with respect to unlikely tube combinations and the use of MPN tables). Several computer programs are vailable for estimation of MPN values using an Excel® (see Note) spreadsheet format, but many are incapable of assessing whether improbable tube combinations have been entered into the program and different approaches have been used for estimation of confidence limits.

A new software program for use in Excel® (see Note) has been written that can handle up to 10 levels of serial dilution. The program has been used to derive the MPN estimates and their parameters shown in the revised Tables C.5, C.6, and C.7. It is highly recommended that this program be used in preference to others, since the results for any specific combination of results derived with 3 dilutions will be the same as those in the published tables. Details of the calculations are described in Reference [55] and the software is freely available for download from

http://standards.iso.org/iso/7218/

If the Excel® spreadsheet highlights a tube combination as improbable, the associated MPN value should not be reported.

NOTE Excel is the trade name of a product supplied by Microsoft. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Pages 57 to 63, Annex B

Delete the existing text and insert Annexes B to D, which start on the next page. The new Annex C replaces the former Annex B.

Annex B
(informative)

Confidence intervals for colony count technique

B.1 Confidence intervals for colony count technique

To assess the validity of results and to avoid overly strict interpretations, it is necessary to estimate the measurement uncertainty, or if not available, to estimate the confidence interval which characterizes the microbial statistical distribution in the sample.

When the value of the measurement uncertainty is not available, the confidence interval δ which characterizes the microbial dispersion, can be calculated using Formula (B.1) (with a 95 % probability):

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

where

$B = V(n_1 + 0{,}1n_2)$

in which

V is the volume of inoculum placed in 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;

ΣC is the sum of the colonies counted on all dishes retained from two successive dilutions;

d is the dilution corresponding to the first dilution retained.

EXAMPLE 1

An enumeration gave the following results (system with one plate per dilution):

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

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

$$N = \frac{\Sigma C}{V \times \left[n_1 + (0{,}1 \times n_2) \right] \times d} = \frac{215 + 14}{1 \times \left[1 + (0{,}1 \times 1) \right] \times 10^{-2}} = \frac{229}{0{,}011} = 20\ 818$$

Rounding off the result as specified in the main text, the number of microorganisms is 21 000 or $2{,}1 \times 10^4$ per millilitre or per gram of product.

With N = 2,1 × 10⁴ per gram, for 229 counted colonies, the confidence interval δ is:

$$\delta = \left[\frac{229}{1{,}1} \pm \frac{1{,}96\sqrt{229}}{1{,}1}\right] \times \frac{1}{10^{-2}}$$

$$\delta = (208{,}18 \pm 26{,}96) \times 10^{2}$$

Thus the confidence interval limits are:

$$\delta_1 = 1{,}8 \times 10^{4} \quad \text{and} \quad \delta_2 = 2{,}4 \times 10^{4}$$

EXAMPLE 2

An enumeration gave the following results (system with two plates per dilution):

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

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

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

Rounding off the result as specified in the main text, the number of microorganisms is 19 000 or $1{,}9 \times 10^4$ per millilitre or per gram of product.

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

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

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

Thus the confidence interval limits are:

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

Table B.1 gives the weighted means and confidence intervals δ for relevant numbers of colonies.

Table B.1 — Weighted means and confidence intervals δ for relevant numbers of colonies

Weighted mean of number of colonies counted on two successive dilutions	System "1 plate per dilution" Confidence interval δ	System "2 plates per dilution" Confidence interval δ
300	268 to 332	277 to 323
150	127 to 173	134 to 166
100	**81 to 119**	**87 to 113**
30	**20 to 40**	**23 to 37**
15	7 to 22	10 to 20
10	4 to 16	6 to 14
7	Not applicable	3 to 10

B.2 Special cases with low numbers

The confidence intervals are given in the Tables B.2 to B.5.

Table B.2 — Confidence intervals for enumeration with the system "one plate per dilution", case of low numbers counted on a single plate

Number of colonies[a]	Confidence limits at approximate 95 % confidence level[b]		Percentage confidence limit
	Lower limit	Upper limit	
1	<1	3	±196
2	<1	5	±139
3	<1	6	±113
4	<1	8	±98
5	<1	9	±88
6	1	11	±80
7	2	12	±74
8	2	14	±69
9	3	15	±65
10	4	16	±62
11	4	18	±59
12	5	19	±57
13	6	20	±54
14	7	21	±52
15	7	23	±51

[a] The number of colonies counted on one plate at one dilution.

[b] Compared to the number of microorganisms calculated and assuming that count is made on one plate at one dilution.

Table B.3 — Confidence intervals for enumeration with the system "one plate per dilution", case of low numbers counted on two single plates at two dilutions

Number of colonies[a]	Number of microorganisms[b]	Confidence limits at approximate 95 % confidencelevel[c]		Percentage confidence limits
		Lower limit	Upper limit	
1	NA[d]	NA	NA	NA
2	NA	NA	NA	NA
3	NA	NA	NA	NA
4	NA	NA	NA	NA
5	NA	NA	NA	NA
6	NA	NA	NA	NA
7	NA	NA	NA	NA
8	NA	NA	NA	NA
9	NA	NA	NA	NA
10	9	3	15	±62
11	10	4	16	±59
12	11	5	17	±57
13	12	5	18	±54
14	13	6	19	±52
15	14	7	21	±51

[a] The number of colonies counted on one Petri dish at two successive dilutions.

[b] Weighted mean from two Petri dishes.

[c] Compared to the number of microorganisms calculated and assuming that count is made on a single plate at two dilutions.

[d] Not available: weighted mean cannot be calculated.

Table B.4 — Confidence intervals for enumeration with the system "two plates per dilution", case of low numbers counted on two plates at one dilution

Number of colonies[a]	Number of microorganisms[b]	Confidence limits at approximate 95 % confidence level[c]		Percentage confidence limits
		Lower	Upper	
1	1	<1	1	±196
2	1	<1	2	±139
3	2	<1	3	±113
4	2	<1	4	±98
5	3	<1	5	±88
6	3	<1	5	±80
7	4	<1	6	±74
8	4	1	7	±69
9	5	2	7	±65
10	5	2	8	±62
11	6	2	9	±59
12	6	3	9	±57
13	7	3	10	±54
14	7	3	11	±52
15	8	4	11	±51
16	8	4	12	±49
17	9	4	13	±48
18	9	5	13	±46
19	10	5	14	±45
20	10	6	14	±44
21	11	6	15	±43
22	11	6	16	±42
23	12	7	16	±41
24	12	7	17	±40
25	13	8	17	±39
26	13	8	18	±38
27	14	8	19	±38
28	14	9	19	±37
29	15	9	20	±36
30	15	10	20	±36

[a] The number of colonies counted. on two Petri dishes at one dilution.

[b] Arithmetical mean from two Petri dishes.

[c] Compared to the number of microorganisms calculated and assuming that count is made on two plates at a single dilution.

Table B.5 — Confidence intervals for enumeration with the system "two plates per dilution", case of low numbers counted on two plates at two dilutions

Number of colonies[a]	Number of microorganisms[b]	Confidence limits at approximate 95% confidence level[c]		Percentage confidence limits
		Lower	Upper	
1	NA[d]	NA	NA	NA
2	NA	NA	NA	NA
3	NA	NA	NA	NA
4	NA	NA	NA	NA
5	NA	NA	NA	NA
6	NA	NA	NA	NA
7	NA	NA	NA	NA
8	NA	NA	NA	NA
9	NA	NA	NA	NA
10	NA	NA	NA	NA
11	NA	NA	NA	NA
12	5	2	9	±57
13	6	3	9	±54
14	6	3	10	±52
15	7	3	10	±51
16	7	4	11	±49
17	8	4	11	±48
18	8	4	12	±46
19	9	5	13	±45
20	9	5	13	±44
21	10	5	14	±43
22	10	6	14	±42
23	10	6	15	±41
24	11	7	15	±40
25	11	7	16	±39
26	12	7	16	±38
27	12	8	17	±38
28	13	8	17	±37
29	13	8	18	±36
30	14	9	19	±36

a The number of colonies counted on two Petri dishes at two successive dilutions.

b Weighted mean from four Petri dishes.

c Compared to the number of microorganisms calculated and assuming that count is made on two plates at two dilutions.

d Not available: weighted mean cannot be calculated.

Annex C
(normative)

Determination of most probable number

Table C.1 — MPN values per test portion and 95 % confidence limits for a series of 10 tubes, calculated in accordance with Reference [29]

Number of positive tubes	Series of 10 tubes			
	MPN /ml or /g	Standard uncertainty of $\log_{10}M$	95 % limits	
			Lower	Upper
1	0,11	0,435	0,02	0,75
2	0,22	0,308	0,06	0,89
3	0,36	0,252	0,11	1,11
4	0,51	0,220	0,19	1,38
5	0,69	0,198	0,28	1,69
6	0,92	0,184	0,40	2,10
7	1,20	0,174	0,55	2,64
8	1,61	0,171	0,75	3,48
9	2,30	0,179	1,03	5,16

Table C.2 — MPN values per test portion and 95 % confidence limits for a series of 15 tubes, calculated in accordance with Reference [29]

Number of positive tubes	Series of 15 tubes			
	MPN /ml or /g	Standard uncertainty of $\log_{10}M$	95 % limits	
			Lower	Upper
1	0,07	0,434	0,01	0,49
2	0,14	0,307	0,04	0,57
3	0,22	0,251	0,07	0,69
4	0,31	0,218	0,12	0,83
5	0,41	0,196	0,17	0,98
6	0,51	0,179	0,23	1,15
7	0,63	0,167	0,30	1,33
8	0,76	0,157	0,37	1,55
9	0,92	0,150	0,47	1,80
10	1,10	0,144	0,57	2,11
11	1,32	0,141	0,70	2,49
12	1,61	0,139	0,86	3,02
13	2,01	0,142	1,06	3,82
14	2,71	0,155	1,35	5,45

Table C.3 — MPN values per test portion and 95 % confidence limits for a series of 20 tubes, calculated in accordance with Reference [29]

Number of positive tubes	Series of 20 tubes			
	MPN /ml or /g	Standard uncertainty of $\log_{10}M$	95 % limits	
			Lower	Upper
1	0,05	0,434	0,01	0,36
2	0,11	0,307	0,03	0,42
3	0,16	0,251	0,05	0,50
4	0,22	0,218	0,08	0,60
5	0,29	0,195	0,12	0,69
6	0,36	0,178	0,16	0,80
7	0,43	0,165	0,20	0,91
8	0,51	0,155	0,25	1,03
9	0,59	0,147	0,31	1,16
10	0,69	0,140	0,37	1,30
11	0,80	0,134	0,44	1,46
12	0,92	0,130	0,51	1,65
13	1,05	0,126	0,59	1,85
14	1,20	0,123	0,69	2,10
15	1,39	0,121	0,80	2,40
16	1,61	0,121	0,93	2,77
17	1,90	0,122	1,09	3,29
18	2,30	0,127	1,30	4,08
19	3,00	0,141	1,58	5,67

Table C.4 — MPN values per test portion and 95 % confidence limits for a series of 25 tubes, calculated in accordance with Reference [29]

Number of positive tubes	Series of 25 tubes			
	MPN /ml or /g	Standard uncertainty of $\log_{10}M$	95 % limits	
			Lower	Upper
1	0,04	0,434	0,01	0,29
2	0,08	0,307	0,02	0,33
3	0,13	0,251	0,04	0,40
4	0,17	0,217	0,07	0,47
5	0,22	0,195	0,09	0,54
6	0,27	0,178	0,12	0,61
7	0,33	0,165	0,16	0,69
8	0,39	0,154	0,19	0,77
9	0,45	0,146	0,23	0,86
10	0,51	0,139	0,27	0,96
11	0,58	0,133	0,32	1,06
12	0,65	0,128	0,37	1,16
13	0,73	0,123	0,42	1,28
14	0,82	0,119	0,48	1,41
15	0,92	0,116	0,54	1,55
16	1,02	0,113	0,61	1,70
17	1,14	0,111	0,69	1,88
18	1,27	0,109	0,78	2,09
19	1,43	0,108	0,88	2,33
20	1,61	0,108	0,99	2,62
21	1,83	0,109	1,12	2,99
22	2,12	0,111	1,29	3,50
23	2,53	0,117	1,49	4,28
24	3,22	0,123	1,77	5,85

Table C.5 — MPN values per gram of sample and 95 % confidence limits

(when three test portions of 1 g, three of 0,1 g and three of 0,01 g are used) Reference [56]

Number positive results for inoculum volume, ml or g			**MPN** /ml or /g	$\log_{10}M$	**Standard deviation of** $\log_{10}M$	**95 % Confidence limits**		**Rarity index**	**Rarity category**
1,00	0,10	0,01				Lower	Upper		
0	0	0	0	NA[a]	NA[a]	0	1,1	1,00	1
0	1	0	0,30	−0,52	0,43	0,04	2,3	0,09	1
1	0	0	0,36	−0,45	0,44	0,05	2,7	1,00	1
1	0	1	0,72	−0,14	0,31	0,17	3,0	0,02	2
1	1	0	0,74	−0,13	0,31	0,18	3,1	0,21	1
1	2	0	1,1	0,06	0,26	0,35	3,7	0,02	2
2	0	0	0,92	−0,04	0,32	0,21	4,0	1,00	1
2	0	1	1,4	0,16	0,26	0,42	4,8	0,04	2
2	1	0	1,5	0,17	0,27	0,43	5,0	0,43	1
2	1	1	2,0	0,31	0,23	0,69	6,0	0,02	2
2	2	0	2,1	0,32	0,24	0,71	6,2	0,07	1
3	0	0	2,3	0,36	0,31	0,55	9,7	1,00	1
3	0	1	3,8	0,59	0,31	0,93	16	0,08	1
3	1	0	4,3	0,63	0,33	0,95	19	1,00	1
3	1	1	7,5	0,87	0,30	1,9	30	0,21	1
3	1	2	12	1,1	0,26	3,6	37	0,02	2
3	2	0	9,3	0,97	0,32	2,2	40	1,00	1
3	2	1	15	1,2	0,27	4,4	51	0,42	1
3	2	2	21	1,3	0,24	7,2	64	0,07	1
3	3	0	24	1,4	0,32	5,6	100	1,00	1
3	3	1	46	1,7	0,34	9,6	220	1,00	1
3	3	2	110	2,0	0,32	25	480	1,00	1
3	3	3	∞	NA[a]	NA[a]	36	∞	1,00	1

[a] Not available.

Table C.6 — MPN values per gram of sample and 95 % confidence limits

(when five test portions of 1 g, five of 0,1 g and five of 0,01 g are used) Reference [56]

No. positive results for inoculum volume, ml **or** g			**MPN** /ml or /g	$\log_{10}M$	**Standard deviation of** $\log_{10}M$	**95 % Confidence limits**		**Rarity index**	**Category**
1	0,1	0,01				**Lower**	**Upper**		
0	0	0	0	NA	NA	0	0,66	1	1
0	1	0	0,18	–0,74	0,43	0,03	1,34	0,09	1
1	0	0	0,20	–0,70	0,44	0,03	1,47	1,00	1
1	0	1	0,40	–0,40	0,31	0,10	1,65	0,02	2
1	1	0	0,40	–0,39	0,31	0,10	1,66	0,21	1
1	2	0	0,61	–0,21	0,25	0,19	1,96	0,02	2
2	0	0	0,45	–0,35	0,31	0,11	1,86	1,00	1
2	0	1	0,68	–0,17	0,25	0,21	2,18	0,03	2
2	1	0	0,68	–0,16	0,25	0,21	2,2	0,35	1
2	1	1	0,92	–0,04	0,22	0,33	2,55	0,02	2
2	2	0	0,93	–0,03	0,22	0,34	2,58	0,06	1
3	0	0	0,78	–0,11	0,26	0,24	2,54	1,00	1
3	0	1	1,1	0,02	0,22	0,38	3,00	0,05	1
3	1	0	1,1	0,03	0,22	0,38	3,02	0,57	1
3	1	1	1,4	0,14	0,20	0,54	3,48	0,03	2
3	2	0	1,4	0,14	0,20	0,54	3,53	0,15	1
3	2	1	1,7	0,23	0,19	0,72	4,02	0,10	2
3	3	0	1,7	0,24	0,19	0,73	4,09	0,03	2
4	0	0	1,3	0,11	0,23	0,44	3,72	1,00	1
4	0	1	1,7	0,22	0,21	0,62	4,4	0,08	1
4	1	0	1,7	0,23	0,21	0,63	4,5	0,92	1
4	1	1	2,1	0,33	0,20	0,85	5,28	0,07	1
4	2	0	2,2	0,33	0,20	0,86	5,41	0,31	1
4	2	1	2,6	0,42	0,19	1,1	6,31	0,03	2
4	3	0	2,7	0,43	0,19	1,1	6,5	0,07	1
4	4	0	3,4	0,53	0,18	1,4	7,8	0,01	2
5	0	0	2,3	0,36	0,24	0,76	7,0	0,77	1
5	0	1	3,1	0,50	0,24	1,0	9,4	0,09	1
5	1	0	3,3	0,52	0,24	1,1	10	1,00	1
5	1	1	4,6	0,66	0,25	1,5	14	0,20	1
5	1	2	6,3	0,80	0,24	2,1	19	0,02	2
5	2	0	4,9	0,69	0,26	1,5	16	1,00	1
5	2	1	7,0	0,84	0,24	2,3	22	0,36	1