



**International
Standard**

ISO 7218

**Microbiology of the food chain —
General requirements and guidance
for microbiological examinations**

*Microbiologie de la chaîne alimentaire — Exigences générales et
recommandations pour les examens microbiologiques*

**Fourth edition
2024-06**

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Published in Switzerland

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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

ISO draws attention to the possibility that the implementation of this document may involve the use of (a) patent(s). ISO takes no position concerning the evidence, validity or applicability of any claimed patent rights in respect thereof. As of the date of publication of this document, ISO had not received notice of (a) patent(s) which may be required to implement this document. However, implementers are cautioned that this may not represent the latest information, which may be obtained from the patent database available at www.iso.org/patents. ISO shall not be held responsible for identifying any or all such patent rights.

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

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

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 463, *Microbiology of the food chain*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This fourth edition cancels and replaces the third edition (ISO 7218:2007), which has been technically revised. It also incorporates the Amendment ISO 7218:2007/Amd 1:2013.

The main changes are as follows:

- the calculations section has been simplified and two further calculators have been added;
- the equipment section has been reorganized into groups with similar purposes and requirements;
- cross-references have been added to other general microbiology standards such as those for media, validation and verification, and uncertainty to reduce repetition;
- information on laboratory quality control and characterization of control microorganisms has been added.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

When conducting microbiological examinations, it is especially important that:

- only those microorganisms present in the samples are detected and/or enumerated;
- these microorganisms do not contaminate the environment.

To achieve this, good laboratory practices are essential, including personal hygiene and aseptic working techniques which exclude extraneous contamination as far as possible.

Only limited information on the precautions to be taken during microbiological examinations is given in this document, so a thorough knowledge of the microbiological techniques and microorganisms involved is essential. It is important that examinations are conducted safely, correctly and as carefully as possible, including monitoring and recording aspects that can affect results, calculating numbers of microorganisms and assessing the uncertainty of test results.

The most common risks and their control in the microbiological laboratory are given in this document. However, work processes in each laboratory can differ and appropriate risk analysis should be considered to ensure good laboratory practices. Periodic evaluation and control of critical points not only maintains safe and hygienic practices but can also improve reliability of test results.

The purpose of this document is to help to ensure the validity of microbiology examinations in the food chain. In particular, to ensure that general techniques for conducting examinations are the same in all laboratories, to achieve consistent results in different laboratories and to contribute to safety of laboratory personnel by preventing risks of infection.

This document includes the main measures necessary for conducting the wide range of microbiological examinations. Additional information is available from the literature listed in the Bibliography (see References [43] to [47]).

In this document, the following verbal forms are used:

- “shall” indicates a requirement;
- “should” indicates a recommendation;
- “may” indicates a permission;
- “can” indicates a possibility or a capability.

In addition, the imperative mood is used to give instructions or where actions are required.

Microbiology of the food chain — General requirements and guidance for microbiological examinations

1 Scope

This document specifies general requirements and gives guidance on microbiological examinations.

It is applicable to:

- the implementation of specific horizontal or vertical International Standards developed by ISO/TC 34/SC 9 or ISO/TC 34/SC 5 for detection or enumeration of microorganisms, named hereafter “specific standards”;
- good laboratory practices for microbiology laboratories testing samples from the food chain;
- guidance for microbiological laboratories testing samples from the food chain on the technical requirements for conforming to ISO/IEC 17025.

The requirements of this general standard supersede corresponding ones in existing specific standards.

Additional instructions for examinations using the polymerase chain reaction (PCR) are specified in ISO 22174.

This document is applicable to examinations for bacteria, yeasts and moulds and can be used, if supplemented with specific guidance, for parasites and viruses. It does not apply to examinations for toxins or other metabolites (e.g. amines) from microorganisms.

This document is applicable to microbiology of the food chain, from primary production stage to food and animal feed products, including the premises where the food or feed production and handling takes place. It is also applicable to the microbiological examination of water where water is used in food production or is regarded as a food in national legislation.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

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

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

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

3.1 food chain

sequence of the stages in the production, processing, distribution, storage and handling of a *food* (3.2) and its ingredients, from primary production to consumption

Note 1 to entry: The food chain includes the environment of primary production, food and feed production, and handling.

Note 2 to entry: The food chain also includes the packaging materials intended to come into contact with food, *feed* (3.3) or raw materials.

[SOURCE: ISO 22000:2018, 3.20, modified — Notes to entry replaced.]

3.2

food

products intended for human consumption

substance (ingredient), whether processed, semi-processed or raw, which is intended for human consumption, and includes beverages and any substance which has been used in the manufacture, preparation or treatment of “food” but does not include cosmetics or tobacco or substances (ingredients) used only as drugs

[SOURCE: CAC/GL 81-2013, 6, modified — Admitted term added. “(ingredient)” added, “beverages” replaced “drink” and “chewing-gum” deleted in the definition.]

3.3

feed

products for feeding animals

single or multiple product(s), whether processed, semi-processed or raw, which is (are) intended to be fed to animals

Note 1 to entry: These products are intended for food-producing animals and non-food producing animals such as pets.

[SOURCE: ISO 22000:2018, 3.16, modified — Admitted term added. “food-producing” deleted in the definition. Note 1 to entry replaced.]

3.4

food or feed production and handling

any operation in the processing (e.g. preparation, cooking, packaging), storage, transport, distribution and service of *food* (3.2) or *feed* (3.3)

[SOURCE: ISO/TS 22002-2:2013, 3.9, modified — “or feed production and” added in the term. “processing (e.g. preparation, cooking, packaging)” replaced “preparation, processing, cooking, packaging”, and “or feed” added in the definition.]

3.5

primary production stage

stage of the *food chain* (3.1) at which the production, rearing or growing of primary products, including harvesting, milking and farmed animal production before slaughter, takes place

Note 1 to entry: It also includes hunting, fishing and the harvesting of wild products.

3.6

horizontal method

method for microbiological examination that is broadly applicable to samples within the *food chain* (3.1), excluding any documented limitations

3.7

vertical method

sectorial method

method for microbiological examination that is specifically applicable to samples (e.g. from *primary production stage* (3.5)), a product or a group of products (e.g. milk and milk products, meat and meat products, fish and fishery products, *feed* (3.3))

3.8

specific standard

standardized reference method for the examination (e.g. detection, enumeration, confirmation or identification) of a specific microorganism or group of microorganisms

Note 1 to entry: A specific standard can describe a *horizontal method* (3.6) or a *vertical method* (3.7).

3.9

general standard

supporting document describing general guidance and requirements necessary for application of *specific standards* (3.8)

3.10

strain

progeny or subculture of a single isolated colony in pure culture that displays the phenotypic characteristics or possesses the molecular attributes/properties as identified with being associated within the classification of the species of that microorganism

3.11

target strain

strain (3.10), defined according to the scope of the method

[SOURCE: ISO 16140-1:2016, 2.74, modified — “method” replaced “reference method that is expected to be detected or enumerated by the alternative method”.]

3.12

target organism

microorganism that is the designated analyte for a microbiological examination

[SOURCE: ISO 22117:2019, 3.1, modified — “microbiological examination” replaced “proficiency testing sample”.]

3.13

laboratory strain

microorganism that is defined to at least the genus and species level, and characterized biochemically, and/or serologically and/or with molecular testing, and preferably originating from the *food chain* (3.1)

3.14

reference strain

microorganism obtained directly from an official culture collection or reference laboratory and defined to at least the genus and species level, catalogued and described according to its characteristics and preferably originating from *food* (3.2), food production areas, *primary production stages* (3.5), animals or water, as applicable

[SOURCE: ISO 22117:2019, 3.4]

3.15

natural background microorganism

microorganism that is naturally present or can be introduced to compete with or mimic the target microorganism

[SOURCE: ISO 22117:2019, 3.2, modified — “natural background microorganism” replaced “background flora” as the term. “Included in a proficiency testing sample” deleted in the definition.]

3.16

matrix

all the components of the sample

[SOURCE: ISO 16140-1:2016, 2.38, modified — “(product)” deleted in the term.]

3.17

biological resource centre

BRC

service provider or repository of the living cells, genomes of organisms and information relating to heredity and the functions of biological systems

Note 1 to entry: BRCs contain collections of culturable organisms (e.g. microorganisms), replicable parts of these (e.g. genomes, plasmids, viruses, cDNAs), viable but not yet culturable organisms, cells and tissues, as well as databases containing molecular, physiological and structural information relevant to these collections and related informatics

[SOURCE: OECD, 2007^[44]]

3.18

microbial (sub)type

group of closely related microorganisms (within a species) distinguished by their shared specific characteristics as determined by, for example, serological testing (serotype) or molecular testing (genotype)

[SOURCE: ISO 16140-6:2019, 3.5]

3.19

challenge testing

study of the growth or inactivation of microorganism(s) artificially inoculated in *food* (3.2)

[SOURCE: ISO 20976-1:2019, 3.5]

3.20

accuracy

accuracy of measurement

measurement accuracy

closeness of agreement between a measured quantity value and a true quantity value of a measurand

[SOURCE: ISO/IEC Guide 99:2007, 2.13, modified — “accuracy” replaced “measurement accuracy” as the preferred term. Notes to entry deleted.]

3.21

resolution

smallest change in a quantity being measured that causes a perceptible change in the corresponding indication

[SOURCE: ISO/IEC Guide 99:2007, 4.14, modified — Notes to entry deleted.]

3.22

uncertainty

uncertainty of measurement

measurement uncertainty

non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used.

[SOURCE: ISO/IEC Guide 99:2007, 2.26, modified — “uncertainty” replaced “measurement uncertainty” as the preferred term. Notes to entry deleted.]

3.23

calibration

set of operations that establish, under specified conditions, the relationship between values indicated by a measuring instrument or measuring system, or values represented by a material measure, and the corresponding known values of a reference standard

3.24

verification

provision of objective evidence that a given item fulfils specified requirements

[SOURCE: ISO/IEC Guide 99:2007, 2.44, modified — Examples and notes to entry deleted.]

3.25

contamination

undesirable presence of microorganisms in the environment, on surfaces (including human skin) or in (or on) laboratory samples

3.26**cross-contamination**

unintentional transfer of microorganisms or their constituents, such as DNA, or metabolites from one area or article to another

Note 1 to entry: This can include, but is not limited to, transfer from:

- the laboratory environment to laboratory samples;
- laboratory personnel to laboratory samples;
- one laboratory sample to other laboratory samples;
- one laboratory area to another;
- the laboratory area to adjacent production areas.

3.27**process control**

internal quality control (IQC) system that is used to confirm acceptable conditions of the entire process have been achieved

Note 1 to entry: This includes the use of a target organism that is tested in accordance with the method from start to finish with each batch of samples, as well as positive, negative and blank samples that are used to confirm the acceptability of media/reagents and consumable supplies, and of the incubation environment (temperature/equipment), and to confirm the competence of personnel.

4 Premises**4.1 General**

This clause gives general requirements, including the principles of design and organization, for the layout of a microbiological laboratory testing samples from the food chain.

NOTE Guidelines referring to PCR in this document apply equally to other nucleic acid amplification methods. Further specifications for laboratories using PCR are given in ISO 22174.

4.2 Biosafety considerations

The laboratory design shall comply with the relevant biosafety requirements for the type of microorganism and potential for causing human illness.

The current four biosafety levels and laboratory design requirements for each are fully described in the WHO Laboratory Biosafety Manual^[47] or the OIE Terrestrial Manual^[45].

NOTE Regional or national regulations can differ in definitions of biosafety levels or risks from microorganisms.

4.3 Laboratory design

The guidelines for laboratory layout described in this clause cover examinations for the detection and enumeration of microorganisms belonging to Biosafety Levels 1 and 2, as only these are routinely handled in food microbiology laboratories. Further details can be found in the WHO Laboratory Biosafety Manual^[47].

NOTE National and regional legislation can require different and/or additional safety measures.

4.4 Laboratory areas**4.4.1 General**

The laboratory comprises separate areas associated with samples and testing (see [4.4.2](#)) and other general areas (see [4.4.3](#)).

4.4.2 Areas associated with samples and testing

It is good practice to allocate separate rooms or clearly designated areas, with dedicated equipment where necessary, for the following activities:

- receipt and storage of laboratory samples before and after testing;
- preparation of laboratory samples and test portions (separate powders and those likely or known to contain high numbers of microorganisms to reduce the risk of cross-contamination, and also commercially sterile foodstuffs to prevent contamination by other samples);
- examination of test portions from the initial suspension, including all dilution, plating, incubation and counting steps of enumeration tests and detection tests up to isolation of presumptive pathogens;
- manipulation of presumptive pathogens, including those from proficiency testing schemes or laboratory spiked samples deliberately contaminated with pathogens;
- handling and storage of reference cultures and other laboratory strains;
- preparation and testing of samples by PCR nucleic acid amplification methods (see ISO 22174 for full details);
- preparation and sterilization of culture media, reagents and necessary equipment;
- storage of culture media and reagents;
- decontamination and disposal of biohazard waste;
- cleaning of glassware and other equipment;
- storage of hazardous chemicals, preferably in specially designated cabinets, cupboards, rooms or buildings.

The areas can be interconnected provided hygiene recommendations (see [5.4](#)) are met and, if space is limited, activities can be separated by time.

4.4.3 General areas

Separate areas are set aside for the following:

- entrances, corridors, stairways and lifts;
- cloakrooms, toilets and staff rooms;
- administration (e.g. secretarial, offices, document archives);
- storage of general laboratory supplies.

4.5 Layout and fittings of the premises

4.5.1 Objectives

The primary objective is to ensure that the environment in which microbiological examinations are carried out is safe and does not adversely affect the validity of test results.

Arrange the premises to avoid risk of cross-contamination, for example:

- construct the laboratory according to the pattern and flow of work (the “no way back” principle);
- carry out procedures in a sequential manner using appropriate precautions to ensure test and sample integrity (e.g. use of sealed containers);
- separate activities by space or time (see [4.4.2](#)).

Avoid conditions such as extremes of temperature outside the accepted range (18 °C to 27 °C), draughts, dust, humidity, steam, noise, vibration, etc., all of which can affect the validity of microbiological test results.

Locate air flows into and out of the laboratory to minimize the risk of contaminating samples under test or any adjacent food processing facilities.

Sufficient space should be available so work areas can be kept clean and tidy. The space required should be proportional to the number of samples and tests handled, the number of staff and the overall internal organization of the laboratory.

4.5.2 Fittings

The test premises should be constructed and equipped as follows to reduce the risk of contamination by dust and therefore by microorganisms:

- The walls, ceilings and floors should be smooth, easy to clean, and resistant to detergents and disinfectants used in laboratories.
- Floors should be slip-resistant.
- Overhead pipes should not cross the premises unless they are sealed or enclosed. Any other overhead structures should be covered or readily accessible for regular cleaning.
- Windows and doors should be closed when testing to minimize draughts and potential airborne contamination. They should be designed to avoid formation of dust traps and to facilitate cleaning.
- The ambient temperature (18 °C to 27 °C) and air quality (microorganism content, dust spreading rate, etc.) should be compatible with testing. Air-conditioning systems should supply filtered air and be regularly cleaned and serviced or maintained.
- Adequate measures, such as special workstations, should be taken to minimize exposure to and cross-contamination from dust when handling dehydrated culture media and dusty or powdered samples.
- When tests are to be conducted in a low-contamination atmosphere, the room should be equipped with a clean laminar airflow and/or safety cabinet.
- If necessary, the laboratory environment should be protected from the harmful effects of solar radiation by the use of shutters or suitably treated window glass. Internally installed blinds are not suitable as they can be difficult to clean and can become a source of dust.

4.5.3 Other arrangements for laboratory premises

Consider general arrangements such as the following:

- availability of water supply, of suitable quality for the intended use;
- availability of electricity;
- availability of gas (piped or bottled);
- sufficient lighting for tasks undertaken in each area of the laboratory;
- laboratory bench tops and furniture manufactured in smooth, impermeable material that is easily cleaned and disinfected;
- laboratory furniture that is movable or designed to allow access for cleaning floors and equipment;
- no furniture, documents or other items than those strictly necessary for testing activities to be kept in the testing areas;
- separate storage for test records and other necessary documents away from samples and test materials;

- provision of handwashing facilities (or special solutions or gels containing alcohol) in each testing room, preferably near the door;
- provision of designated handwashing facilities, preferably with hands-free operation, at the entrance/exit to pathogen testing areas;
- provision for hanging used laboratory clothing when exiting laboratory areas;
- availability of an autoclave for destruction of contaminated waste materials and culture media, unless another appropriate system such as removal and incineration is in place (see [6.2.2](#));
- provision of safety systems for fire and electrical emergencies;
- provision of safety showers and eyewash facilities, where necessary;
- provision of first aid facilities.

4.5.4 Cleaning and disinfection

Consider methods, frequency and controls for cleaning and disinfection according to risk and check the following points:

- The floors, walls, ceilings, laboratory bench tops, furniture and junctions between these should be regularly maintained and repaired to avoid cracks which can harbour contamination.
- Carry out regular cleaning and disinfection to keep the premises in a condition suitable for conducting tests. Contaminated or potentially contaminated surfaces should be decontaminated using a disinfectant known to be bactericidal and fungicidal (see [Annex A](#)).
- Use dedicated cleaning equipment for all microbiology laboratories, with separate equipment for high-risk areas such as pathogen laboratories.

NOTE Rooms and equipment can be decontaminated by fumigation with formaldehyde vapour if appropriate.

- Maintain the ventilation systems and filters and change the filters when necessary.
- Monitor the microbiological contamination level of laboratory work surfaces, staff contact surfaces and the air regularly (at a frequency depending on previous monitoring results and risk to validity of test results).
- Surface contamination may be estimated by applying a contact plate, containing suitable neutralizing agents against sanitizers (e.g. lecithin, sodium thiosulfate) or by swabbing (see ISO 18593).
- Contamination level of laboratory air may be monitored by exposing (e.g. for 15 min to 30 min) an open Petri dish containing a non-selective agar medium, such as plate count agar, or a selective agar appropriate for the target microorganisms sought (e.g. moulds). Active air sampling equipment may also be used.

5 Personnel

5.1 General

General requirements on the competence of laboratory staff can be found in ISO/IEC 17025.

5.2 Competence

For each method or technique, define objective criteria for the assessment of appropriate competence, both initially and on an ongoing basis.

Competence can be assessed by internal quality control (IQC) or proficiency testing (PT) (see [Clause 16](#)).

NOTE A method of investigating the cause of poor performance (pipetting, poor homogeneity of the initial suspension, dilution preparation, colony counting, etc.) for enumeration by counting colonies is given in ISO 14461-1.

5.3 Verification of ongoing staff competence

Verification of ongoing staff competence should be carried out at a documented frequency against objective parameters. This includes participation in regular IQC, PT (see ISO 22117), use of (certified) reference materials or by self-assessment tests for enumeration of microorganisms as described in ISO 14461-2.

Any deviations from expected results or unsatisfactory results should be investigated and personnel retrained.

5.4 Hygiene

Take the following personal hygiene precautions to avoid contaminating samples, culture media and/or reagents, and to avoid the risk of infecting laboratory personnel or those in adjacent areas:

- Wear properly fastened laboratory clothing that is clean and in good condition. Use clothing made from fabric which limits the risks of flammability where open flames are in use. Consider dedicated, fully fastening laboratory coats, preferably with long sleeves and elasticated cuffs, and laboratory shoes for designated pathogen handling areas. Do not wear laboratory clothing outside the laboratory work areas (and changing rooms).
- Store outside clothing and personal possessions, such as phones, headphones and jewellery, before entering the laboratory as these can be a source of contamination.
- Wear protection for hair and beards, if required and necessary.
- Keep fingernails clean and preferably short.
- Wash hands thoroughly under running water, preferably delivered hands free, before and after microbiological examinations and immediately after visiting the toilets. Use liquid, foam or powdered soap, preferably delivered by an automatic dispenser. Dry hands with single-use paper or cloth towels and discard these into an adjacent sealed bin. Hot-air hand dryers are not suitable for use in testing areas of microbiology laboratories but can be used in other areas. Use of a hand sanitiser after drying is recommended. These precautions are applicable both to laboratory staff and visitors.
- When working with exposed samples, cultures, culture media, reagents, etc. and when manipulating tests, avoid speaking, coughing, etc.
- Staff with skin infections or other illnesses likely to spread microorganisms which can contaminate samples and invalidate test results shall take the necessary precautions, such as wearing disposable gloves or covering affected areas.
- Do not eat or drink in the laboratory and do not put food for personal consumption in laboratory refrigerators, freezers or microwaves.
- Mouth pipetting is strictly prohibited.

6 Equipment and consumables

6.1 General

In accordance with good laboratory practice, keep all apparatus and equipment clean and in good working condition. Equipment shall be constructed and installed to facilitate operation and to allow for ease of maintenance, cleaning, decontamination and calibration. Dedicated equipment, such as trolleys, pipettors, etc., should be allocated to pathogen testing areas.

Check that all equipment is suitable for the intended purpose and conforms to specified requirements before use. Ensure equipment used for measurements is capable of achieving the accuracy and/or uncertainty required to produce a valid result.

Calibrate critical equipment that directly affects the validity of test results and/or associated uncertainty in a metrologically traceable manner. For non-critical measurement equipment, verification is sufficient.

NOTE The decision chart in ISO 20836:2021, Figure 1, includes guidance on whether a metrological traceable calibration is necessary or not.

To maintain confidence in the status of the calibration, establish a documented calibration schedule to minimize invalid test results.

Maintain equipment regularly to ensure safety and suitability for the intended use and perform intermediate checks when necessary to give confidence in the ongoing performance of the equipment. The frequency of calibration and verification checks of each item of equipment is, in most cases, not specified in this document, since it is determined by each individual laboratory using a risk-based approach. The frequency depends on many factors, including the type of equipment and the level of laboratory activity, and is in accordance with the manufacturer's instructions. In a limited number of cases, a frequency has been specified because it is considered essential.

Throughout this clause, requirements for accuracy and/or uncertainty of measuring equipment are given. These are based on the practical tolerance required to demonstrate suitable control of equipment in routine use. The tolerance stated is related to the metrological uncertainty of the device (see ISO/IEC Guide 99). Any performance criteria given in this clause apply to the equipment concerned and not to the whole method of analysis.

6.2 Sterilization and other heating equipment

6.2.1 General

This equipment includes autoclaves (see [6.2.2](#)), culture media preparators (see [6.2.3](#)), steamers, including boiling-water baths (see [6.2.4](#)), sterilizing ovens (see [6.2.5](#)), microwave ovens (see [6.2.6](#)), hotplates, induction cookers and heating mantles (see [6.2.7](#)), and gas (Bunsen) burners or wire incinerators (see [6.2.8](#)).

6.2.2 Autoclave

6.2.2.1 Description

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

The autoclave should be equipped with:

- at least one safety valve and a safety lock;
- 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 uncertainty associated with the monitoring thermocouple);
- a temperature probe or a recording thermocouple;
- a timer and temperature recorder.

6.2.2.2 Use

With steam sterilization, all air is expelled before the pressure can build up. If the autoclave is not fitted with an automatic evacuation device, it is necessary to expel the air until a continuous jet of steam is emitted. Load the autoclave to ensure a free flow of steam to replace the air.

For the sterilization of culture media, the saturated steam in the chamber shall be at a temperature of $121\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$, or other temperatures specified by the manufacturers in the preparation instructions or in the test method.

For the destruction of cultured microorganisms and for decontaminating equipment and/or culture media after use, the saturated steam in the chamber shall be at a temperature of at least $121\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$.

Do not autoclave unsuitable materials and media, especially those that can pose a safety risk, e.g. due to decomposition or build-up or corrosive or volatile compounds.

To avoid cross-contamination, use separate sterilization cycles to sterilize clean equipment and/or culture media and to decontaminate used equipment and/or used culture media. It is preferable to use separate autoclaves, which can be located in separate areas, for these two processes.

After autoclaving, allow all materials and equipment to cool within the autoclave before removal.

For safety reasons, do not remove the contents until the chamber temperature has dropped below $80\text{ }^{\circ}\text{C}$.

Sterilization cycles for large loads (such as those used by commercial manufacturers) may be evaluated using F_0 values, taking into account the heat treatment during heating and cooling. The heat treatment should be defined for each particular load to be treated to ensure suitable conditions irrespective of the container type or placement in the autoclave (see ISO 17665).

6.2.2.3 Maintenance

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

6.2.2.4 Calibration and verification

Keep the autoclave in good operating condition and schedule regular inspections by 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 commissioning 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 (e.g. steam chemical integrator or biological indicator) should be included at the centre of the load to show that the target temperature was reached where a traceable record of process efficiency is not available.

6.2.3 Culture media preparator

6.2.3.1 Description

A culture media preparator is principally designed for sterilization of large volumes of media ($>1\text{ l}$). It consists of a heating vessel, water jacket and continuous stirring device, and is also fitted with a temperature gauge, pressure gauge, timer and safety valve.

A temperature sensor is positioned in contact with the culture media to demonstrate adequate heat treatment according to the temperature tolerances established for each medium.

In addition, the unit should have a safety lock to prevent opening until a temperature of $< 80\text{ }^{\circ}\text{C}$ is reached.

6.2.3.2 Use

Follow the manufacturer's instructions at all times.

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

6.2.3.3 Maintenance

Wash the preparator and rinse thoroughly with purified water (see 6.7.1) between each culture media batch.

NOTE Rinsing with a volume fraction of 70 % ethanol can be useful to remove staining from strongly coloured indicators present in certain culture media, followed by thorough rinsing with purified water.

Maintain the culture media preparator in good operating condition and schedule regular inspections by qualified personnel in accordance with the manufacturer's instructions.

6.2.3.4 Calibration and verification

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

Initial commissioning should include performance studies for each operating cycle and each load configuration used in practice. Repeat this process after significant repair or modification.

For each load, keep a traceable record of process efficiency or, as a minimum, place a temperature sensor in contact with the culture media produced to verify the heating process.

6.2.4 Steamers, including boiling-water baths

6.2.4.1 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.

6.2.4.2 Use

The main uses are as follows:

- melting solid media;
- preparation of heat-labile culture 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 that is without pressure may also be used.

6.2.4.3 Maintenance

Keep steamers and boiling-water baths clean.

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

6.2.5 Sterilizing oven

6.2.5.1 Description

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

6.2.5.2 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 or for sterilizing unsuitable materials (see 6.2.2.2).

Clean all glassware and metal items before sterilization in the oven.

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

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 resolution.

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 $170\text{ °C} \pm 10\text{ °C}$.

After sterilization, allow glassware to cool in the oven before removal to prevent cracking.

6.2.5.3 Maintenance

Clean internal surfaces when required.

6.2.5.4 Calibration and verification

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

The oven shall be fitted with a calibrated temperature-monitoring device or thermocouple 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.

For each load, as a minimum, a process indicator (e.g. steam chemical integrator or biological indicator) should be included at the centre of the load to show that the target temperature was reached where a traceable record of process efficiency is not available. Monitor the temperature of the oven and record during each use.

6.2.6 Microwave oven

6.2.6.1 Description

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

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 give better heat distribution.

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

6.2.6.2 Use

It is advisable to use the equipment to heat liquids or melt agar media only.

Do not heat culture media containing heat-sensitive components in a microwave unless it has been verified that this method of heating has no effect on medium performance.

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

When melting solid media, the use of a low power setting (e.g. defrost cycle) and placing the bottle in 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.

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

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

6.2.6.3 Maintenance

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

Inspect the oven door seals and check for any other damage which can lead to radiation leakage at regular intervals according to the manufacturer's instructions. Checks with a radiation meter by competent personnel or servicing agent are recommended.

6.2.6.4 Verification

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

6.2.7 Hotplate, induction cooker and heating mantle

6.2.7.1 Description

Hotplates, induction cookers and heating mantles are thermostatically controlled heating devices, some of which incorporate magnetic stirring systems.

6.2.7.2 Use

Hotplates, induction cookers and heating mantles equipped with magnetic stirring systems are used for heating relatively large volumes of liquid such as during preparation of culture media.

Do not use hotplates, induction cookers and heating mantles without stirring systems for preparation of culture media.

6.2.7.3 Maintenance

Clean up any spillages as soon as the unit has cooled.

6.2.8 Gas burner or wire incinerator

6.2.8.1 Description

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

Wire incinerators (electronic) include a narrow ceramic chamber designed to contain any aerosols generated when decontaminating at high temperatures.

6.2.8.2 Use

Gas burners and wire incinerators are 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.

Wire incinerators are preferred when handling pathogenic bacteria to prevent splatter and avoid risk of cross-contamination.

The use of gas burners should be avoided in protective cabinets for safety reasons and because they can interfere unacceptably with the laminar airflow. Sterile disposable equipment is recommended as an alternative.

Gas burners also produce much heat and air turbulence in the laboratory and aseptic techniques can be achieved by using disposable equipment instead.

6.2.8.3 Maintenance

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

6.3 Temperature controlled equipment and monitoring devices

6.3.1 General

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

6.3.2 Incubator

6.3.2.1 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. Heat distribution may be by convection or fan-assisted forced air.

Refrigerated incubators which cycle between incubation and refrigeration when the incubation period has been completed are also available.

6.3.2.2 Use

Incubators are primarily used to incubate inoculated culture media at a constant temperature. However, they can also be used for other purposes. These include melting laboratory samples (e.g. chocolate), prewarming pre-enrichment broths before inoculation (see [12.1](#)) or tempering sterile molten culture media before use, if the time taken for the culture media to reach the correct temperature has been evaluated as appropriate.

All 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. If the correct temperature is not achieved throughout, mark the areas which cannot be used for incubation.

If the ambient temperature is close to or higher than that required for incubation, it is necessary to use an incubator fitted with a cooling system.

Protect incubators from direct sunlight to minimize heat gain.

When loading incubators, ensure free air circulation (see [11.2.5](#)).

If possible, incubators should not be completely filled in a single operation as this extends the time for the contents to reach temperature equilibrium in all types of incubator.

Avoid opening incubator doors frequently and leaving them open for long periods.

6.3.2.3 Maintenance

Clean the inner and outer walls of the incubator regularly. If applicable, remove dust from the ventilation system.

Ensure that the cleaning and disinfection techniques will not affect test results, particularly by leaving inhibitory residues.

6.3.2.4 Verification

Use a temperature-monitoring device of appropriate resolution. The instrument display may be used for checks during use if it has been shown to be accurate.

Check the temperature stability and homogeneity of the temperature distribution at the working temperature throughout the usable volume of the incubator with a number of temperature-monitoring devices of known accuracy and uncertainty and appropriate temperature range. More monitoring devices are required for large incubators and incubation rooms, depending on the chamber volumes.

Use the information from this temperature mapping/profiling to define the acceptable operating range of the incubator and the optimum position of the monitoring device or recording thermocouple used to monitor working temperatures.

For example, to achieve a target temperature of $37\text{ °C} \pm 1\text{ °C}$ when the profiling data/temperature mapping shows a range of $36,8\text{ °C}$ to $37,2\text{ °C}$ across the incubator, take the observed temperature variation of $\pm 0,2\text{ °C}$ into account to define the operating limits of the incubator. Amend the lower operating range to $36,2\text{ °C}$ and the upper limit to $37,8\text{ °C}$ to ensure all areas of the incubator achieve the target temperature.

When re-defining the operating range, the uncertainty of the monitoring device should also be taken into account, although this is usually minimal.

If the monitoring device is fixed elsewhere than at the centre of the temperature range, adjust the incubator temperature so that the device reads the target temperature and amend the permitted operating range accordingly.

Temperature mapping is necessary before initial use and at a defined frequency to show any deviation in use. It is also necessary after each significant repair or modification.

In addition, temperature profiling of (larger) enrichment broths shall be checked before implementing a method or changing the test portion size, to ensure the time taken to reach target incubation temperature is considered with the incubation times stated in the specific standard (see [12.2](#)). Use a separate incubator for pre-warming broths if necessary, so that the recovery time cannot affect any other test results.

Check and record the incubator temperature to confirm it is stable and within the maximum permissible tolerance during the incubation period. Alternatively, dataloggers or electronic monitoring systems may be used.

6.3.3 Thermostatically controlled bath

6.3.3.1 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 a stability of $\pm 0,5\text{ °C}$ or better to be achieved. The working temperatures and required maximum permissible tolerance are stipulated in each individual application or specific standard.

A cooling system is necessary to maintain a temperature near or below ambient temperature.

6.3.3.2 Use

The main uses are as follows:

- incubation of inoculated culture media at a constant temperature;
- maintenance of sterile molten culture media during media preparation;
- tempering of sterile molten culture media for use in specific standards;
- preparation of initial sample suspensions or solutions at a controlled temperature;

- heat treatment of initial sample suspensions at a controlled temperature (e.g. pasteurization or for spore counts).

Where precise temperature control is required, the bath shall be equipped with a circulating-water pump and an automatic temperature-regulation system (including a cut-out when it is running dry). 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 culture 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 incubation.

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

Maintain the depth of immersion to prevent entry of water through the closure.

Devices made of non-corroding materials to maintain stability of the containers can be required (e.g. racks or weight rings).

Dry all containers after removal from the bath and before further use.

6.3.3.3 Maintenance

Baths should be filled with liquid as recommended by the manufacturer. For incubation of cultures, distilled or deionized water should be used and a suitable microbial growth inhibitor can be added.

Check the level of the liquid regularly 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.

6.3.3.4 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 temperature control. For usage where temperature is critical (e.g. tempering agar for pour plates or incubation of cultures), use the information to define the acceptable operating range (see [6.3.2.4](#)).

Use a temperature-monitoring device (see [6.3.7](#)) of suitable resolution (see [6.3.2.4](#)) independent of the automatic temperature-regulation system.

The digital display can also be used, provided that the accuracy (uncertainty) and resolution are verified.

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

6.3.4 Heating blocks

6.3.4.1 Description

Heating blocks are solid metal or ceramic blocks with wells, which deliver consistent heat to small vessels, such as microtubes or cuvettes, and their contents. The operating temperature can be precisely set across the block.

6.3.4.2 Use

Heating blocks can be used to melt or boil contents, to control and optimize enzyme reactions, and to incubate or inactivate microorganisms. Monitor and record the temperature at each use.

6.3.4.3 Maintenance

Clean the block and wells regularly and after any spillage.

6.3.4.4 Verification

Verify the temperature of the heating block across the working area in a selected number of wells and use the information to modify the acceptable operating range if necessary (see [6.3.2.4](#)).

6.3.5 Refrigerators and cold-storage rooms

6.3.5.1 Description

These are chambers which allow maintenance of cold storage.

For general laboratory purposes, these maintain a temperature of $5\text{ °C} \pm 3\text{ °C}$.

For storage of samples from the food chain before testing, these should maintain a temperature of $5\text{ °C} \pm 3\text{ °C}$ unless otherwise required in specific standards (see [Clause 9](#)).

6.3.5.2 Use

To avoid cross-contamination, use different chambers, or at least different containers, to achieve physical separation for storage of:

- uninoculated culture media and reagents;
- test samples;
- cultures of microorganisms;
- cultures retained after incubation.

Load refrigerators and cold-storage rooms so that appropriate air circulation is maintained and the potential for cross-contamination is minimized.

6.3.5.3 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, if not automatic;
- cleaning and sanitization of the inside of the chambers;
- cleaning and sanitizing of door seals and checking their integrity.

6.3.5.4 Verification

Check and record the temperature of each chamber to confirm it is stable and within the maximum permissible tolerance, using a thermometer or a permanently installed probe.

Alternatively, maximum and minimum thermometers, dataloggers or electronic monitoring may be used.

The resolution and accuracy (uncertainty) required for the temperature-monitoring device are dependent on the purpose for which the unit is used and the specified temperature tolerances (see [6.3.2.4](#) for guidance).

6.3.6 Freezer and deep freezer/ultra-low temperature freezer

6.3.6.1 Description

A freezer is a chamber which maintains frozen storage at a temperature, unless otherwise specified, below $-15\text{ }^{\circ}\text{C}$ and preferably below $-18\text{ }^{\circ}\text{C}$ for samples of frozen food.

A deep freezer/ultra-low temperature freezer is a chamber which maintains deep-frozen storage at a temperature, unless otherwise specified, below $-70\text{ }^{\circ}\text{C}$.

6.3.6.2 Use

Use different chambers, or at least different containers, to ensure physical separation for storage of:

- uninoculated reagents;
- frozen food samples for testing;
- stock and reference cultures of microorganisms.

Load freezers so that the required temperature is maintained, in particular if non-frozen products are introduced.

6.3.6.3 Maintenance

See [6.3.5.3](#).

6.3.6.4 Verification

Check the temperature of each chamber regularly with a suitable temperature-monitoring device (see [6.3.5.4](#)).

6.3.7 Temperature-monitoring devices, including automatic recorders

6.3.7.1 Description

Temperature-monitoring devices are used to monitor temperatures across the range of laboratory activities. These devices include alcohol-in-glass thermometers, thermocouples, platinum resistance thermometers and thermal data logging systems. Mercury-in-glass thermometers are no longer available for purchase and their use is discouraged due to health and safety concerns. 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. Do not use thermometers if the mercury or alcohol column is broken.

WARNING — Mercury is hazardous to health. Remove any spillages.

Devices of adequate accuracy and uncertainty that conform to an appropriate international or national specification may also be used as working temperature-monitoring devices after verification of their performance.

6.3.7.2 Use

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 uncertainty of the temperature-monitoring device, including among other factors the resolution and uncertainty of calibration, should be at least four times smaller than the range of the required maximum permissible tolerance. For example, for a tolerance of $\pm 1\text{ }^{\circ}\text{C}$, this should not exceed $0,5\text{ }^{\circ}\text{C}$; for a tolerance of $\pm 0,5\text{ }^{\circ}\text{C}$, this should not exceed $0,25\text{ }^{\circ}\text{C}$.

Secure temperature-monitoring devices in air incubators (e.g. by placing them 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 total immersion thermometers with only the bulb immersed or use equivalent means to ensure stability. For electronic probe thermometers, immerse the probe according to the manufacturer's instructions.

Immerse temperature-monitoring devices placed in water baths in the water in accordance with individual specifications (e.g. if a partial immersion thermometer is used), immerse it to the depth specified for that thermometer, usually 76 mm or 100 mm.

6.3.7.3 Maintenance

Maintain temperature-monitoring devices in a clean and sound condition in accordance with the manufacturer's instructions.

6.3.7.4 Calibration and verification

Before initial use, fully calibrate reference temperature-monitoring devices against traceable national or international standards across the range for which they are required. Recalibrate regularly and at a frequency defined by the laboratory and in accordance with manufacturer's instructions. Carry out intermediate single point (e.g. ice point or working temperature) checks to verify performance. Use these for reference purposes only and not for routine monitoring.

Verify working temperature-monitoring devices in a way that allows traceability to national or international standards before initial use and at regular intervals. Check these at the ice point and/or against a reference device in the working temperature range.

NOTE Calibrations are fully metrologically traceable by definition, whereas verifications are not.

6.3.8 Balances and gravimetric diluters

6.3.8.1 Description

Electronic balances of appropriate range and resolution are mainly used for weighing the test portion of samples to be examined and the components of culture media and reagents. In addition, they may be used for checking 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 test portion at a set ratio.

6.3.8.2 Use and tolerance

The test portion is weighed on the balance to the tolerance specified in the application and a known volume of diluent added.

For gravimetric diluters, the diluter is set to dispense sufficient diluent for the ratio required (e.g. 9 to 1 for decimal dilutions) with a tolerance of $\pm 2\%$.

For both instruments, the tolerance on mass weights is $\pm 5\%$ and the tolerance on volume weights is $\pm 2\%$ as this is a critical application having a direct effect on test results.

A food microbiology laboratory shall be equipped with balances of the required range and resolution for the different items to be weighed.

Unless otherwise stated, the resolution of the balance or gravimetric diluter shall be good enough to read at least 1 % of the mass. For example, to weigh 10 g, the balance shall be capable of being read to 0,1 g; to weigh 1 g, the balance shall 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.

6.3.8.3 Maintenance

Clean and sanitize the equipment after use or following spillage during weighing with an appropriate and non-corrosive disinfectant (see [Annex A](#)).

6.3.8.4 Calibration and verification

Calibration shall be checked across the entire range by a qualified person at a frequency dependent on use, using standard weights traceable to national or international standards. This should include checks for linearity, reproducibility and repeatability.

Follow the manufacturer's instructions and the technical specification for calibration and verification of gravimetric diluters.

Regularly verify the performance of the balance system with check weights and the volume dispensed from the gravimetric dilutor during use and after cleaning to ensure the range of use is covered.

NOTE In-house check weights can be verified immediately after calibration of the balance.

6.4 Defined volume inoculation equipment

6.4.1 Pipettes and pipettors

6.4.1.1 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.

6.4.1.2 Use

Discard pipettes that are damaged or broken.

Fit sterile Pasteur or graduated pipettes and pipettor tips with a non-absorbent cotton wool plug or filter and ensure the pipette bulb or pipettor barrel is not contaminated when used to manipulate microbial cultures and sample dilutions. Change filters used to prevent contamination of all types of pipettes regularly.

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 on disposable Pasteur pipettes can be found in ISO 7712.

6.4.1.3 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. If this is not possible in the laboratory, return to the manufacturer or to a calibration laboratory for re-assembly and recalibration.

6.4.1.4 Calibration and verification

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

The calibration of pipettes/pipettors is described in ISO 835 and ISO 8655 (all parts).

Calibrate new pipettors on receipt and at regular intervals, depending on the frequency and nature of use (see ISO 8655-1).

Perform intermediate gravimetric checks using distilled or deionized water to ensure that volumes dispensed with pipettes/pipettors remain within the defined limits of ± 2 % for critical applications, such as preparation of dilutions, and ± 5 % for other applications which do not have a direct effect on test results.

6.4.2 Dispensers

6.4.2.1 Dispenser for culture media and reagents

6.4.2.1.1 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.

6.4.2.1.2 Use

Equipment used for dispensing finished culture media and reagents shall be clean and free of inhibitory substances. Use separate tubing for selective culture media to minimize leaching/carryover.

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.

6.4.2.1.3 Cleaning and maintenance

Clean the outer surface of the dispenser regularly and if a spillage occurs. Wash and rinse thoroughly all parts of the dispenser that come into contact with the contents and sterilize them if required for use to dispense sterile liquid. Do not use disinfectants on surfaces that come into contact with the contents to be dispensed as they can impart inhibitory properties.

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

6.4.2.1.4 Verification

The volume delivered by a dispenser for liquid or molten culture media shall achieve an accuracy of ± 5 % of the designated volume.

The accuracy required for dispensing measured volumes of dilution fluid and other critical applications shall be ± 2 % of the designated volume.

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 8655 (all parts).

6.4.3 Spiral platers

6.4.3.1 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 Archimedes spiral (exponential or logarithmic mode). The volume per segment 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 segment is known and is constant. For the loading and dispensing of liquids, a vacuum source and a motorized plunger (if the instrument uses a disposable microsyringe), or an equivalent system, is required.

NOTE Some systems can also be used to prepare dilutions automatically and others are capable of both dilution and different plating modes, but these are not discussed in this document.

6.4.3.2 Use

The equipment is used to dispense a liquid sample, sample suspension or dilution onto an appropriate agar plate 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 so the total number in the inoculum can be calculated.

Sanitize the dispensing system and then rinse with sterile distilled or deionized water, 0,9 % sterile saline or another solution specified by the manufacturer before and after use, and between each sample.

NOTE Sanitizing is not necessary for equipment using sterile, disposable micro-syringes.

Sanitization may be achieved by flushing (e.g. with a solution containing 0,5 % to 2 % mass fraction of active chlorine or 70 % ethanol). Other solutions may be used where specified by the manufacturer. Efficacy of the above reagents should be verified by the user according to the context and microorganism tested. Some systems rinse with sterile 0,9 % saline and prepare serial dilutions before plating.

Use equipment that operates according to a different principle (e.g. disposable micro-syringes with plunger) 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.

Use plates 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.

6.4.3.3 Maintenance

Remove any spillages immediately and clean the equipment on a regular basis.

Service the equipment and verify performance regularly according to usage. In the event of problems, the manufacturer can perform additional verification checks.

6.4.3.4 Verification

Centre an agar plate on the turntable.

Verify the dispensing pattern with washable ink or a dye solution at a frequency according to the manufacturer's instructions. Ensure the spiral pattern is complete with no breaks and is most dense near the centre of the plate where deposition begins, becoming steadily less dense to the point of stylus lift-off. Centre the clear portion of the plate which should be approximately 2,0 cm in diameter. Verify the position of the stylus according to the manufacturer's instructions.

If the pattern is not correct, follow the manufacturer's instructions.

Verify the sterility of the spiral plater by plating sterile water or diluent before examination of each series of samples and at the end of the day. Also check for the absence of antimicrobial residues following sanitization.

NOTE Checks before each series of samples are not necessary for spiral platers which use sterile syringes.

Perform a gravimetric check of the volume dispensed regularly, using distilled or deionized water, by dispensing the volume 10 times and checking the total weight. The mass of the volume dispensed for this critical application shall be within ± 2 % of that expected.

The volumes deposited on various segments of the grid are given in the operator's manual accompanying the spiral plater. For precise calibration, the grid area volumes shall be checked by the manufacturer.

6.4.4 Serial diluters

6.4.4.1 Description

There are many types of serial diluters which can be used to prepare serial dilutions (e.g. decimal dilutions) in accordance with ISO 6887-1 by dispensing and mixing liquids (culture media, samples or bacterial suspensions, etc.). Dilutions are performed in different consumable containers (tubes, bags, cups, beakers, etc.) appropriate for the specific diluter. Some steps may be manual and others automated. The dilution system can be part of another piece of equipment having other functions, such as those used to inoculate different dilutions for plate counts.

The diluent is dispensed into the container of liquid (e.g. sample or initial suspension) to be diluted and mixed. The resulting dilution is then transferred (automatically or not) into further diluent to perform a series of sequential dilutions. For aspiration and dispensing, any type of pipette or syringe may be used.

6.4.4.2 Use

Take appropriate precautions during use of the serial diluter to minimize the risk of contamination and to comply with the manufacturer's instructions.

Monitor the volumes distributed at a documented frequency to check accuracy.

6.4.4.3 Maintenance

Decontaminate or clean/sterilize the equipment and moving parts using an appropriate sanitizer or disinfectant according to the manufacturer's instructions after each use and at least every day.

6.4.4.4 Calibration and verification

Ensure new serial diluters are calibrated on receipt and at regular intervals, depending on the manufacturer's instructions and according to the frequency and nature of use.

To check volumes dispensed, perform intermediate gravimetric checks using appropriate diluent to ensure volumes remain within the defined limits of ± 2 % for critical applications, such as preparation of dilutions, and ± 5 % for other applications which do not have a direct effect on test results.

Check the efficiency of homogenization, initially and at defined frequencies, by carrying out comparative tests with another approved homogenization system, such as a manual vortex.

6.5 Protective cabinets

6.5.1 Description

A protective cabinet is a workstation with horizontal or vertical laminar airflow to remove dust and other particles, such as microbes, from the air. It should be designed to prevent or limit contact between the operator and infectious material and/or to protect material in the cabinet.

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 laboratories, the number of particles shall not exceed 4 000 per cubic metre.

NOTE This maximum number corresponds to ISO Class 5 in ISO 14644-1:2015, Table 1.

Cabinets commonly used in food microbiology laboratories are of various types, as follows:

- Class I biosafety cabinets are open-fronted exhaust-protective cabinets that are intended to protect the operator and the environment but do not protect the work from extraneous contamination. Potentially infected aerosols are contained within the cabinet and trapped when impacting 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.
- Class II biosafety cabinets protect the work, 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 group 2 and 3 pathogens.
- 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 cultures of microorganisms, inoculated samples or preparation of tissue cultures. They may be used for adding test portions to diluents or suspensions to culture media, and for pouring agar plates.
- Vertical laminar airflow cabinets protect the work by use of vertical laminar flow of HEPA-filtered air. They also protect the operator by using internally recirculated air. They are particularly suitable for providing an aseptic environment for handling sterile products and for protecting the operator when handling powders.
- PCR workstations are used to protect the work from free nucleic acids. An ultraviolet (UV) light is fitted for rapid decontamination of the workspace between activities.
- Chemical fume hoods can also be needed outside the testing laboratory for safety reasons to handle certain noxious or flammable chemicals.

Use Class II protective cabinets for all work involving handling of certain pathogens and contaminated powders.

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

6.5.2 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 cabinet. Position all equipment and materials so that disturbance to the airflow over the working area is minimized.

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

6.5.3 Cleaning and disinfection

Clean and disinfect the working area after use with appropriate and non-corrosive disinfectant (see [Annex A](#)) 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 wiped clean with a disinfectant-soaked cloth regularly, taking care not to damage the filter medium.

Safety cabinets should be fumigated before changing the filter or servicing. It is also advisable after spillages of cultures or inoculated samples.

After cleaning the cabinets, UV lamps may be used for disinfection. These should be cleaned regularly to remove any dust and dirt that can block the germicidal effectiveness of the light. UV light intensity should be checked when the cabinet is recertified to ensure that light emission is according to the manufacturer's instructions and the lamps replaced if necessary.^[47]

6.5.4 Maintenance and inspection

The efficiency of a protective cabinet shall be checked by a qualified service agent 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.

Also, periodic checks on the number of airborne microorganisms present should be carried out during operation of the filters using appropriate methods. 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 and monitor the results for changes. Other methods may be used.

6.6 Homogenizers, blenders, mixers and shakers

6.6.1 Homogenizers and blenders

6.6.1.1 Description

This equipment is used to prepare a homogeneous initial suspension from the test portion.

The following apparatus may be used:

- a peristaltic blender with sterile bags, often with a device for adjusting speed and time (e.g. Stomacher^{®1)});
- 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;
- a vibrational mixer with sterile bags (e.g. Pulsifier^{®2)});
- another homogenizing system with equivalent efficiency.

6.6.1.2 Use

The usual operating time of a peristaltic homogenizer is 1 min to 3 min (see the ISO 6887 series for specific applications).

Do not use this type of apparatus for certain food, 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).

1) 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.

2) 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.

Operate the rotary homogenizer 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 to minimize heat gain.

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 before processing.

Manual mixing or shaking with glass beads of approximately 6 mm diameter can be used for preparation of the initial suspensions of certain viscous or thick products, in particular certain dairy products (see the ISO 6887 series and specific standards).

6.6.1.3 Maintenance

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.

NOTE Homogenizers and blenders can be operated on trays to contain any leakage or spills.

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

6.6.2 Vortex mixers

6.6.2.1 Description

This instrument facilitates the homogeneous mixing of liquid culture 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).

6.6.2.2 Use

Press the base of the closed 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.

Ensure that spillage does not occur during mixing by adjusting the speed as necessary and controlling the tube by holding it approximately one-third of its length below the top and so avoid the liquid rising too high in the tube.

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

6.6.2.3 Maintenance

Keep equipment clean. If spillage occurs, decontaminate the equipment using an appropriate laboratory disinfectant (see [Annex A](#)).

6.6.2.4 Verification

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

6.7 Stills, deionizers and reverse-osmosis units

6.7.1 Description

These devices are used to produce purified water (i.e. distilled, deionized, demineralized or produced by reverse osmosis) of the required quality (see 6.7.4) for preparation of microbiological culture media or reagents and for other laboratory applications.

Purified water should be free from substances likely to inhibit or influence the growth of the microorganisms under test conditions, e.g. traces of chlorine, ammonia and metal ions (see ISO 11133).

6.7.2 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.

6.7.3 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, then rinsed thoroughly with freshly distilled water to remove residual acid. Deionizers and reverse-osmosis units should be maintained in accordance with the manufacturer's instructions.

6.7.4 Verification

Check the water regularly and particularly when used after storage for satisfactory conductivity; this shall be no more than $25 \mu\text{S cm}^{-1}$ (equivalent to a resistivity $\geq 0.04 \text{ M}\Omega\cdot\text{cm}$) and should be below $5 \mu\text{S cm}^{-1}$ at 25°C , for preparation of culture media and reagents (see ISO 11133).

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.

6.8 Separation and concentration equipment

6.8.1 Immunomagnetic separator (IMS)

6.8.1.1 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.

6.8.1.2 Use

Follow the manufacturer's instructions for use and those given in specific standards (e.g. ISO 16654 for *Escherichia coli* O157).

6.8.1.3 Maintenance

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

6.8.1.4 Verification

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 into routine use.

It is important to be aware of the potential for cross-contamination during manual separation procedures and to take appropriate steps to avoid this happening. Use of screw-capped containers can reduce the risk from aerosols and sample cross-contamination associated with using flip-cap containers.

6.8.2 Centrifuge

6.8.2.1 Description

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

6.8.2.2 Use

In some applications, concentration of target microorganisms is achieved by centrifuging liquid samples to produce a deposit, which can be resuspended in liquid and subjected to further examination. Always balance the centrifuge by placing samples on opposite sides.

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.

6.8.2.3 Maintenance

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

Centrifuges should be serviced regularly.

6.8.2.4 Calibration and verification

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

6.8.3 Filtration systems

Filtration systems as described in ISO 8199 can be used to concentrate target microorganisms in water and other liquids, such as beverages, which are filterable.

6.9 Modified atmosphere equipment

6.9.1 Description

This can be a jar, bag or box that can be hermetically sealed or any other appropriate equipment which enables modified atmosphere conditions (e.g. anaerobiosis) to be maintained for the total incubation time of the inoculated culture media. Other systems of equivalent performance, such as anaerobic cabinets, are also suitable for incubating larger quantities of inoculated culture media.

6.9.2 Use

The composition of the atmosphere required can be achieved by 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 % to 13 % volume fraction carbon dioxide. Microaerobic (capnaerobic) incubation requires an atmosphere of 5 % to 7 % volume fraction oxygen and approximately 10 % volume fraction carbon dioxide.

Conditions can need modification depending on the requirements of specific microorganisms.

6.9.3 Maintenance

Follow the manufacturer's instructions for installation and maintenance.

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

Regularly clean and sanitize the equipment according to the manufacturer's instructions.

6.9.4 Verification

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

6.10 Other equipment

6.10.1 pH meter

6.10.1.1 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 immersed in 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 $\pm 0,1$ 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.

6.10.1.2 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 as detailed in reference standards specific to the product to be tested (e.g. ISO 5546 for pH of casein).

Adjust the pH meter according to the manufacturer's instructions to measure the pH value at a standardized temperature (e.g. 25 °C). Read the pH value once it has stabilized and record the value to two decimal places.

NOTE The reading can 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 and fresh buffers, equilibrium is normally achieved within 30 s.

6.10.1.3 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;
- the response time and stability.

Rinse the electrodes with distilled or deionized water after each use. To reduce soiling and ageing of the electrodes, regularly clean them thoroughly in accordance with the manufacturer's instructions.

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

6.10.1.4 Calibration and verification

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 buffer solutions should be traceable to national standards 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 pH range of the standards used shall encompass the pH value to be measured.

After calibration of the pH meter with the two traceable standard buffer solutions, a third standard buffer solution should be used to verify performance in "read" mode and 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 checks.

6.10.2 Colony-counting device

6.10.2.1 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 including use of a camera and a monitor.

6.10.2.2 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.

6.10.2.3 Maintenance

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

6.10.2.4 Verification

Automated colony counters should be checked before each use with the validation plate supplied by the manufacturer, which includes a known number of countable particles.

Parallel manual checks should be made on a regular basis to ensure that accurate counts are obtained when using automated colony counters. Differences should ideally be 2 % to 5 % but up to 10 % has been considered acceptable.^[56]

6.10.3 Timers and timing devices

6.10.3.1 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.

6.10.3.2 Use

Analogue and digital hand-held 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 the application.

6.10.3.3 Maintenance

Regularly clean and check timers for correct functioning.

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

6.10.3.4 Verification

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

6.10.4 Optical microscope

6.10.4.1 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 $\times 10$ (dry lens) to about $\times 100$ (oil immersion with spring-loaded turret) are used to obtain an overall magnification of $\times 100$ to $\times 1\,000$. Phase contrast and dark field microscopy are also invaluable for the examination of so-called "wet preparations".

6.10.4.2 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 sub-stage condenser, the slide and the objective lens to the eyepiece so that spherical and chromatic aberrations do not occur.

6.10.4.3 Maintenance

Follow the manufacturer's instructions concerning storage, cleaning and servicing. Prevent condensation occurring where humidity is high as this can 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 contact with the operators' skin from the eyepiece lens.

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

6.10.5 Glass washers, glassware and other laboratory ware

6.10.5.1 Glass washer

6.10.5.1.1 Description

Laboratory glass washers are electronically controlled machines for washing general laboratory glassware. These 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.

6.10.5.1.2 Use

Many types of glass washer are available. In general, install and use glass washers according to the manufacturer's instructions.

6.10.5.1.3 Maintenance

Follow the manufacturer's instructions concerning cleaning and servicing.

6.10.5.1.4 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 (see [6.10.5.2.4](#)).

6.10.5.2 Glassware and other laboratory ware**6.10.5.2.1 Description**

Various items of glassware and other laboratory ware are used in microbiology laboratories. These shall be of suitable design and prepared in such a manner to guarantee cleanliness and/or sterility until use.

6.10.5.2.2 Use

Use all glassware and other laboratory ware correctly for their intended purpose.

6.10.5.2.3 Maintenance

Appropriate closures should be placed loosely on all tubes and bottles, whether filled or empty, to allow free access of steam so sterilization can be achieved.

If necessary, place fragile glassware to be sterilized (e.g. pipettes) in special containers, such as metal cans, or wrap in an appropriate material (special paper, aluminium foil, etc.).

Protect clean glassware and other laboratory ware from dust during storage, in conditions which maintain cleanliness and/or sterility.

6.10.5.2.4 Verification

Use laboratory grade water for rinsing reusable glassware after washing (see [6.7.4](#)). Check for alkaline or acidic residues using a universal pH indicator solution. A pH within the range 6,5 to 7,5 should be achieved.

When sterilizing equipment intended for microbiology, put a suitable expiry date on each package and store such laboratory prepared equipment in clean conditions.

6.10.6 Disposable equipment and consumables

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

Verify that such equipment is suitable for use in microbiology. In particular, check manufacturers' certificates of sterility and check for freedom from substances that inhibit the growth of microorganisms if certificates are not provided.

Store disposable equipment in accordance with the manufacturer's instructions and ensure packaging remains intact, with no deterioration.

6.10.7 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.

Before routine use, calibrate (if possible) and check the equipment to ensure it meets laboratory requirements and complies with the relevant standard specifications. Establish a schedule of calibrations and/or checks for key quantities or values where these properties have a significant effect on the test result.

Any reconfigurations or modifications made by the laboratory to the software shall be verified to ensure that the modified software gives the correct result.

7 Sterilization/decontamination and disposal of laboratory materials

7.1 Sterilization

7.1.1 General

Record the temperature and duration of sterilization. Appropriate sterilization indicators, such as autoclave tape, may be used to distinguish between sterilized and unsterilized materials.

7.1.2 Sterilization by dry heat

Robust laboratory equipment such as glassware, metal sampling implements, etc. may be heated in a sterilizing oven for at least 1 h at $170\text{ °C} \pm 10\text{ °C}$ (see [6.2.5](#)).

7.1.3 Sterilization by moist heat (steam)

Moist steam under pressure is the most effective method of sterilization of laboratory glassware and other materials. The temperature of the autoclave chamber shall remain at $121\text{ °C} \pm 3\text{ °C}$ for at least 15 min (see [6.2.2](#)).

7.2 Decontamination and disinfection

7.2.1 Decontamination of glassware and materials before use

In general, it is preferable to sterilize items of laboratory equipment using dry heat (see [7.1.2](#)) or moist heat (see [7.1.3](#)).

In certain situations, such as field sampling, chemical decontamination^[45] of cleaned equipment can be appropriate.

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

Ensure that any chemical residues do not affect the recovery of microorganisms and that after such treatment the equipment is free from inhibitory substances.

7.2.2 Decontamination of glassware and materials after use

Place materials for decontamination and disposal in containers (e.g. autoclavable plastic bags and/or buckets to prevent spillage).

Autoclaving is the preferred method for all decontamination processes (at least 30 min at $121\text{ °C} \pm 3\text{ °C}$).

NOTE Alternative methods, other than autoclaving, can be used, e.g. chemical sterilization and incineration for molecular waste (see ISO 22174).

Load the autoclave to allow good steam circulation and heat penetration into the load and take care to loosen caps/lids and open bags.

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

During laboratory testing, decontamination by immersion in freshly prepared disinfectant solutions may be used for small and corrosion-resistant equipment or disposables, such as pipettes, loops and slides. However, autoclave all such materials before final disposal as waste.

Most disinfectants have some toxic effects (see the manufacturer's safety data sheet (SDS) and [Annex A](#)). Wear gloves and eye protection, as a minimum, when preparing dilutions from concentrated disinfectant.

7.3 Waste management

Correct disposal of contaminated materials does not directly affect the quality of sample testing, but it is a matter of good laboratory practice.

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

- non-contaminated waste (e.g. food samples) that can be disposed of with general waste;
- scalpels, needles, knives, syringes and broken glass;
- contaminated materials for autoclaving and subsequent reuse in the laboratory;
- contaminated material for autoclaving and disposal, or for disposal only if the material is to be incinerated.

Autoclave materials contaminated with Biosafety Level 3 microorganisms and their containers before they are incinerated.

7.4 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. pipette washer, glass washer (see [6.10.5.1](#)).

After washing, ensure re-usable equipment is free of residues that can affect the subsequent growth of microorganisms.

8 Preparation and use of culture media and reagents

Culture media, diluents and reagents should be prepared in accordance with ISO 11133 and/or specific standard(s).

Follow the requirements and guidance in ISO 11133 for all aspects of use of microbiological media and reagents required for specific standards.

Methods for performance testing of different types of microbiological culture media and reagents are given in ISO 11133. Recommended control strains to be used for each culture medium and reagent are also quoted in ISO 11133 or in specific standards for detection, enumeration or confirmation of microorganisms.

Ready-to-use media that have been tested in accordance with ISO 11133 by the supplier may only need reduced testing by the user laboratory if transport and storage conditions are followed (see ISO 11133).

9 Laboratory samples

9.1 Sampling techniques and sampling plans

9.1.1 General

Although extremely important for the interpretation of test results, sampling techniques and sampling plans are not a part of this document.

However, general guidance is given in this clause to ensure that samples received at testing laboratories are suitable for microbiological examination.

9.1.2 Sampling

The laboratory should receive a sample which is representative of the product, is sufficient for the requested tests and has not been damaged or changed during transport and storage. A documented chain of custody should be established for regulatory work, as samples are transferred, monitored and controlled through each step in the collection and testing process. This helps to provide credibility that sample integrity has been maintained.

Protect the sample against extraneous contamination from the air, the sample container, the sampling devices used and improper handling. A sample container should not be more than three-quarters full to avoid leakage and to allow proper mixing of the sample in the laboratory.

Identify samples clearly and completely and record the sample information.

For certain samples, the temperature at the time of collection and upon receipt is required. This can also be useful to the laboratory for the interpretation of results.

Packaged samples should be submitted to the laboratory in the original, unopened container. If the product or container is too large for submission, transfer a portion aseptically to a sterile sample container and record the product details. Open the sterile sample container for just long enough to transfer the laboratory sample, then close it immediately.

Further details of sampling certain products are given in specific standards (e.g. ISO/TS 17728, ISO 17604, ISO 13307, ISO 18593, ISO 707, ISO 6887-3).

9.2 Sample transport

All laboratories are responsible for checking that samples are received intact and in their original condition, so transport procedures should minimize any alteration in the number of microorganisms present. This applies both to samples collected or transported by the laboratory and also to advising clients on correct transport conditions.

Pack the samples so that breakages or spillages are avoided.

Deliver samples to the laboratory promptly and ensure the original storage conditions are maintained as closely as possible.

Samples stable at ambient temperatures may be packed in a clean container using appropriate packing material to avoid damage.

Product information labels should indicate whether refrigeration or freezing is required.

Do not use loose water ice as this can cause sample contamination if the container breaks or leaks. Instead use portable refrigeration equipment or cold blocks, around but not in contact with the packaged samples.

Unless otherwise required by specific standards (e.g. ISO 6887-3), the following temperatures during transport are recommended:

- stable products: ambient temperature (18 °C to 27 °C);

- frozen or deep-frozen products: below $-15\text{ }^{\circ}\text{C}$, preferably below $-18\text{ }^{\circ}\text{C}$;
- other products not stable at ambient temperature: $5\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$;
- surface samples: refrigerated at $1\text{ }^{\circ}\text{C}$ to $8\text{ }^{\circ}\text{C}$ (see ISO 18593 and ISO 17604).

For some critical samples, such as waters used in food production and environmental surface samples (e.g. swabs, wipes, sponges), the time elapsing between sampling and examination is specified to ensure valid test results (see ISO 19458, ISO 18593 and ISO 17604).

When no conditions are specified, it is recommended that the parties concerned agree on the duration and temperature of transport.

9.3 Sample receipt

Check the condition of the samples on receipt at the laboratory.

If the condition of any sample is unsatisfactory or if there is insufficient sample for the requested testing, the laboratory should refuse the samples and/or notify their client.

In special circumstances, such samples may be tested, but only after discussion and agreement with the client. Include disclaimers about the validity of the results in the test report.

Register the samples received at the laboratory and record all relevant sample details so that their progress through to the time of drafting the test report can be monitored. The identity and coding of samples and records shall ensure traceability throughout all stages in the laboratory.

If necessary (e.g. if the containers are dirty), disinfect the exterior surfaces of the containers using an appropriate disinfectant (see [Annex A](#)).

Check sample containers for evident physical defects which can affect sample integrity.

Note the following information:

- date (and time, where relevant) of receipt;
- details of sampling (sampling date and time and, if relevant and known, sample condition);
- client name and address.

On receipt of perishable samples, record the temperature of transport or the temperature of a simulated sample included for this purpose. If neither option is available, take the temperature with an infrared thermometer or a probe in contact with the sample packaging, or mark the position where the temperature of the sample was taken so this area is avoided when taking the test portion.

Examine the samples as soon as possible after receipt and preferably within 24 h, or as agreed with the parties concerned.

For highly perishable products (such as shellfish) or where specified (such as waters and swabs), test within 24 h of sampling. For other perishable products (such as fish or raw milk), ideally test within 36 h.

Freezing of samples other than already frozen products before testing is not permitted as this can affect test results by changing the microbial load.

9.4 Sample handling

9.4.1 General

Handle samples at all stages through the laboratory to minimize any changes in the number of microorganisms present.

9.4.2 Storage before examination

Store samples awaiting examination under conditions which minimize any alteration in the number of microorganisms present.

The following short-term storage temperatures are recommended:

- stable products: ambient temperature (18 °C to 27 °C);
- frozen or deep-frozen products: below –15 °C, preferably below –18 °C;
- other products not stable at ambient temperature, including spoiled food, unless otherwise stated in specific standards: 5 °C ± 3 °C (see ISO 6887-3);
- surface samples: refrigerated at 1 °C to 8 °C (see ISO 18593 and ISO 17604).

9.4.3 Test portions

Refer to the relevant part of the ISO 6887 series or specific standards for rules for taking the test portion and preparing the homogenate and initial suspension.

9.4.4 Storage of laboratory samples after examination

Except in special cases when samples cannot be retained, such as waters or surface samples, keep the laboratory samples or a representative portion until all results have been obtained, or longer if required by the client.

Pack in sterile containers (e.g. a plastic bag) and replace in the storage conditions specified in [9.4.2](#).

It is not accepted practice to retest stored samples, because the microbial distribution in laboratory samples is heterogeneous and the microbial population can change during storage. Thus different results can be obtained for both qualitative and quantitative retests.

NOTE For qualitative tests capable of detecting low numbers of the target organism, a “not detected” result in the retest does not negate detection of the target in the initial test.

However, if retesting is necessary (e.g. in outbreak investigations) or is requested by the client, report the retest results separately and clearly marked as a retest. Ideally another sample of the same product should be obtained from the client for further testing.

9.5 Pre-testing of samples

For some microbiological examinations, knowledge of physical sample parameters such as pH or water activity can be required before testing. Perform any necessary pre-testing on sub-samples taken aseptically.

Guidance on pH testing of individual food commodities is available in specific national, regional and international standards (e.g. ISO 5546 for pH of casein).

General guidance and specific requirements for testing the water activity of food and feed are given in ISO 18787, together with precision data obtained from interlaboratory studies. Additional information is given in the manufacturer’s instructions for the specific water activity measurement equipment in use. The manufacturer’s instructions should be followed.

10 Examination

10.1 Hygienic precautions during sample preparation and examination

10.1.1 General

Take all necessary precautions to ensure sample preparation and examination in the laboratory are conducted under aseptic conditions. Detection/enrichment techniques are particularly sensitive to accidental contamination from the laboratory environment.

10.1.2 Basic precautions

Basic precautions before beginning sample examination include the following:

- Laboratories should be designed with physical separation between critical areas such as: sample check-in; storage and set up areas; pre-enrichment and culture transfer areas; and culture media and reagent preparation, sterilization and storage areas (see [Clause 4](#)).
- Ensure that the work area is clean, that all possible sources of contamination have been removed or reduced to the minimum, that there are no draughts (i.e. that the doors and windows are closed) and avoid unnecessary movement of personnel during the examination.
- Clean the surrounding work area and swab with an appropriate disinfectant (see [Annex A](#)) before sample preparation or testing begins.
- Ensure all necessary items are on hand before starting work.
- Use a biological safety cabinet for handling samples likely to contain pathogenic bacteria.
- Separate activities assessed as “low-risk”, such as sample receipt or culture media preparation, and those assessed as “high-risk” by time or location to reduce the potential for cross-contamination. This is particularly important with high-risk samples such as powders, raw meat and raw eggs, and high-risk activities involving handling of cultures, such as transfers, isolation procedures and biochemical/serological identification.
- To avoid contamination of the environment and test portions, handle powdered or highly contaminated samples in a separate room or area and preferably in a protective cabinet.
- Wash hands immediately before testing and again during testing if they become contaminated.

10.1.3 Sample handling

Sample handling precautions are necessary to reduce the risk of cross-contamination (see the ISO 6887 series for sample-specific details) and include the following:

- Before opening packaged samples, swab the exterior area around the intended opening point with 70 % (by volume) alcohol (or another appropriate disinfectant, see [Annex A](#)) and allow to evaporate. Aseptically open the packaging and remove the sample or a test portion using sterile instruments (can opener, scissors, spoon, forceps, pipette, etc.).
- Take special care when handling spoiled samples showing gas build up (e.g. blown cans).
- Open and prepare one sample at a time. Finish and clean up before going on to the next sample.
- When inoculating tubes with cultured material or enrichment cultures, open only one tube at a time for inoculation.

10.1.4 Sample handling tools and implements

Use suitable tools and implements for different sample types as detailed in the ISO 6887 series and take the following general precautions:

- sterilize all implements and protect them before and during use from exposure to contamination;
- place used implements in a suitable vessel for subsequent sterilization and/or disposal;
- use disposable implements whenever possible where there is a risk of “carry over” contamination;
- if the whole contents of a pack of disposable pipettes, Petri dishes, etc., is not used during the course of an examination, close the pack and seal it tightly to minimize contamination;
- when removing sterile pipettes from their pack, do not allow the tips to touch the outside surfaces or any remaining pipettes to reduce potential contamination;
- do not allow pipettes to touch the outside of dilution bottles or tubes, especially the rims or necks.

10.1.5 Spillages

Immediately mop up any spillages using cotton pads or other appropriate material impregnated with 70 % (by volume) alcohol or any other appropriate disinfectant (see [Annex A](#)), then clean and disinfect the entire work surface before continuing. Do not use a spray bottle as these can create aerosols.

The incident should be documented to assist with investigation of any subsequent cross-contamination issues.

10.1.6 Process controls

When using process control strains (see [16.2.2](#)) alongside testing, particularly positive controls of the target organism, reduce the potential for laboratory cross-contamination and false-positive results by:

- inoculating the controls in a separate area from the test samples or separate in time;
- inoculating all process controls at the end of testing;
- choosing specific positive control organisms that are easily distinguishable from typical sample isolates (e.g. by choosing uncommon strains);
- using a genetically altered control strain with easy-to-detect characteristics, such as luminescence or fluorescence.

NOTE The use of genetically altered control strains is restricted by some national or regional regulations.

10.1.7 Aerosols

Aerosols are a major cause of environmental contamination and of laboratory-acquired infection and shall be minimized during testing.

Aerosols can be formed when:

- opening Petri dishes, tubes and bottles containing inoculated cultures;
- using shakers, vortex mixers, blenders, syringes, centrifuges, etc.;
- evacuating pipettes;
- flame-sterilizing inoculation loops or needles still carrying wet contaminated inocula;
- opening and reconstituting ampoules containing freeze-dried cultures.

Take appropriate precautions where possible. A culture medium blank can be exposed alongside testing and incubated with batches of samples to check for this type of contamination.

10.1.8 Molecular methods

Additional precautions are necessary for PCR methods (see ISO 22174).

10.2 Preparation of initial suspension and dilutions

10.2.1 General

Prepare the initial suspension and dilutions in accordance with the relevant part of the ISO 6887 series.

The time which elapses between the end of preparation of the initial suspension and the moment the inoculum comes into contact with the culture medium should ideally be less than 20 min and shall not exceed 45 min unless specific conditions (e.g. soaking of dehydrated samples to reduce osmotic shock) are given in the specific standard.

The initial suspension and dilution steps may be followed by an enrichment step as described in specific standards.

10.2.2 Concentration

10.2.2.1 Centrifugation or membrane filtration

If enumeration of low numbers of microorganisms is required this may be improved, in terms of both sensitivity and precision, by concentrating the test portion using centrifugation or membrane filtration.

Evaluate whether it is possible to filter food suspensions before choosing the concentration step.

If using centrifugation, resuspend the centrifuged deposit in a known volume of diluent before testing.

For each combination (food and microorganism) verify whether the addition of a concentration step is necessary and valid. ^[55]

The performance of the overall method, in terms of sensitivity, selectivity, linearity and repeatability, should be compared with that of the specific standard (without concentration) conducted in parallel.

10.2.2.2 Immunoseparation

If low numbers of target microorganisms are present in the sample, separation and concentration of microorganisms may be achieved with immunomagnetic beads coated with specific antibodies. Spread the beads, together with the captured target microorganisms, directly onto the culture medium specified in relevant standards.

Before use, check that the immunomagnetic beads coated with the specific antibodies used for this concentration step are suitable and give expected results in the laboratory with control samples containing the target microorganisms. Such checks are especially important if the procedure has not been validated in accordance with the ISO 16140 series.

11 Enumeration (quantitative) methods

11.1 General

When assessing the microbiological quality and/or safety of food, feed, environmental or other samples taken from the food chain, it is often not enough to know only which microorganisms are present. In most cases, the quantitative aspect is equally important, and enumeration of the microorganisms is required. This may be achieved in various ways: through direct examination (microscopy), by inoculating solid or liquid culture media, with flow cytometry, by PCR methods, etc. However, this clause covers only enumeration using solid and liquid culture media.

Enumeration on solid culture media is based on the fact that many microorganisms produce visible colonies in or on solid culture media that can be seen with the naked eye or with a simple magnifying system.

However, if the matrix contains particles, which can be confused with colonies when counting the plates, these should first be separated by allowing the suspension to settle or by using filter bags. In some cases, an inoculated plate can be refrigerated at $5\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$ for comparison after incubation (see [11.2.5](#)).

If the level of bacteria is very low (i.e. $< 10\text{ cfu/ml}$), enumeration on or in solid media cannot be used without, if possible, first separating the target microorganisms from the matrix, e.g. using immunoseparation or concentration by filtration or centrifugation (see [10.2.2](#)). Another solution in the case of expected low numbers can be to increase the volume of the inoculum (e.g. $\text{XX ml} > 1\text{ ml}$) used in pour plating, but the ratio between the inoculum volume and quantity of agar added for a 1 ml inoculum shall be maintained.

In such cases, enumeration with liquid media is often a suitable alternative (see [11.3](#)).

11.2 Enumeration using a solid medium

11.2.1 General

The choice between the pour or surface plating methods described in [11.2.2](#) and [11.2.3](#), respectively, depends on the requirements of specific standards and the objective of the analysis.

Label or otherwise identify the Petri dishes with the sample number, dilution, date if necessary, and any other relevant information.

Select dilutions to prepare and inoculate to ensure that plates containing the appropriate number of colonies are obtained (see [11.2.6.1](#)) and to overcome any possible inhibitory properties of the sample.

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

For pour or spread plate enumeration techniques on solid media, use one plate per dilution with at least two subsequent dilutions for laboratories which operate in accordance with the principles of ISO/IEC 17025.

NOTE The use of the initial suspension at different volumes (1 ml and 0,1 ml) is also considered as “subsequent dilutions”.

If only one dilution is performed or if a laboratory does not operate in accordance with these principles, then use two plates.

11.2.2 Pour plate technique

Pour plating is the preferred technique for enumeration methods where:

- a low limit of determination is specified or required;
- products are expected to contain spreading colonies that obscure growth of other organisms (e.g. milk and milk products likely to contain spreading *Bacillus* spp.);
- products are expected to contain oxygen sensitive bacteria (e.g. some lactic flora that develop during shelf-life or modified atmosphere storage).

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 and then dispense the contents.

Remove the tempered culture medium at $44\text{ }^{\circ}\text{C}$ to $47\text{ }^{\circ}\text{C}$ from the water bath (or incubator) and, if necessary, wipe the bottle dry with a clean towel to prevent water from contaminating the plates. Avoid spilling the culture medium on the outside of the container or on the inside of the plate lid when pouring. This can require holding the bottle in a near horizontal position or avoiding setting the bottle down between pouring steps.

Pour the molten culture medium into each Petri dish (generally 18 ml to 20 ml of agar in 90 mm Petri dishes or as required in 140 mm dishes, to obtain at least 3 mm thickness). Avoid pouring the molten culture medium directly onto the inoculum as this can cause heat shock to the microorganisms in the inoculum. Immediately mix the molten culture medium and the inoculum carefully to obtain a homogeneous distribution of the microorganisms within the medium. Allow to cool and solidify quickly by placing the Petri dishes on a cool horizontal surface and invert them only when the culture medium has set (solidification time should not exceed 10 min).

If the presence of spreading colonies (e.g. *Proteus* spp.) is expected in the product to be examined or if stated in specific standards, overlay the solidified plates with sterile non-nutritive culture medium or the culture medium used in the test (generally 5 ml of agar in 90 mm Petri dishes) to prevent or minimize spreading.

When low numbers (i.e. < 10 cfu/ml) are expected, the volume of the inoculum in pour plating can be increased (e.g. XX ml > 1 ml), but the ratio between the inoculum volume and quantity of culture medium added for a 1 ml inoculum shall be maintained.

11.2.3 Surface plating techniques

11.2.3.1 General

Methods of plating designed to produce only surface colonies on culture medium plates differ from the pour plate method in a number of ways, such as:

- the morphology of surface colonies is easily observed, improving the ability to distinguish between different types of colony;
- colonies are more easily removed for sub-culturing;
- heat labile microorganisms are not exposed to tempered culture medium;
- improved growth of obligate aerobes.

Use pre-poured plates, containing a minimum of 3 mm of the culture medium, which are level and free from air bubbles and have been dried before use to remove surface moisture.

To facilitate uniform spreading, dry the surface of pre-poured plates in accordance with ISO 11133 or as stated in specific standards so that the inoculum is absorbed within 15 min.

11.2.3.2 Spread plate 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 solid samples, to the agar plate (90 mm or 140 mm in diameter, respectively). Repeat this step for the next decimal dilution, to give a dilution step of 10^{-1} , in the case of liquid samples, and 10^{-2} in the case of solid samples. If higher counts are expected, repeat for further decimal dilutions (see [11.2.6](#)).

To detect lower microbial counts if required, the limit of detection may be increased by a factor of 10 by testing 1,0 ml of liquid samples or 1,0 ml of the initial suspension of solid samples. This is achieved by spreading 1,0 ml of the inoculum over the surface of a large (140 mm diameter) Petri dish or over the surfaces of three 90 mm diameter Petri dishes (see [11.2.7.2.7](#)).

Using a sterile spreader or spatula made of glass, plastic or stainless steel, spread the inoculum evenly over the culture medium surface as quickly as possible without touching the side walls of the Petri dish. Use a separate spreader for each dilution, unless working from the highest to the lowest dilution. Allow the inoculum to absorb with the lids in place for about 15 min at room temperature.

NOTE A spreader can be made from a glass rod about 3,5 mm in diameter, shaped like a hockey stick about 20 cm long, bent at right angles at about 3 cm from one end and flattened at the ends by heating.

In some cases, as stated in the specific standard, the inoculum may be deposited on a membrane, before spreading as previously described.

11.2.3.3 Spiral plate method

11.2.3.3.1 General

The spiral plate method^[53] has been in use for many years for routine monitoring of aerobic colony counts in food expected to contain higher levels of microorganisms ($\geq 4\ 000$ cfu/g or ≥ 400 cfu/ml) and has been extensively evaluated.

The method uses dedicated equipment, a spiral plater (see 6.4.3), to deposit an inoculum of 50 μ l on the surface of plates quickly and easily. Generally, fewer or no dilutions are required and duplicate plates (see 11.2.1) are not needed because a single spiral plate represents multiple dilutions. It is, however, not suitable for larger or spreading colonies which can merge, making counting plates accurately impossible.

NOTE 1 Some spiral platers deposit other volumes, such as 100 μ l, but there is little validation data available for volumes other than 50 μ l.

Transfer, using a sterile pipette, an adequate amount (e.g. 3 ml to 5 ml) of the sample homogenate to a sterile disposable beaker. The inoculum of 50 μ l of a liquid sample or a suspension, in the case of other products, is deposited continuously on the surface of a rotating culture medium plate in the form of an Archimedes spiral, producing multiple successive dilutions on a single plate. The volume dispensed is decreased while the dispensing system (stylus or disposable sterile micro-syringe) moves from the centre to the edge of the plate, so that an exponential relationship exists between the volume deposited and the radius of the spiral.

NOTE 2 Other plating modes are available on some spiral platers but only the exponential (logarithmic) mode is discussed in this document.

Exponential spiral plating as an option in surface plating enumeration methods shall only be used instead of spread plating if this is indicated in the relevant specific standard, based on supporting data from a comparative validation study (see ISO 17468), and together with the applicable range of results. In addition, perform verification for routine use in each individual laboratory in accordance with ISO 16140-3.

11.2.3.3.2 Preparation of culture medium plates

An automatic dispenser with a sterile delivery system is recommended for the preparation of culture medium plates, to help ensure that the plates are level.

Pour the same quantity of culture medium into all the plates so that the same height (a minimum of 3 mm) is presented to the spiral plater stylus tip to maintain the correct contact angle.

Alternatively, commercially prepared ready-to-use culture medium plates may be used.

Before use, dry all plates as described in ISO 11133 or in the specific standard and ensure they have reached room temperature.

11.2.3.3.3 Plating procedure

Place a pre-poured culture medium plate on the turntable and launch the plating procedure. The inoculum is collected by the stylus and differentially dispersed as the stylus tip rides over the surface of the rotating plate. The stylus will return to the starting position and the inoculated plate can be removed.^[53]

11.2.4 Enumeration of yeasts and moulds

Yeasts and moulds are usually 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 culture medium (see the ISO 21527 series). Dry pre-poured culture medium plates before inoculating (see ISO 11133).

Some yeasts and moulds can be infectious or can elicit allergic responses, sometimes even in healthy individuals, so it is important to be cautious when working with them. Plates should be kept in incubators or other containers, not in an open room. Plate lids should be removed as infrequently as possible and only for essential purposes such as preparation of slides for microscopic examination. Care should be taken when

manipulating cultures to avoid dispersal of conidia and other cells. Work benches and incubators should be disinfected routinely to avoid spores spreading and contaminating other tests.

Incubate Petri dishes in an upright position undisturbed until ready to be counted, as movement can release mould conidia or spores to develop into satellite colonies, causing overestimation of the population.

11.2.5 Incubation

For bacterial counts, unless otherwise stated in specific standards, immediately invert dishes once pour plates have set and surface-inoculated plates have dried and place them in an incubator set at the appropriate temperature. If excessive dehydration can occur (e.g. at 55 °C or in strong air circulation), wrap the dishes loosely in plastic bags or place them in clean containers before incubation. An open container of water can also be placed in the incubator to counteract dehydration.

Petri dishes should not be stacked 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.

During the incubation period, minor variations in the incubation temperature can be unavoidable and are acceptable (e.g. during the usual operations of loading or unloading the incubator). However, it is important that variations caused by these operations are kept to a minimum so that they do not have a significant effect on the test result.

It is sometimes necessary for the laboratory to refrigerate the inoculated dishes at $5\text{ °C} \pm 3\text{ °C}$ for a maximum of 24 h before incubation. If this is done, the laboratory shall verify that this practice does not affect the resulting counts.

After incubation, the dishes should normally be examined immediately. They may, however, be stored, unless otherwise stated in specific standards, for up to 48 h at $5\text{ °C} \pm 3\text{ °C}$ in a refrigerator. Refrigerated storage for longer periods is only acceptable if it has been verified as having no effect on the numbers, appearance or subsequent confirmation of the colonies. With certain culture media containing indicator dyes, refrigerated plates should be allowed to equilibrate at room temperature before examination, to ensure that the correct colour is regained.

11.2.6 Calculation and expression of results obtained with solid culture media

11.2.6.1 Plate counts

Following the period of incubation stated in the specific standard, count the colonies (total colonies, typical colonies or presumptive colonies) from the Petri dish from the first, more concentrated (lower) dilution with no more than 300 total colonies or no more than 150 typical or presumptive colonies (for a 90 mm diameter Petri dish) or the maximum number as stated in the specific standard.

Also count the colonies from a Petri dish if the number of colonies is within the interval between the maximum countable number plus 1 (e.g. 301 for 300 colonies) and the upper limit of the confidence interval for the maximum countable number (e.g. 334 for 300 colonies) (see [Annex B](#)).

If higher dilutions have been prepared, also select Petri dishes from the second subsequent dilution, including for calculation of results, plate(s) with 0 colonies, i.e. no growth observed (see [11.2.7.2.7](#), Example 3).

When Petri dishes with different diameters are used, the maximum number of colonies shall be increased or decreased in proportion to the surface area of the dishes (or membranes) (e.g. 110 colonies for 55 mm dishes or 730 colonies for 140 mm dishes instead of 300 colonies for 90 mm).

Consider spreading or chains of 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 the theoretical number by extrapolation and report as an estimated result. Discard the count if more than one-quarter is overgrown.

If the level of determination has been lowered (for low microbial counts) by inoculating a number of Petri dishes with a higher volume of the same dilution, consider all these Petri dishes as only one count.

Special cases can occasionally occur (e.g. the ratio of dilution factors used for two successive dilutions can be quite different), and it is therefore necessary for the results to be examined and interpreted by a qualified microbiologist and, if necessary, rejected.

Acceptability limits of the differences between the number of colonies from parallel duplicate plates can be established according to the probability of compliance estimated using the formula given in ISO 14461-2:2005, 7.1, which has been incorporated into the colony count techniques (CCT) calculator available at: <https://standards.iso.org/iso/7218/ed-4/en/> together with the verification report for this calculator.

Similarly, acceptability limits of the differences between the number of colonies from two subsequent 10-fold dilutions can be established using the formula given in ISO 14461-2:2005, 7.2, which has also been incorporated into the CCT calculator.

11.2.6.2 Spiral plating

For spiral plate counting, use counting grids according to the manufacturer's instructions.

Centre the incubated plate over the grid. Choose any segment and count the colonies from the outward edge into the centre until 20 colonies have been counted. Continue to count the remaining colonies contained in the area (i.e. segment or subdivision of segment) in which the 20th colony was observed. Count in the same area on the opposite side of the plate and divide the total count of the two areas by the volume known to be deposited on the counted areas. This gives the number of colonies per millilitre of inoculum.

Count only areas with well-isolated colonies. If the total number of colonies counted in the area containing the 20th colony exceeds 75 or the area does not contain discrete colonies, the count is generally not accurate because of coincidence error due to crowding of colonies, and this area is not considered.

If less than 20 colonies, the lower limit to achieve acceptable variance for spiral plating, [44][46][52][54] is counted on the segment selected, count the total plate and follow the general instructions for plate count.

11.2.6.3 Counting yeast and mould colonies

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 or full identification is needed, examine wet mounts or stains of cells from at least five colonies per sample to confirm that bacteria are not present.

11.2.6.4 Expression of results

The final number (N) of colony forming units (cfu) per g or per ml, calculated according to the formula used for general or special cases, should be expressed as a number between 1,0 and 9,9 times the appropriate power of 10, or a whole number with two significant figures. When doing this, if the third figure is less than five do not modify the preceding figure, and if the third figure is greater than or equal to five, increase the preceding figure by one unit.

For most microbiology plate count methods, other than spiral plating (see 11.2.6.2), 10 colonies are generally accepted as the lowest reliable count and therefore the lower limit of determination. For results below the limit of determination, the presence of the target organism can be detected but not quantified sufficiently.

Therefore, if the plate (or both plates) from the first dilution retained contains in total less than 10 colonies, express the final result as an "estimated number" (N_E).

NOTE 1 The term "estimated number" means a less precise estimate of the true value.

If the total number of colonies is < 4 (i.e. 1, 2 or 3), the precision of the result is extremely low. The final result indicates only that microorganisms are present and is expressed as less than $4/Vd$ per gram or per ml because the estimated numerical value is statistically unreliable.

NOTE 2 At an average count of three colonies per test portion, provided that the Poisson distribution prevails, the chance of detecting the presence of the analyte (i.e. observing at least one colony) equals 95 %. For further details of the statistical background, see ISO 8199.

11.2.7 Calculations for enumeration methods

11.2.7.1 General

Formulae for the calculations for all enumeration methods using solid media are given in [11.2.7.2](#) and [11.2.7.3](#) alongside worked examples.

11.2.7.2 Plate count calculations

11.2.7.2.1 General

Calculations for the general case, counts after confirmation and some special cases are given in [11.2.7.2.2](#) to [11.2.7.2.7](#). Only 10-fold dilutions are considered for the calculation.

For a result to be valid (see [11.2.1](#)), it is generally considered necessary to retain at least two countable plates (duplicates from the same dilution or plates from two successive dilutions).

11.2.7.2.2 Plate count calculation: General case (counting of total colonies or typical colonies without confirmation)

Calculate the number N of microorganisms in colony forming units per gram or millilitre (cfu/g or ml) of the test portion using [Formula \(1\)](#):

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

where

$\sum C$ is the sum of the colonies counted on all the dishes retained from one or two successive dilutions;

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

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

n_2 is the number of dishes retained at the second dilution ($n_2 = 0$ if it is not performed);

d is the dilution corresponding to the first dilution retained ($d = 1$ for liquid product undiluted).

EXAMPLE Counting in a pour plate method with one Petri dish per dilution, has produced the following results:

— At the more concentrated dilution retained (10^{-2}): 168 colonies.

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

$$N = \frac{168 + 14}{1 \times [1 + (0,1 \times 1)] \times 10^{-2}} = \frac{182}{0,011} = 16\,545$$

Rounding off the results, the number of microorganisms is $N = 17\,000$ or $N = 1,7 \times 10^4$ cfu/g or ml.

where

ΣC is $168 + 14 = 182$;

V is 1 ml of dilution plated in each Petri dish;

n_1 is 1 Petri dish retained for the 10^{-2} dilution;

n_2 is 1 Petri dish retained for subsequent 10^{-3} dilution;

d is $0,01 = 10^{-2}$ dilution corresponding to the first dilution retained.

11.2.7.2.3 Plate count calculation: Count after confirmation

When the method requires confirmation, a given number A (generally five) of presumptive colonies are confirmed from each of the dishes retained for counting.

After confirmation, calculate, for each of the dishes, the number of colonies (a) complying with identification criteria using [Formula \(2\)](#):

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

where

A is the number of presumptive colonies selected for confirmation from each Petri dish;

b is the number of colonies confirmed from A ;

C is the total number of presumptive colonies in the Petri dish.

In addition, replace ΣC by Σa in [Formula \(1\)](#).

NOTE If Σa is < 10 , the final result is expressed as estimated.

EXAMPLE Counting from a surface plating method has produced the following results:

- At the more concentrated dilution retained (10^{-1}): 75 colonies. Of the 75 colonies, 5 colonies have been tested, 3 of which were confirmed:

$$a = \frac{b}{A} \times C = \frac{3}{5} \times 75 = 45$$

- At the subsequent dilution retained (10^{-2}): 4 colonies. Of the 4 colonies, 4 colonies have been tested, 1 of which was confirmed:

$$a = \frac{b}{A} \times C = \frac{1}{4} \times 4 = 1$$

Replace ΣC by Σa in [Formula \(1\)](#):

$$N = \frac{45 + 1}{0,1 \times [1 + (0,1 \times 1)] \times 10^{-1}} = \frac{46}{0,011} = 4\,182$$

Rounding off the results, the number of microorganisms is $N = 4\,200$ or $N = 4,2 \times 10^3$ cfu/g or ml.

where

Σa is $45 + 1$ (instead of $\Sigma C = 75 + 4$);

V is 0,1 ml of dilution plated in each Petri dish;

n_1 is 1 Petri dish retained for the 10^{-1} dilution;

n_2 is 1 Petri dish retained for subsequent 10^{-2} dilution;

d is $0,1 = 10^{-1}$ dilution corresponding to the more concentrated dilution retained.

11.2.7.2.4 Plate count calculation, special case: No colonies detected

If the Petri dishes do not contain any colonies (or presumptive colonies have not been confirmed), report the result using [Formula \(3\)](#):

$$N < \frac{1}{V \times d} \quad (3)$$

where

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

d is the dilution corresponding to the first dilution inoculated ($d = 1$ for liquid product undiluted).

EXAMPLE Counting from a pour plate method has produced no colonies in the 10^{-1} dilution:

$$N < \frac{1}{1 \times 10^{-1}} < 10$$

where

V is 1 ml inoculated in each Petri dish;

d is $0,1 = 10^{-1}$ the dilution corresponding to the first dilution inoculated.

11.2.7.2.5 Plate count calculation, special case: More than the maximum number of typical colonies

If all Petri dishes contain more than the maximum number of typical colonies stated in the specific standard (usually 300 or 150 colonies), report the result using [Formula \(4\)](#):

$$N > \frac{C}{V \times d} \quad (4)$$

where

C is the maximum number of colonies stated in the specific standard;

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

d is the dilution corresponding to the highest (most diluted) dilution ($d = 1$ for liquid product undiluted).

For colonies after confirmation, estimate a according to [Formula \(2\)](#) and replace C by a .

EXAMPLE Counting in a spread plate method has produced more than 150 colonies (maximum number of colonies stated in the method) in the two dilutions performed (10^{-1} and 10^{-2}):

$$N > \frac{150}{0,1 \times 10^{-2}} > 150\ 000 > 1,5 \times 10^5$$

where

C is 150 (maximum number of colonies stated in the method);

V is 0,1 ml inoculated in each Petri dish;

d is $0,01 = 10^{-2}$ the dilution corresponding to the highest (most diluted) dilution performed.

11.2.7.2.6 Plate count calculation, special case: Presence of presumptive colonies with more than the maximum number of total (typical and atypical) colonies

If all Petri dishes contain more than the maximum number of total colonies stated in the specific standard and presumptive colonies are present but not confirmed in all Petri dishes, results are not conclusive and shall not be reported.

If several dilutions have been performed, all containing more than the maximum number of total colonies (e.g. 300 or 150) and presumptive colonies are confirmed, follow [11.2.7.2.3](#) or [11.2.7.2.5](#).

If one dilution (d_1) contains more than the maximum number of total colonies (e.g. 300 or 150) with presumptive colonies confirmed, and the subsequent dilution performed (d_2) does not contain presumptive colonies or are not confirmed, report results as:

“Less than $1/Vd_2$ and more than $1/Vd_1$ cfu/g or ml.”

where

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

d is the dilution corresponding to the dilutions retained.

11.2.7.2.7 Plate count calculation, special case: Lowered limit of determination (plating a set of Petri dishes)

If each plate of the set of Petri dishes contains less than the maximum number stated in the specific standard, report the results according to the general case [see [Formula \(1\)](#)], but substituting V by V_{set} , using [Formula \(5\)](#):

$$N = \frac{\sum C}{V_{\text{set}} \times [n_1 + (0,1 \times n_2)] \times d} \quad (5)$$

where V_{set} is the total volume of inoculum applied to the set of Petri dishes in ml. When the method requires confirmation, estimate a according to [Formula \(2\)](#) and replace C by a .

EXAMPLE 1 For a spread plate method, three Petri dishes with a total volume of 1 ml from the first dilution (10^{-1}) and one Petri dish with 0,1 ml from the same dilution (10^{-1}) have produced the following results:

- At the higher volume spread (1 ml from 10^{-1} dilution in three Petri dishes considered as only one Petri dish): Petri dish A: 6 colonies; Petri dish B: 8 colonies; Petri dish C: 9 colonies. Total (A+B+C): 23 colonies.
- On the subsequent Petri dish spread with a lower volume (0,1 ml from 10^{-1} , equivalent to 1 ml of the subsequent 10^{-2} dilution): 4 colonies.

$$N = \frac{(6+8+9)+4}{1 \times [1 + (0,1 \times 1)] \times 10^{-1}} = \frac{27}{0,11} = 245$$

Rounding off the results, the number of microorganisms is $N = 250$ or $N = 2,5 \times 10^2$ cfu/g or ml.

where

$\sum C$ is $(6 + 8 + 9) + 4 = 27$;

V_{set} is 1 ml of dilution plated in total in the first set of plates;

n_1 is 1 Petri dish (the three Petri dishes are considered as only one);

n_2 is 1 Petri dish retained for the subsequent dilution;

d is $0,1 = 10^{-1}$ dilution corresponding to the more concentrated dilution retained.

If the set of Petri dishes contains no colonies, follow the special case but replace V (total volume inoculated in each Petri dish) by V_{set} (total volume of inoculum applied to the set of Petri dishes).

EXAMPLE 2 Counting in a spread plate method has produced no colonies in the 10^{-1} dilution inoculated in a set of three Petri dishes (1 ml in total). Report the result N as follows:

$$N < \frac{1}{1 \times 10^{-1}} < 10$$

where

- V is 1 ml inoculated in the set of three Petri dishes;
- d is $0,1 = 10^{-1}$ the dilution corresponding to the first dilution inoculated.

If at least one of the Petri dishes from the first set of plates contains more than the maximum number stated in the specific standard, discard the first set of Petri dishes and proceed from the subsequent dilution to estimate the number N of microorganisms following the corresponding general or special case.

EXAMPLE 3 For a spread plate method, three Petri dishes with a total volume of 1 ml from the first dilution (10^{-1}), one Petri dish with 0,1 ml from the same dilution (10^{-1}) and a Petri dish with 0,1 ml from the second dilution (10^{-2}), have produced the following results:

- At the higher volume spread (1 ml from 10^{-1} dilution on three Petri dishes): > 150 colonies in at least one plate.
- On the subsequent Petri dish spread with a lower volume (0,1 ml from 10^{-1}): 33 colonies.
- On the subsequent 10-fold dilution (0,1 ml from 10^{-2}): 0 colonies (no growth).

$$N = \frac{33}{0,1 \times [1 + (0,1 \times 1)] \times 10^{-1}} = \frac{33}{0,011} = 3\,000$$

The number of microorganisms is $N = 3\,000$ or $N = 3,0 \times 10^3$ cfu/g or ml.

11.2.7.3 Spiral plate calculations

11.2.7.3.1 General

Calculations for spiral plate results require a different approach. Details for the general case, counts after confirmation and two special cases are given in [11.2.7.3.2](#) to [11.2.7.3.5](#), respectively.

11.2.7.3.2 General case (counting of total colonies or typical colonies without confirmation)

If the number of colonies is greater than or equal to 20 on the first countable segment selected on one side of the plate (see [11.2.6.2](#)), calculate the number N of microorganisms per gram or per millilitre of test sample, using [Formula \(6\)](#):

$$N = \frac{C \times 1\,000}{V \times d} \quad (6)$$

where

- C is the number of colonies on the counted areas of the counting grid;
- V is the volume inoculated in those areas, in μl ;
- d is the dilution of the suspension inoculated (e.g. 10^{-1} for the initial suspension).

EXAMPLE A total of 54 colonies have been counted on the two segments of the grid where 18 μl from the 10^{-1} dilution have been inoculated:

$$N = \frac{54 \times 1\,000}{18 \times 10^{-1}} = 30\,000$$

The number of microorganisms is $N = 30\,000$ or $N = 3,0 \times 10^4$ cfu/g or ml.

where

C is 54 colonies;

V is 18 µl;

d is 10^{-1} .

NOTE Spiral plating is not suitable for enumerating low numbers of colonies. However, when low counts occur in a segment (between 1 and 19 colonies) results can be estimated from the total number of colonies on the plate using the general plate count formula. If less than 20 colonies are counted on the total plate, the confidence interval of the result obtained is wide, but results can be expressed as estimated. ^{[43][46]}

11.2.7.3.3 Spiral plate calculation: Count after confirmation

When the method used requires confirmation, a given number A (generally five) of presumptive colonies is identified from each of the dishes retained for counting.

After confirmation, calculate, for each of the dishes, the number of colonies (a) complying with identification criteria using [Formula \(7\)](#):

$$a = \frac{b}{A} \times C \quad (7)$$

where

A is the number of presumptive colonies selected for confirmation from the counted areas in each Petri dish;

b is the number of colonies confirmed from A ;

C is the total number of colonies in the Petri dish.

In addition, replace C by a from [Formula \(6\)](#).

EXAMPLE A total of 54 colonies has been counted on two areas of the grid where the inoculum was 18 µl from the 10^{-1} dilution. From these 54 colonies, 5 colonies have been tested, 2 of which were confirmed:

$$a = \frac{b}{A} \times C = \frac{2}{5} \times 54 = 21,6$$

Replace C by a from [Formula \(6\)](#):

$$N = \frac{21,6 \times 1\,000}{18 \times 10^{-1}} = 12\,000$$

The number of microorganisms is $N = 12\,000$ or $N = 1,2 \times 10^4$ cfu/g or ml.

where

a is 21,6 colonies replacing $C = 54$ colonies;

V is 18 µl;

d is 10^{-1} .

11.2.7.3.4 Spiral count calculation, special case: No colonies detected

If the Petri dish does not contain colonies, report the result using [Formula \(8\)](#):

$$N < \frac{1\ 000}{V_t \times d} \quad (8)$$

where

V_t is the total volume deposited on the plate, in μl ;

d is the dilution of the suspension inoculated (e.g. 10^{-1} for the initial suspension).

EXAMPLE No colonies have been detected in a spiral plate inoculated with 50 μl from the initial suspension (10^{-1}).

The final count (N) is:

$$< \frac{1\ 000}{50 \times 10^{-1}} = < \frac{1\ 000}{50 \times 0,1} = < 200$$

where

V_t is 50 μl ;

d is 10^{-1} .

11.2.7.3.5 Spiral count calculation, special case: More than the maximum number of typical colonies

If the first area in the selected segment contains confluent (i.e. uncountable) colonies or the number of colonies is more than 75, record the results using [Formula \(9\)](#):

$$N > \frac{C \times 1\ 000}{V_t \times d} \quad (9)$$

where

C is 150 (the maximum number of colonies estimated on the two segments, i.e. 75×2);

V_t is the total volume deposited on the first area of the wedge in μl ;

d is the dilution of the suspension inoculated (e.g. 10^{-1} for the initial suspension).

EXAMPLE More than 75 colonies have been estimated on both areas of the grid where 5 μl have been inoculated from the dilution 10^{-2} .

The final count (N) is reported as follows:

$$N > \frac{150 \times 1\ 000}{5 \times 10^{-2}} = > \frac{150\ 000}{5 \times 0,01} = > 3\ 000\ 000$$

The number of microorganisms is $N > 3\ 000\ 000$ or $N > 3,0 \times 10^6$ cfu/g or ml.

where

C is $> 75 \times 2$ colonies;

V is 5 μl ;

d is 10^{-2} .

11.3 Enumeration using liquid media

11.3.1 Principle

Conventional most probable number (MPN) methods only detect viable microorganisms.

The dilution series used for the method shall be such that a proportion of tubes is positive and a proportion negative (otherwise greater than or less than results are obtained).

Use of the MPN test includes the following four assumptions:^[52]

- if even a single microorganism (or clump of microorganisms) is present in a tube, then it will grow, and the growth will be detected and (where appropriate) confirmed;
- the source material for the inoculations is mixed thoroughly enough so the microorganisms are randomly distributed, without attracting or repelling each other;
- the process of dilution does not cause clumps of microorganisms to break apart and so preparation of only one initial suspension is necessary from which all other test portions are obtained;
- the number of microorganisms in any tube is statistically independent of the number in any other tube.

The principle of the MPN method is the dilution of a sample to such a degree that the inocula sometimes, but not always, contains viable target microorganisms. The “outcome” (i.e. the number of tubes producing growth at each dilution) gives an estimate of the initial concentration of bacteria in the sample.

11.3.2 General MPN procedure

To obtain estimates over a broad range of possible concentrations, several test portions and serial dilutions may be used, with incubation of several tubes, bottles, flasks or microplate wells at each dilution. The MPN of microorganisms present in the original sample, and the precision of the estimate, is calculated by statistical procedures from the number of positive and negative tubes (or flasks, bottles, microplate wells) observed per dilution after incubation.

Test portions are inoculated into a liquid medium designed to support the growth of a particular microorganism or group of microorganisms. The medium used may also inhibit proliferation of non-target microorganisms.

Various criteria can be used to determine whether target microorganisms are present, growth of these has occurred and/or a target-specific metabolic activity has taken place.

These include visual detection of turbidity, gas production, colour changes and subsequent isolation of the microorganisms on a selective agar medium, together with incubation atmosphere and temperature.

Molecular methods such as DNA probes, conventional and real-time PCR may also be used to determine presence of the target microorganism after incubation. The composition of the growth medium and the criteria for discriminating between positive and negative results are defined in the specific standards.

Molecular detection approaches applied directly to multiple tubes or microplate wells of a homogenate or dilutions, rather than as a confirmation method on a culture, can detect moribund or dead microorganisms in addition to live ones. Using the MPN procedure, only a qualitative value is attributed, i.e. the result is either positive or negative.

11.3.3 Limitations of MPN

Addition of the test portion to a selective growth medium should not reduce the selective properties to avoid growth of non-target microorganisms. Known limitations of specific standards with specific matrices are given in the scope of the standard.

Special preparation techniques may be required for so-called “challenging” matrices such as spices, cocoa, bouillon, etc. as they can contain growth-inhibiting substances (see the ISO 6887 series). Addition

of neutralizing compounds, use of higher dilution factors, centrifugation, filtration or immunomagnetic separation to remove target microorganisms from the matrix are options.

Limitations can also be due to the natural background microorganisms of the matrix: heavily contaminated environmental samples, fermented products or products with probiotic bacteria can require the use of a highly selective growth medium, a two-stage culture procedure or molecular confirmation of the presence of the target organism in the primary growth medium.

For potentially interfering matrices, or those with a high concentration of non-target microorganisms, spiking experiments using representative microorganisms should be performed to verify that the method gives acceptable results.

Derivation of an MPN value assumes that a well-mixed homogenate has been prepared. If an MPN is used for the analysis of solid samples, take only one test portion to prepare the initial suspension and use aliquots for subsequent dilutions.

11.3.4 Inoculation procedure

Unless otherwise stated in specific standards, volumes of less than or equal to 1 ml are normally added to 5 to 10 times the volume of single strength liquid media. Test portions greater than 1 ml to 100 ml are normally added to equal volumes of double-strength liquid media.

For volumes greater than 100 ml, more concentrated liquid media may be used. For large liquid samples, sterile dehydrated culture media may be dissolved in the cold (or pre-warmed to 30 °C) sample before testing.

The time lapse between preparing the initial suspension of a sample and inoculation of the last tube, flask, bottle or well should be less than 30 min.

11.3.5 Choice of MPN configuration

11.3.5.1 General

Make a choice of MPN configuration according to:

- the expected number of microorganisms in the sample under investigation;
- regulatory requirements;
- the precision needed;
- any other practical considerations.

The uncertainty of the test results depends on the number of positive test portions observed in a similar way to the uncertainty of a colony count depends on the number of colonies on a plate. Uncertainty changes as a function of the square root of the number of tubes (or other containers) used, since precision increases with increasing numbers of replicate tests. But it is necessary to quadruple the number of tubes to halve the uncertainty. When systems use only a few replicate tubes, the relative uncertainty is high.

Use tubes, flasks or bottles of sufficient size for the total volume of medium plus inoculum and, if appropriate to the method, still leave a headspace above the inoculated medium.

Multi-well plates are only suitable for small volumes.

11.3.5.2 Single dilution system

When the expected concentration of microorganisms is low 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 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 no-growth tubes is probably too high.

11.3.5.3 Multiple dilution system

When the concentration of microorganisms in the sample is unknown, or if greater variation is anticipated in concentration between different samples, it can 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.

11.3.5.4 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 an indication of the order of magnitude of the concentration. If more precision is required, five or more parallel tubes should be chosen.

11.3.5.5 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, flask or bottle can be lost or broken leading to a non-symmetrical system. Also, some commercial test kits are based on non-symmetrical systems.

11.3.6 Incubation

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

Use the incubation temperature and duration stated in the specific standard.

For some microorganisms, a two-stage incubation procedure and/or a confirmation step can be necessary. Refer to the specific standards for details but note that this can add a complication to the derivation of the MPN values.^[48]

11.3.7 Interpretation and expression of results

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

11.3.8 Determination of MPN values using MPN calculators

It is recommended that an MPN calculator be used to determine MPN values from the combination of positive and negative tubes, flasks bottles or microplate wells at each dilution.

These calculators allow input of the results from all portions tested rather than restricting the use to a certain number of dilutions and replicates, as is so with MPN tables.

The output of the calculator should include an estimate of the 95 % confidence intervals for the MPN, together with an indication of the probability of occurrence of the combination of results yielding the MPN (this may be as a rarity index, rarity category, or both; see 11.3.9).

Calculators that fulfil these requirements for 10 g and 100 g test portions are available at: <https://standards.iso.org/iso/7218/ed-4/en/>

These are written in Excel®³⁾ and can be used for up to 10 levels of serial dilution. Details of the calculations are described in Reference [58].

Guidance on the use of the 10 g calculator is also given on the above link, together with the verification document.

Guidance on the use of the 100 g calculator, which was designed specifically for testing shellfish in accordance with ISO 16649-3, is included in a generic protocol for carrying out such tests. This is also available on the above link, together with the verification document.

Use positive and negative results from all tubes or wells that have been tested at all dilutions in the MPN calculator to determine the MPN value. It is not appropriate to select a subset of dilutions from which to determine the value.

11.3.9 Rarity categories

The MPN calculators also yield the decimal logarithm of the MPN value, its standard deviation (SD), $s_{(\log_{10} M)}$, the lower and upper confidence limits of the approximate 95 % confidence interval, together with a rarity index and a rarity category. The rarity value (based on work by Blodgett, see References [48], [49] and [52]) provides a simpler approach to assessment of the likelihood that an observed result is obtained in a test.

Some combinations of positive tubes are more likely to occur than others (e.g. a combination of positive results 0-0-3 is much less likely to occur than the combination 3-2-1). A rarity index is used to quantify this probability.

The rarity index is a value between 0 and 1. It is 1 if the result of the serial dilution test is the most likely 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 [51], the results are classified into the following three categories of rarity:

- Category 1: the MPN value would be highly likely to occur if its rarity value falls within the range 0,05 to 1,00, $0,05 \leq n \leq 1,00$ (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, $0,01 \leq n \leq 0,05$ (i.e. such a result would be likely to occur by chance with a frequency less than 5 % and should only be reported with caution).
- Category 3: the MPN value would be expected to occur extremely rarely if its rarity value falls within the range 0 to 0,01, $0 \leq n \leq 0,01$ (i.e. such a result would be expected to occur by chance less than once in 100 tests).

If the MPN calculator highlights a tube combination as improbable (i.e. Category 3), do not report the associated MPN value. Where possible, request a repeat sample.

11.4 Estimates of uncertainty of test results

Quantitative (enumeration) test results have an uncertainty (imprecision) associated with them which can be estimated for microbiological examinations and taken into account when reporting.

Studies and reviews of the various aspects of this topic applicable to microbiology testing are available and some examples are given in References [50], [57] and [58].

ISO 19036 details the theory of uncertainty applied to quantitative microbiological examinations and includes methods for laboratories to derive estimates of uncertainty of enumeration results using both solid and liquid media.

3) 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.

12 Detection (qualitative) methods

12.1 General

Qualitative methods only detect (or not) a particular target microorganism in a given quantity of product and give no direct indication of the numbers present.

The quantity of product tested is linked to the microbiological criteria and typical examples include 10 g or 25 g. The test portion size can be increased, e.g. to 375 g, by taking a larger single test portion or by pooling. Either samples, test portions or (pre-) enriched test portions can be pooled. Further details are given in specific standards and the ISO 6887 series.

This clause gives general information on methods involving culture of the microorganisms sought, but PCR methods are also available. Further details are given in specific standards and ISO 22174.

12.2 Principle

Unless otherwise stated in a specific standard, mix (liquid products) or homogenize (other products) a quantity P of the test portion to be examined with $9 \times P$ ml or $9 \times P$ g of an elective and/or a selective broth.

To facilitate the recovery of stressed microorganisms in samples from the food chain, samples are usually pre-enriched in a non-selective broth followed by selective enrichment and isolation on selective/differential solid media. The use of two different enrichment broths, as well as two or more agar media, increases method sensitivity.

Enrichment broths should be incubated to allow air circulation between them. If containers are used to limit the risk of spillage, the temperature should be verified for homogeneity. Requirements for incubating larger test portion sizes are included in [6.3.2.4](#).

After incubation, spread a loopful of the culture obtained over the surface of a selective agar medium to obtain isolated colonies. Unless otherwise stated, the incubated enrichment broths may only be refrigerated before plating if the impact of refrigeration on results has been evaluated and then only if noted as a deviation to the method in the test report.

A number (generally up to five per agar plate) of the colonies obtained after incubation are then confirmed using appropriate techniques. The selection of colonies for confirmation should include all presumptive colony types.

13 Confirmation and identification methods

13.1 General

Confirmation and identification methods are used to confirm presumptive isolates from both qualitative and quantitative methods. (Sero)typing is used for further characterization of the isolates when relevant (e.g. serotypes known to be pathogenic).

The following two method principles are available for confirmation of presumptive isolates:

- confirmation methods that provide positive or negative results (e.g. latex agglutination tests, nucleic acid hybridization, molecular amplification);
- identification methods that also provide the identity of an isolate as a confirmation result (e.g. biochemical galleries, mass spectrometry, sequencing).

Use the reference tests given in specific standards and the general tests according to [Annex C](#). As an alternative to the serological and biochemical tests specified in these standards, other methods described in this clause may be used, unless otherwise stated in the specific standards.

Any alternative method used shall be based on a different measurement principle (e.g. DNA, protein, immunological, biochemical based analysis) than the principle used in the detection or enumeration method,

or shall use different markers (e.g. different antibodies, primers). For example, a PCR detection method can be confirmed by another PCR method based on different primers or probes than the detection method.

When alternative methods are used in a reference procedure, verify that they are suitable, as shown by evaluation studies provided by the manufacturer or published in the international scientific literature, preferably relating to food microbiology.

A control certificate should be obtained for each batch of tests, with an indication of the strains used. If the reference tests are replaced by alternative methods based on different principles (e.g. molecular amplification or hybridization, mass spectrometry or sequencing), see the appropriate part of the ISO 16140 series for detail of validation procedures.

Verification of validated alternative confirmation and (sero)typing methods only requires implementation verification in accordance with the appropriate part of the ISO 16140 series.

Only pure cultures should be used for biochemical and serological confirmation, although it is possible to conduct these directly on discrete colonies from selective agar plates in some cases (e.g. ISO 6579-1). For nucleic acid hybridization and molecular amplification formats, the use of mixed cultures may be acceptable if sufficient target organism DNA is present.

13.2 Preparation of a pure culture

Begin preparation of a pure culture by selecting a single colony on or in an agar medium. Inoculate the selected colony onto a non-selective agar medium. After appropriate incubation, select a well-isolated colony for subsequent confirmation tests. Repeat the operation if necessary, until a pure culture is achieved.

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

13.3 Confirmation methods

13.3.1 Latex agglutination test

A rapid method employing latex particles coated with group-specific antibodies is commercially available for confirmation of microorganisms such as *Salmonella* spp. or coagulase-positive staphylococci. The antigens in a microbial suspension are tested against a range of latex reagents.

When the latex test is used for serotyping (e.g. of *Escherichia coli* O157), perform it after the confirmation/identification of the genus and species.

When using the reagents, suitable positive and negative controls should be included if internal latex controls are not provided.

13.3.2 Nucleic acid hybridization or molecular amplification methods

Molecular hybridization is the formation of a complementary nucleic acid duplex by association of single strands.

PCR and isothermal amplification are enzymatic procedures which combine the *in vitro* amplification of specific DNA segments by a process of denaturation, annealing of specific primers and synthesis of DNA with the detection of specific amplified products during the amplification process.

The laboratory shall specify control strains used to verify the maintenance of primers, probes and molecular reactions performance. Where appropriate, colonies should be taken from the specific agar medium that is used for the growth of the isolated colony and be suspended in ultrapure water (or another suitable diluent if validated for use in a specific method) before nucleic acid extraction.

For PCR, the requirements given in ISO 22174 should be followed. In addition, general details on performance characteristics for PCR are given in ISO 22118.

NOTE No specific standard is available describing the use of isothermal amplification for confirmation, but the above references can provide some guidance.

13.3.3 Slide agglutination tests

When serological characterization is required, perform it on isolated colonies after confirmation/identification of the genus and species.

Antigen-antibody reactions in microbial culture suspensions cause bacterial cells to clump together and form flocculent masses or dense granules. In the case of bacteria of the family *Enterobacteriaceae*, the reaction between the “H” (i.e. flagellar) antigen and its homologous antiserum results in flocculent clumping, whereas the reaction involving the “O” (i.e. somatic) antigen results in more dense, granular clumping.

Before agglutination with antisera, a test should be carried out to determine whether the bacterial cells agglutinate in 3 % (by mass) sodium chloride solution. If the bacterial cells agglutinate, the strain is autoagglutinable and should not be tested with antisera.

Commercially available antisera are of the following two types:

- polyvalent antisera which react with microorganisms of a particular genus or with groups of serovars and which are suitable for preliminary screening;
- specific monoclonal antibodies, the use of which allows identification of a particular serovar.

When using the reagents, include suitable positive and negative controls alongside the tests.

13.4 Identification methods

13.4.1 Biochemical galleries

Biochemical galleries may be used for identification of isolated colonies.

Verify that the galleries are suitable, as shown by evaluation studies provided by the manufacturer or published in the international scientific literature, preferably relating to food microbiology.

The manufacturer should also specify control strains for the laboratory to verify performance of new batches of galleries in routine use.

Ensure the galleries include, as a minimum, the biochemical tests described in specific standards or are supplemented by other suitable tests and follow the manufacturer’s instructions for use.

13.4.2 DNA sequencing

This technology generates sequences which are unique signatures for each microorganism. Accurate microbial identification at genus or species level is obtained by comparing the sequence(s) to a database or library.

DNA sequencing methods, including Sanger sequencing and next generation sequencing (NGS), may be used for identification of isolated colonies. Sequencing can be applied either directly to nucleic acid purified from the isolate or to PCR amplicons generated from the isolate using either specific or generic primers. Certain sequencing methods may also be used for identification in complex microbial ecosystems, e.g. enrichment broths, environmental or food samples.

Sanger sequencing is a method of DNA sequencing based on the selective incorporation of chain-terminating dideoxy nucleotides by DNA polymerase during DNA replication.

NGS gather various high-throughput technologies which determine a portion of the nucleotide sequence of an individual genome. These NGS technologies are capable of processing multiple DNA sequences in

parallel. The most widely used genes for identification purposes are ribosomal RNA (rRNA) encoding genes (rDNA), as they are recognized phylogenetic markers. Other housekeeping genes are recognized as well as phylogenetic markers and can therefore be used (e.g. *rpoB* or *gyrA*). Some possible target sequences can be of interest to discriminate some specific *phyla* or serogroup (e.g. *panC* to discriminate *phyla* in the *Bacillus cereus* group). The generated sequences are compared to the available database using dedicated software. Various technology principles are available. For these, use the workflow recommended by the manufacturer.

NGS provides high-throughput access to microbial whole genome sequences (WGS) for use in microbiology of the food chain. WGS are digital representations of the biological potential of the sequenced microorganism at single base resolution and have advantages over other technologies, such as serology. For general requirements and guidance, see ISO 23418.

Sequencing methods should be validated for use for this purpose, as shown by evaluation studies published in the international scientific literature, preferably relating to food microbiology, and in accordance with the appropriate part of the ISO 16140 series.

13.4.3 Mass spectrometry

This technology generates characteristic mass spectral fingerprints which are unique signatures for each microorganism. Accurate microbial identification at genus or species levels is obtained by comparing the spectrum to a commercial database.

The most used mass spectrometry technology is matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI ToF MS).

For bacteria and yeasts, the usual workflow is as follows:

- collect a portion of an isolated colony on a culture medium plate and smear a thin layer on one spot of the MS target;
- overlay the spot with a saturated solution of alpha-cyano-4-hydroxycinnamic acid matrix and allow to dry;
- introduce the target into the mass spectrometry instrument for spectrum acquisition.

Depending on the microorganisms, specific inactivation and/or extraction protocols from solid or liquid cultures can be needed to obtain a spectrum.

Each sample is subjected to multiple laser shots, so the sample molecules vaporize into the vacuum while being ionized at the same time. A spectrum is generated and compared to a database for organism identification via dedicated software.

Various technology principles are available. For these, use the workflow recommended by the manufacturer.

Use the appropriate media and control strains specified by the manufacturer to verify the performance of the system and the reagents. When using the reagents, suitable positive and negative controls should be used.

Mass spectrometry should be validated for use for this purpose, as shown by evaluation studies published in the international scientific literature, preferably relating to food microbiology, and in accordance with the appropriate part of the ISO 16140 series.

14 Selection and characterization of control microorganisms

14.1 General

Microorganisms and their derivatives are used for performance testing of culture media, quality control of tests, method validation and verification, and challenge testing. They are also used for preparation of samples for distribution in PT schemes. These microorganisms are selected based on their end use. Typically, the positive control possesses the same characteristics as the target in a test, while the negative control is one that lacks those defining characteristics. Their inclusion in tests is used to confirm the validity of the test results.

14.2 Characterization of microorganisms

14.2.1 General

Microorganisms have traditionally been characterized phenotypically. With advances in molecular testing, microorganisms can now be characterized using such techniques. It is important to note that identifications do not always fully align when both molecular and phenotypic methods are used. This is because the techniques detect different targets: molecular techniques target different entities (e.g. DNA, RNA, proteins, carbohydrates), whereas phenotypic techniques are based on the physical appearance and biochemical reactions of the living microorganism. Also, the presence of a gene for a particular trait does not mean that the trait will be expressed.

It is important, therefore, that the microorganism is characterized using techniques that are appropriate for its end use.

14.2.2 Phenotypic characterization

Microorganisms are typically characterized by their appearance on culture media, microscopic morphology and their reactions to Gram staining and motility tests (see [Annex C](#)), in addition to biochemical and serological tests. These characteristics have been utilized to isolate and identify (and, in some cases, define) the target organisms in culture-based methods.

14.2.3 Molecular characterization

Microorganisms are characterized by their molecular properties (e.g. DNA, RNA, proteins, carbohydrate patterns). These molecular characteristics are utilized to identify and/or characterize the target organisms.

14.3 Selection of control microorganisms

The selection of test controls is critical as microorganisms, including strains within the same species, can be genetically and phenotypically variable.

The control strains used shall be fit for their intended purpose. Tests based on biochemical reactions require phenotypic characterization whereas tests based on the molecular make-up of the target require molecular characterization.

It is good practice to use reference strains from recognized biological resource centres (BRCs) that have been fully characterized and shown to possess the properties required for their end-use.^[44] Suitably characterized laboratory strains from related sources (food, feed, environment, clinical samples from outbreaks, etc.), sometimes referred to as “wild strains”, can also be used in addition to strains from culture collections. These laboratory strains should be maintained to ensure minimal genetic drift (see ISO 11133).

The extent of characterization required depends on how the control microorganisms are to be used. For example, as follows:

- Performance testing (quality control; QC) of culture media: The purpose is to demonstrate the ability of a culture medium to support the growth of the target microorganism(s) and inhibit growth of non-target microorganisms. As media quality underpins all tests, the controls used should be well-characterized strains from culture collections.
- Test (or process) controls: Microbiological tests exploit one or more microbial characteristics to differentiate between the microorganism(s) in the target group and those that are not. Controls are used to demonstrate that the tests have been correctly performed. Strains from culture collections are normally used for this purpose. However, laboratory strains may also be used if they are thought to provide more insight into the suitability of the test method. These strains should be shown to possess the properties sought in the test. For example, a laboratory strain for use as a control in a test for *E. coli* should be a Gram – rod that grows on the selective medium used and is able to grow at 44 °C and produce indole.
- Method validation and verification: During method validation the test method is challenged with a variety of target and non-target organisms to demonstrate that it can detect or enumerate the target in

selected matrices. The method performance characteristics are also determined to show they meet the requirements for the intended use. In addition to strains from culture collections, laboratory strains, especially if they are from sources relevant to the matrix tested, are useful as they can provide insight into the performance of the method when challenged with relevant biodiversity. Such strains should be characterized to a sufficient taxonomic level and possess the specific properties sought in the test (e.g. growth according to the test conditions, motility or not, development of characteristic colonies on the test culture media). They should be maintained to enable future testing. Strains used for verification studies should be well-characterized, to the level specified for the test target. Ideally, these strains are obtained from culture collections.

NOTE For further information about method validation and verification, see [Clause 17](#) and the ISO 16140 series.

- Challenge testing: The purpose of challenge testing is to determine the growth or the inactivation of microorganisms of interest in certain food matrices. The selection of strains should consider variations in growth or inactivation kinetics amongst strains. Laboratory strains, especially if they are from sources relevant to the matrix tested, are useful. Strains used should be well-characterized, with known cardinal values (e.g. temperature, pH, a_w , minimal inhibitory concentration for preservatives) for growth studies to ensure they are fit for purpose. These characteristics can be determined experimentally or obtained from published, peer-reviewed data. Such strains should be maintained to enable future testing. For further information about challenge testing, see the ISO 20976 series.
- Proficiency testing (PT): The main purpose of PT schemes is to evaluate laboratory performance. Samples with the target (microorganism or derivative) are distributed to participants for testing. Results of testing are compared with the assigned value, usually based on consensus values from participant laboratories (see ISO 22117), to determine trueness/bias. In most instances, PT scheme samples are prepared by spiking matrices with the target organism. The spike may be typical or atypical target strains or microbial (sub)types and, in some instances, include non-target strains introduced to assess the ability to detect or enumerate the target in the presence of background microorganisms. In all cases, the spike shall be well-characterized to ensure that the target can be reliably detected/identified using suitable but different methodologies. Strains/derivatives from recognized BRCs should be used. However, wild or laboratory strains isolated from the matrices may be used in PT schemes to provide a more relevant test. Such strains should be sufficiently characterized in accordance with the appropriate specific standards. For general requirements on PT, see ISO/IEC 17043 and for information on microbiology see ISO 22117.

15 Test report

Specify in the test report some or all of the following information, depending on the requirements of the specific standard and of the client:

- all details necessary for the complete identification of the sample(s);
- the International Standard method used (including its year of publication) the temperature of incubation, if necessary;
- the results obtained (including a reference to the clause which explains how these were calculated);
- any operating details not included in the specific standard used, as well as those regarded as optional;
- details of any incidents or deviations from the method that can influence the results (e.g. sample condition unsatisfactory or insufficient sample, sample retested);
- whether further tests are to be carried out by a reference laboratory or sub-contractor and, if such tests have been carried out, what the results were;
- if appropriate or requested by the client, all information necessary for the interpretation of the test results;
- when necessary (e.g. for official or regulatory samples) or if requested by the client, an estimate of the uncertainty of quantitative test results (see ISO 19036);
- the date of the test.

NOTE Uncertainty estimates can also be necessary when reporting statements of conformity to specification limits.

16 Laboratory quality control in microbiology

16.1 General

Microbiological examinations involve many complex operations from sampling to the calculation and reporting of results, all of which shall be carried out competently in order to give valid test results.

These operations are discussed in [Clauses 4 to 17](#), and are illustrated in [Figure 1](#).

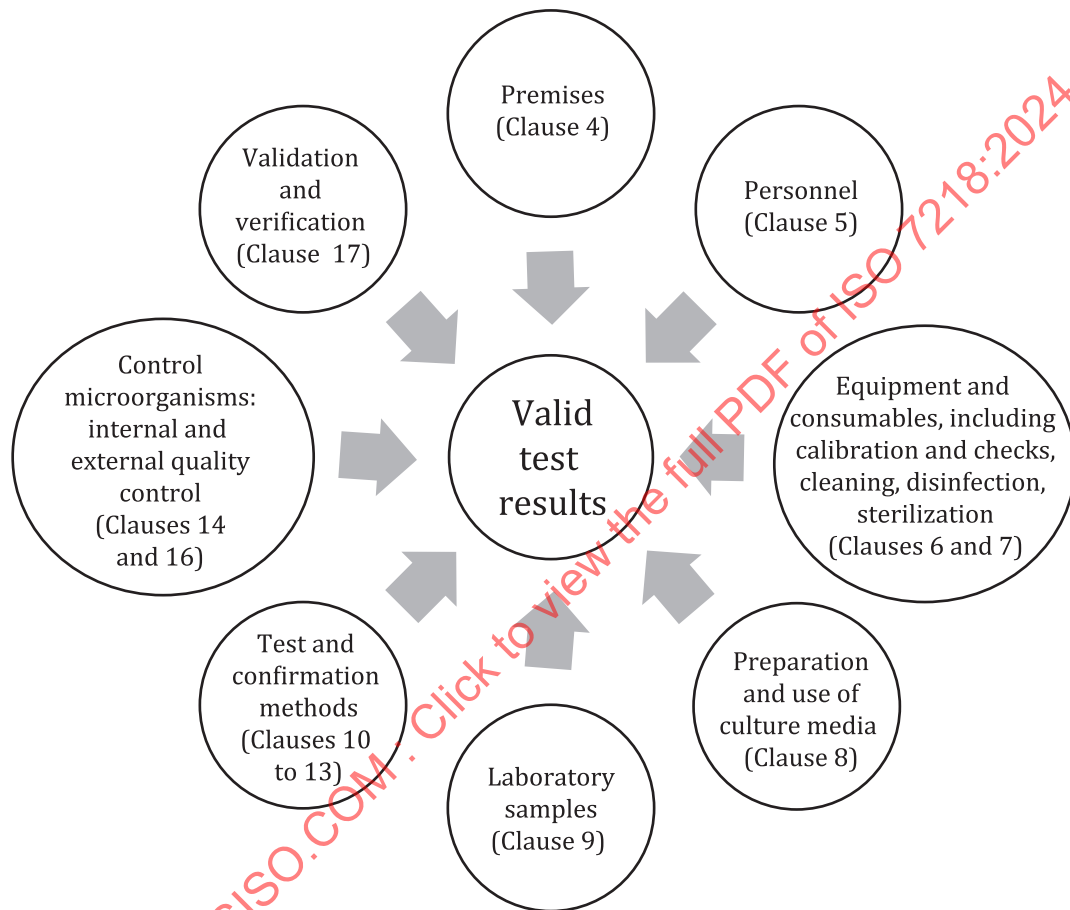


Figure 1 — Factors affecting the validity of microbiological test results

Quality control is a key part of a laboratory quality assurance programme to ensure the reliability of test results. The quality assurance programme should cover all equipment and consumables, culture media and reagents, as well as training and competence of personnel.

Quality control of each step of the testing process from beginning to end at a defined frequency gives confidence in the validity of test results. Additional key components of the quality assurance programme are IQC carried out alongside sample examination and external quality assurance (EQA) through participation in an appropriate PT scheme.