International Standard 7218

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION•МЕЖДУНАРОДНАЯ ОРГАНИЗАЦИЯ ПО СТАНДАРТИЗАЦИИ•ORGANISATION INTERNATIONALE DE NORMALISATION

Microbiology — General guidance for microbiological examinations

Microbiologie — Directives générales pour les examens microbiologiques

First edition — 1985-09-01

UDC 579.678 **Ref. No. ISO 7218-1985 (E)**

Descriptors: microbiological analysis, micro-organisms, sampling, food products.

Price based on 14 pages

Foreword

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Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council. They are approved in accordance with ISO procedures requiring at least 75 % approval by the member bodies voting.

International Standard ISO 7218 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*.

Printed in Switzerland

Microbiology — General guidance for microbiological examinations

0 Introduction

When performing microbiological examinations, it is especially important

a) that only the micro-organisms which are present in the samples are isolated or enumerated;

b) that the micro-organisms do not contaminate the environment.

In order to achieve this, it is necessary to pay attention to personal hygiene and to use working techniques that ensure, as far as possible, aseptic conditions.

Since, in this International Standard, it is possible to give only a few examples of the precautions to be taken during microbiological examinations, a thorough knowledge of microbiological examinations and of the micro-organisms involved is essential.

Ultimately, it is the analyst who should judge whether manipulations are safe and can be considered to be good laboratory practice.

Many manipulations may, for instance, unintentionally lead to cross-contamination and the analyst should always verify the accuracy of the results given by his technique.

In order to be able to perform examinations correctly, it is necessary to take certain precautions when constructing and equipping a laboratory. A number of such precautions are referred to in clause 4.

Certain precautions have to be taken not only for the sake of hygiene but also to ensure good reproducibility of results. It is not possible to specify all precautions to be taken in all circumstances, but the principal measures to be taken during the preparation, sterilization and storage of media and apparatus are described in clause 5.

If the guidance given in this International Standard is observed, this will also contribute to the protection of the health of personnel. For further information on this subject, reference should be made to the documentation listed in the bibliography, and in particular to references [1], [2] and [3].

1 Scope and field of application

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

The purpose of this International Standard is to help ensure the validity of examinations, to ensure that general techniques used for carrying out the examinations are the same in all laboratories and to contribute to the protection of the health of laboratory personnel by avoiding risks of infection.

2 Reference

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

3 Personal hygiene

From the point of view of personal hygiene, the following precautions need to be taken to prevent contamination of samples and culture media in particular, but also to avoid risks of infection to laboratory personnel:

— Strictly observe the usual hygienic precautions. Keep nails short. Wear hair and beard protection when necessary.

— Wash hands thoroughly with warm water and liquid or powdered soap before and after microbiological examinations and also immediately after each use of toilets. Use disposable single-use paper or cloth towels.

— Wear clean laboratory clothing, without holes or tears. This clothing shall not be worn elsewhere.

— Do not eat, drink or smoke in microbiological laboratories.

— Do not speak, cough, etc. during inoculation of plates and tubes.

— Do not allow personnel with serious infections of the hands or face to carry out microbiological examinations when there is a possibility that this could lead to contamination.

4 Installations and equipment

4.1 Work areas

4.1.1 There shall be sufficient room to ensure that work areas can be kept clean and orderly.

Approximately 20 m^2 of work area should be provided for each analyst, including auxiliary rooms (for special work such as weighing, centrifuging, incubation and refrigeration).

4.1.2 Special rooms shall be provided for

a) cleaning glassware and other utensils, and, if possible, sterilization of used glassware and incubated culture media;

b) preparation and sterilization of media;

c) checking the sterility of foodstuffs in cases where a laminar flow hood is not used.

4.1.3 A separate room shall be provided for the preparation of samples if there is a risk of cross-contamination, for example for powdered products containing a high number of micro-organisms (especially in the case of raw materials).

For the determination of pathogenic micro-organisms, for example *Salmonella*, it is strongly advised to provide a separate room, or at least to provide a cabinet for that purpose, to avoid not only cross-contamination but also infection of personnel.

4.1.4 When installing a laboratory, measures should be taken to reduce the risks of contamination by dust, and hence micro-organisms, for example

a) cupboards that reach the ceiling;

b) tables, window-ledges, refrigerators, ovens, inoculation cabinets installed in such a way that there are no dust-traps and that they are easy to clean (to facilitate cleaning, movable equipment may be used);

c) windows and doors that close tightly (in order to avoid air draughts);

d) window blinds, etc., installed on the outside;

e) closed cupboards for storage of documents used during analyses, and of samples, media, reagents, etc.;

NOTE — Periodicals and books etc. which are not frequently used should be kept in a special room (an office for example) outside the room used for analysis.

f) walls, ceilings, floors and furniture with smooth surfaces, easy to clean and, if necessary, to disinfect, and with curved joins between walls and ceilings, and between walls and floors;

g) no exposed water pipes crossing rooms;

h) filters for the air entering ventilation systems.

It is possible to check that the measures taken are effective simply by exposing, during an examination, open Petri dishes with plate count agar (PCA). The number of colonies that have developed after incubation for 3 days at 30 °C is counted and, if this number is unacceptably high, the causes of this excessive contamination shall be determined and eliminated.

4.1.5 When examinations are required to be carried out in atmospheres having no more than very slight contamination, the room shall be equipped, for example, with:

a) UV lamps, to be used outside working hours;

b) special cabinets (laminar flow or completely closed).

In such rooms, special clothing shall be worn. Check the correct functioning of UV lamps and cabinets at regular intervals, once per month for example. Check the count of micro-organisms in the air at frequent intervals by means of special equipment for the sampling and examination of air.

4.1.6 Working places shall be protected against direct sunlight.

4.1.7 Ensure that work benches and apparatus remain in good repair (for example cracks in work benches can trap dirt and hence constitute a source of contamination).

4.1.8 Rooms shall be well lit: at least 500 lx at 1 m from the floor and 1 200 lx ("artificial daylight") on work benches.

4.2 Incubators (cabinets, rooms)

Use special air incubators or water-baths when the temperature has to be constant and accurate to within a few tenths of a degree.

4.2.1 Incubators shall be well insulated and properly equipped with heating units and large incubators shall have forced air circulation, so that the temperature everywhere is correct to the nearest 1 °C.

4.2.2 The ambient temperature shall always be lower than that of the incubator, or the latter shall have a cooling system.

4.2.3 The temperature shall be checked at least every working day.

For this purpose, each incubator shall contain at least one thermometer, the bulb of which is submerged in glycerol contained in a closed flask, and a maximum and minimum thermometer. Large cabinets and incubating rooms shall be equipped with several thermometers and maximum and minimum thermometers, suitably distributed.

4.3 Water-baths

4.3.1 Use water-baths of the required accuracy.

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

There shall always be a thermometer of adequate precision, independent of the automatic control system.

Check the temperature at least every working day.

4.3.2 In order to avoid the development of micro-organisms in the water, clean the water-bath regularly and fill it with recently distilled or demineralized water. Also a disinfectant may be added or the bath heated to a temperature of at least 90 °C for a few minutes.

4.3.3 In order to prevent contamination

— do not immerse tubes, bottles and flasks to too great a depth;

— when cotton wool plugs are used, ensure that they do not become moist (use non-absorbent cotton wool).

4.3.4 In order to attain the correct temperature of incubation, ensure that the liquid level in tubes, bottles or flasks is below that of the water-bath.

4.3.5 Regularly count the number of micro-organisms in the water.

4.4 Autoclave for sterilizing apparatus and culture media

The autoclave shall have a thermometer and a manometer, and shall be capable of attaining a temperature of at least 121 ± 1 °C.

If the autoclave is used for the sterilization of apparatus, it shall be provided with a device for the evacuation of air, in order to reach a pressure of 13,7 kPa; for the sterilization of culture media, it is necessary to remove the air until a jet of steam is emitted.

The air inlet shall be provided with a filter to prevent contamination from the air during drying.

4.5 Sterilizing oven

The oven shall enable apparatus to be sterilized at a temperature of 170 to 175 °C.

5 Preparation of apparatus and media

5.1 Sterilization and preparation of apparatus

5.1.1 Preparation

All apparatus to be used shall be clean.[1)]

Apparatus made of plastics material and supplied sterile does not need to be sterilized.

Before sterilization, stopper test tubes, bottles and flasks with cotton wool or by means of an appropriate closure. Stopper pipettes with cotton wool if there is a risk of contamination of the sample (mixing by repeated aspirations) or a risk of infection to the analyst.

If necessary, place the apparatus to be sterilized in special containers or wrap it in special paper or aluminium foil.

5.1.2 Sterilization

Sterilize the apparatus according to one of the following methods:

a) sterilization by dry heat: heat in a hot air sterilizer for at least 1 h at 170 to 175 °C;

b) sterilization in moist heat: heat in a special autoclave, provided with a device for drying under vacuum, for at least 20 min at 121 ± 1 °C.

Place the apparatus to be sterilized in the autoclave in such a way as to allow free circulation of the steam. Before starting sterilization, evacuate the autoclave in order to reduce the pressure to at least 13,7 kPa[2)]. Sterilize as indicated in the first paragraph. After sterilization and evacuation of the steam, create a vacuum three times in succession, each time for a period of 3 min, so as to obtain complete drying of the equipment.

In order to be certain that the apparatus has been properly sterilized, sterilization indicators (for example special papers) can be used.

5.1.3 Treatment of used apparatus

5.1.3.1 Sterilization/decontamination

All used apparatus and incubated culture media shall be sterilized or decontaminated, even if they have not been used for the determination of pathogenic micro-organisms.

Sterilize Petri dishes and tubes, bottles or flasks containing the agars or the broths used, as well as apparatus that has come into contact with the cultures of micro-organisms during the manipulations, for 20 min at 121 °C.

Wash pipettes after removing them from the disinfectant solution [see clause 7 g)] and after removing the cotton wool plugs.

Pasteur pipettes shall be used once only.

5.1.3.2 Washing

Wash apparatus only after it has been sterilized or decontaminated.

Empty containers of their contents. Wash plugs carefully with hot water, and glassware with a detergent solution or successively with a solution of an alkaline detergent [for example 0,125 % (m/m) sodium carbonate solution] and with a dilute acid [for example hydrochloric acid, c(HCl) = 0,1 mol/l].

Wash metal capsules in hot water only.

Rinse all apparatus with distilled or demineralized water.

1) New apparatus delivered non-sterile should be washed — see 5.1.3.2.

2) It should be noted that the sterilization pressure is here expressed in absolute units and in kilopascals.

5.2 General techniques for the preparation and sterilization of culture media

5.2.1 Basic components

In order to improve the reproducibility of the results, it is recommended that, for the preparation of culture media, dehydrated basic components or complete dehydrated medium be used. The manufacturer's instructions shall be rigorously followed.

Basic components shall be kept in a cool dry place and sheltered from light.

The chemical products used for the preparation of the culture media shall be of recognized analytical quality.

5.2.2 Distilled water

The water used shall be distilled water, free from substances that could inhibit the growth of micro-organisms under the test conditions. If distilled water is prepared from chlorinated water, neutralize the chlorine before distillation.

If possible, use glass-distilled water.

NOTE — Water demineralized by passing through an ion-exchange column often contains large numbers of micro-organisms, thus this procedure is not suitable for preparing water to be used directly.

5.2.3 Preparation of media

After use, close bottles containing dehydrated culture media as quickly as possible in order to prevent the absorption of moisture by the powder. Do not use such culture media when they have become a compact mass.

Prepare culture media in accordance with the relevant International Standard or with the manufacturer's instructions.

Measure the pH of the medium so that after sterilization it is at the pH required. This measurement should be made with a pH meter. Adjust the pH, if necessary,[1] with 40 g/l sodium hydroxide solution, or 36,5 g/l hydrochloric acid.

Distribute liquid culture media in the quantities as needed, so that no transfer after sterilization is necessary.

For liquid media, use in general 10 ml in tubes of dimensions 16 mm × 160 mm or 20 ml in tubes of dimensions 20 mm × 180 mm or larger quantities in appropriate bottles or flasks but not exceeding 500 ml per bottle or flask. When it is desired to observe the possible production of gas, tubes containing liquid medium can be fitted with a Durham tube.

The distribution of culture media can be carried out manually or by using an automatic syringe.

Distribute agar media intended for the preparation of Petri dishes in quantities of 15 ml in test-tubes of dimensions 16 mm × 160 mm, or 18 mm × 180 mm, or larger quantities in appropriate bottles or flasks, but not exceeding 100 ml per bottle or flask.

NOTE — Devices for the preparation and distribution of media exist, in this case refer to the specifications for use of the apparatus.

5.2.4 Sterilization of media

Follow the instructions given in the International Standard concerned (or the manufacturer's instructions); generally, sterilization of culture media requires 15 min at 121 °C (245 kPa).

Make certain that, when culture media are sterilized, the time required to reach the sterilization temperature and the time necessary for cooling are not too long, in order to avoid degradation of certain components of the medium. Do not overload the autoclave.

Degradation is more marked when a large volume of culture media is sterilized, because the period of heating has to be longer than for a small volume. Unless otherwise indicated, do not sterilize volumes of more than 500 ml in the case of liquids or more than 100 ml in the case of agar media. After sterilization, do not reduce the pressure too rapidly, to prevent boiling of the media.

As in the case of sterilization of apparatus, sterilization indicators may be used.

Check media regularly after sterilization, notably pH, sterility and efficiency.

5.2.5 Storage of prepared culture media

If the prepared culture media are not used immediately, they shall, unless otherwise specified in the relevant International Standard, be stored in the dark at 0 to 4 °C, for no longer than 1 month, in conditions which do not produce any modification in their composition.

Never use media that have become dehydrated.

5.2.6 Liquefaction of agar culture media and preparation of agar plates

Melt the culture medium by placing it in a boiling water-bath or by any other means giving similar results (for example a steam flow-through autoclave, or a microwave oven). Avoid heating it for too long by removing it from the water-bath as soon as it has melted.

Keep the culture medium in the molten state by leaving it in a thermostatically controlled water-bath at 45 to 50 °C until it is used.

Never use a culture medium at a temperature higher than 50 °C. Do not keep a medium in the molten state for longer than 4 h and never melt a medium more than once.

Pour agar into the dishes in such a way that a layer at least 2 mm thick is obtained (for example, for dishes of diameter 90 mm, 10 ml of agar are required).

1) Normally, culture media prepared from dehydrated preparations will have the desired pH and will not require adjustment.

Unless otherwise indicated, before inoculating the surface of media, dry them preferably with the cover removed (see figure 1) and the agar surface downwards, in an incubator, maintained at 50 °C for 30 min, until all droplets of water have disappeared from the surface of the medium.

Do not dry any further.

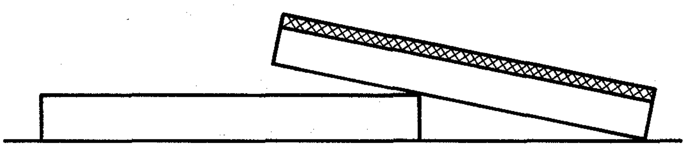


Figure 1

5.2.7 Loading of incubators

Do not overload incubators. Leave at least 25 mm between stacks, and between stacks and the walls and shelves. Each stack shall be composed of not more than six dishes.

6 Sampling

Carry out sampling in accordance with the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

Keep the product to be sampled and the samples to be examined under conditions which avoid any change in the number of micro-organisms present.

To count micro-organisms in a liquid product, either the product itself or dilutions can be examined. In the case of solid products, it is always necessary to prepare a solution or suspension (initial suspension); this solution and/or its dilutions are then examined. For simplification, these will always be referred to as the inoculum.

For the preparation of dilutions, see ISO 6887.

7 Hygienic precautions during examinations

Precautions shall be taken in order to ensure as much as possible aseptic working conditions, for example

a) Make sure that the work area is clean and that there are no air draughts.

b) Clean the work surface with an adequate disinfectant before and after working.

c) Open test-tubes and bottles close to a flame, keeping them as strongly inclined as possible.

d) Work as rapidly as possible without unnecessary movements.

e) If the contents of a bag of disposable pipettes, Petri dishes etc. are not used entirely during one operation, make sure the bag is closed adequately after taking out the appropriate number of units.

f) Sterilize inert metal loops and wires, etc. in a flame, before and after use. Carefully dry wet loops over the flame before they are sterilized to avoid the projection of material. When working with pathogenic micro-organisms, use a protective bell jar (see figure 2) or another form of protection [see, for example, Barlow (1972) *Soc. Appl. Bacteriol. Tech. Serv.* No. 1];

g) Place used pipettes, spatulas, etc., in special containers with a disinfectant (for example sodium hypochlorite solution) before cleaning and/or sterilization.

h) Place used Petri dishes, culture media, and all other material that may contain micro-organisms, in special containers.

j) Place plastics material that is not to be reused in plastic bags for burning or sterilization.

k) Take out books, journals, laboratory administration documents, media, reagents, etc., only for as long as is necessary.

m) Immediately remove any spilt contaminated media or products with cotton swabs impregnated with 70 % (*V*/*V*) ethanol or another disinfectant and then clean and disinfect the work surface before continuing work.

Handling pathogenic or toxic material may require special precautions described in specialized literature. Some examples are

— working in closed areas or under laminar flow hoods, especially when opening Petri dishes;

— using safety pipettes, etc.

The risk of infection or contamination depends on the type of micro-organisms and the manipulations involved in the examination.

Contaminated aerosols are an important cause of contamination of the environment and of infection. The formation of aerosols shall therefore be avoided as much as possible. Aerosols can be formed when opening plates, tubes, bottles and flasks, when using mixers, syringes, centrifuges, etc., and similarly when using blow-out pipettes and sterilizing wet inoculation loops or needles. When opening ampoules containing freeze-dried cultures, micro-organisms can also be spread in the air.

8 Preparation of the test sample

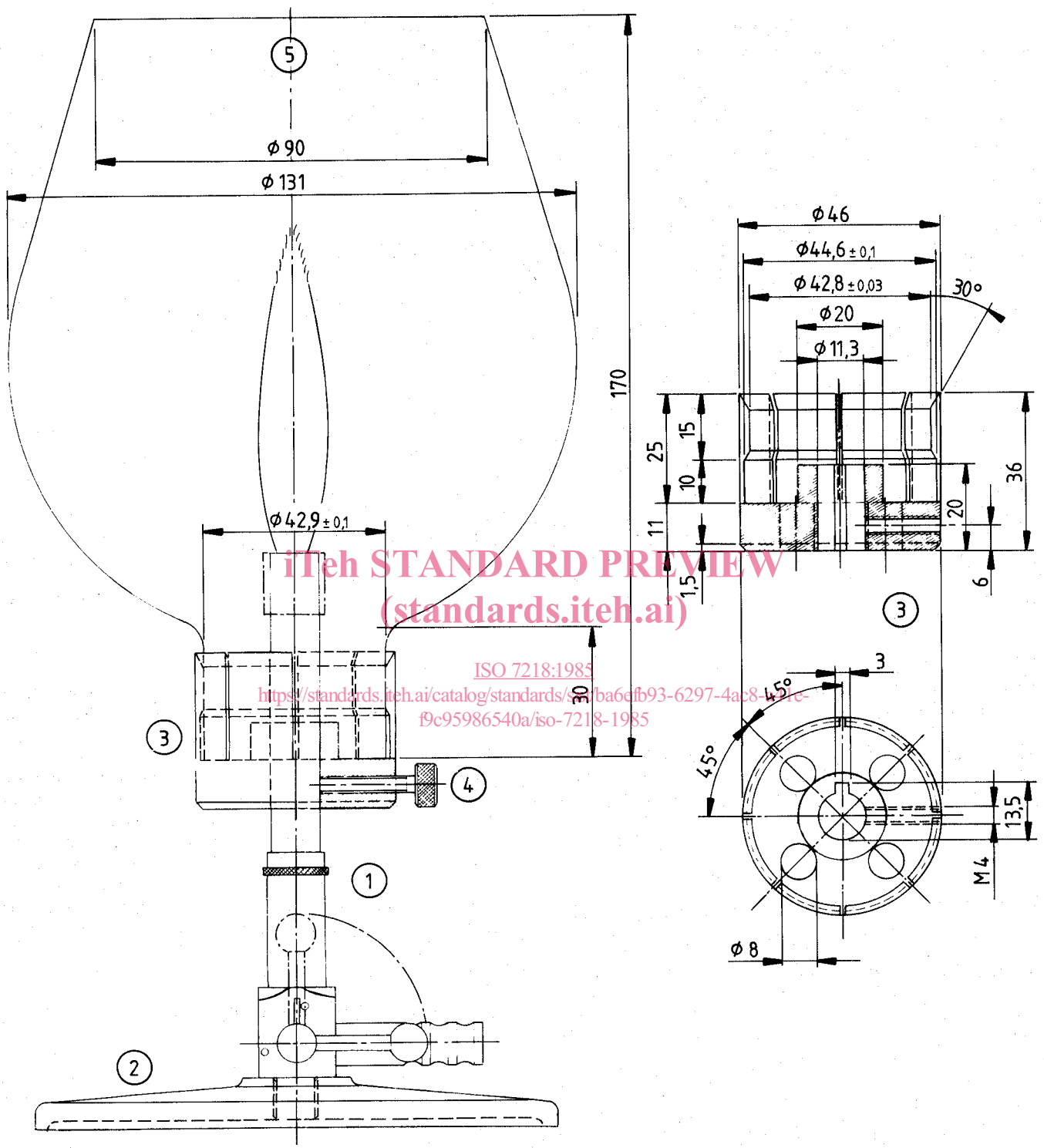
Mark samples, containers, plastic bags, bottles, test-tubes, Petri dishes, etc. in such a way that they can be easily identified during all the stages of the examination so as to eliminate any possibility of confusion.

Handle samples in such a way as to avoid any risk of contamination. In order to do this, take the following precautions:

a) In the case of packed products, clean the outside of the packing at the place where it will be opened with 70 % (*V*/*V*) ethanol. Flame if possible.

b) Any instrument used for opening the packing unit (can opener, scissors, etc.) shall be sterile (these instruments shall be sterilized after having been individually packed in a suitable material).

Dimensions in millimetres



(2), (3) and (4) in nickel-brass

(5) Transformed 1 000 ml round-bottom flask

Figure 2 — Gas burner with protective glass bell jar

9 Procedure

9.1 Test portion, initial suspension and dilutions

To avoid contamination of the environment and the test portion, it is recommended that special rooms or hoods be used for working. If these are not available, products containing few micro-organisms, for example pasteurized products and precooked foods, shall be examined first and more contaminated ones afterwards.

The protection of the environment against pollution is particularly important for the weighing and taking of samples from heavily contaminated powder products.

For checking the sterility of a product, sampling shall be performed under strictly controlled environmental conditions, for example under a fume hood with, if possible, laminar air flow.

For the preparation of dilutions, see ISO 6887.

9.2 Counting techniques

9.2.1 Counting when using a solid medium

9.2.1.1 Technique by incorporation in solid medium

Prepare the medium (in accordance with 5.2.6), the Petri dishes, the diluent and the dilutions to be examined (according to 9.1) in quantities and numbers corresponding to the plan of inoculation specified in the relevant International Standard.

Distribute in the dishes labelled in accordance with clause 8 the prescribed volumes of the dilutions to be examined.

Pour into each dish the volume of medium specified in 5.2.6. The time which elapses between the distribution of the inoculum in a dish and pouring of the medium shall not exceed 15 min. Immediately mix the melted medium and the inoculum carefully so as to obtain a homogeneous distribution of the micro-organisms in the medium. Allow to cool and solidify by placing the Petri dishes on a cool horizontal surface.

Make provision for a second layer of non-nutritive agar or agar identical to the culture medium used in the analysis in order to avoid the growth of invading colonies.

After complete solidification of the medium, place the dishes to be incubated as indicated in 9.2.1.3.

9.2.1.2 Technique by spreading on the surface

Uniformly spread the inoculum on the surface of the medium contained in the Petri dishes prepared in accordance with 5.2.6. Spreading may be carried out by means of a sterile glass or inert wire spreader.

When the inoculum has been completely absorbed by the medium, proceed with the incubation as described in 9.2.1.3.

9.2.1.3 Incubation

Incubate the inoculated dishes upside down at the appropriate temperature. Generally an air oven will be sufficient. If excessive dehydration is expected (for example at 55 °C or in the case of strong air circulation), wrap the dishes loosely in plastic bags before incubation.

If only small temperature variations are allowed, put the dishes into a watertight container which is placed in a thermostatically controlled water-bath.

NOTE — In some cases, it may be useful to inoculate duplicate plates, which are then kept at 4 °C for comparison with incubated dishes when counting, in order to avoid mistaking particles of the product being examined for colonies.

After incubation, the plates shall be examined immediately, if possible. Otherwise, they may be stored, unless otherwise indicated, for up to a maximum of 48 h at 4 °C.

9.2.1.4 Interpretation of the results

9.2.1.4.1 Selecting plates for examination

Plates that do not meet the requirements of the specific method shall be discarded. In general, plates are discarded when there are not well-separated colonies on at least half the plate.

9.2.1.4.2 Expression of results

For a result to be valid, in general it is considered that at least one dish should be counted containing 15 to 150 colonies per dish.

Calculate the number of micro-organisms present in the test sample as the weighted mean from two successive dilutions using the formula

$$\frac{\Sigma C}{V(n_1 + 0{,}1n_2)d}$$

where

ΣC is the sum of colonies on all plates counted;

d is the dilution from which the first counts were obtained (for example 10^{-2});

n_1 is the number of plates in the first dilution counted;

n_2 is the number of plates in the second dilution counted;

V is the volume of inoculum applied to each plate.

9.2.1.4.3 Confidence intervals

See table 1.