International Standard ISO 6579

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

Microbiology — General guidance on methods for the detection of *Salmonella*

Microbiologie — Directives générales concernant les méthodes de recherche des Salmonella

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards institutes (ISO member bodies). The work of developing International Standards is carried out through ISO technical committees. Every member body interested in a subject for which a technical committee has been set up 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.

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.

International Standard ISO 6579 was developed by Technical Committee ISO/TC 34, *Agricultural food products*, and was circulated to the member bodies in February 1979.

It has been approved by the member bodies of the following countries :

Australia	Hungary	Poland
Bulgaria	India	Portugal
Canada	Indonesia	Romania
Chile	Israel	South Africa, Rep. of
Cyprus	Kenya	Thailand
Czechoslovakia	Korea, Rep. of	Turkey
Egypt, Arab Rep. of	Malaysia	United Kingdom
Ethiopia	Mexico	USA
France	Netherlands	Yugoslavia
Germany, F. R.	Philippines	

The member body of the following country expressed disapproval of the document on technical grounds :

New Zealand

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Microbiology — General guidance on methods for the detection of *Salmonella*

0 Introduction

This International Standard is intended to provide general guidance for the examination of products not dealt with by existing International Standards and for the consideration of bodies preparing reference microbiological methods of test for application to foods or to animal feeding stuffs. Because of the large variety of products within this field of application, these guidelines may not be appropriate for some products in every detail, and for some other products it may be necessary to use different methods. Nevertheless, it is hoped that in all cases every attempt will be made to apply the guidelines provided as far as possible and that deviations from them will only be made if absolutely necessary for technical reasons.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which the guidelines have been followed and the reasons for deviation from them in the case of particular products.

The harmonization of test methods cannot be immediate, and, for certain groups of products, International Standards and/or national standards may already exist that do not comply with these guidelines. In cases where International Standards already exist for the product to be tested, they should be followed[1], but it is hoped that when such standards are reviewed they will be changed to comply with this International Standard so that, eventually, the only remaining departures from these guidelines will be those necessary for well established technical reasons.

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Salmonella* are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials.

1 Scope

This International Standard gives general guidance on methods for the detection of *Salmonella*.

2 Field of application

Subject to the limitations discussed in the introduction, this International Standard is applicable to products intended for human consumption or feeding of animals.

3 Definitions

For the purpose of this International Standard, the following definitions apply.

3.1 *Salmonella* : Micro-organisms which form typical colonies on solid selective media and which display the biochemical and serological characteristics described when tests are carried out in accordance with this International Standard.

3.2 detection of *Salmonella* : Determination of the presence or absence of these micro-organisms, in a particular mass, when tests are carried out in accordance with this International Standard.

4 Principle

In general, the detection of *Salmonella* necessitates four successive stages (see also annex A).[2]

4.1 Pre-enrichment in non-selective liquid medium

Inoculation of buffered peptone water (also used as diluent) with the test portion, and incubation at the specified temperature (see 9.2) for 16 to 20 h.

4.2 Enrichment in selective liquid media

Inoculation of a tetrathionate medium and of a selenite cystine medium with the culture obtained in 4.1.

Incubation of the tetrathionate medium at 43 °C, and incubation of the selenite cystine medium at the specified temperature (see 9.3.2), for 24 h, and then for 48 h.

1) For meat and meat products, see ISO 3565, *Meat and meat products — Detection of* Salmonella *(Reference method)*. For milk and milk products, a method will form the subject of ISO 6785.

2) *Salmonella* may be present in small numbers and are often accompanied by considerably larger numbers of other members of *Enterobacteriaceæ* or of other families. Therefore, selective enrichment is necessary; furthermore, pre-enrichment is often necessary to permit detection of injured *Salmonella*.

4.3 Plating out and recognition

From the cultures obtained in 4.2, inoculation of two selective solid media :

— brilliant green/phenol red agar, unless the International Standard appropriate to the product to be examined, or other specific considerations (for example the isolation of lactose-positive *Salmonella*), require substitution of some other medium as the one for obligatory use;

— any other solid selective medium (see 7.2.4.2).

Incubation at the specified temperature (see 9.4.4), and examination after 24 h and, if necessary, after 48 h to check for the presence of colonies which, from their characteristics, are considered to be presumptive *Salmonella*.

4.4 Confirmation

Subculturing of colonies of presumptive *Salmonella*, plated out as described in 4.3, and confirmation by means of appropriate biochemical and serological tests.

5 Sampling

Carry out sampling in accordance with the International Standard appropriate to the product concerned, if available.

6 Apparatus and glassware

Usual microbiological laboratory equipment, and in particular

6.1 Apparatus

6.1.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave).

Apparatus that will enter into contact with the culture media, the dilution fluid or the sample, except for apparatus that is supplied sterile (particularly plastic apparatus), shall be sterilized either

— by being kept at 170 to 175 °C for not less than 1 h in an oven;

— by being kept at 121 ± 1 °C for not less than 20 min in an autoclave.

An autoclave is also necessary for the sterilization of culture media and reagents. It shall be capable of being controlled at 121 ± 1 °C, and at 115 ± 1 °C, as indicated in annex B.

6.1.2 Drying cabinet, oven, or incubator, ventilated by convection (for drying the surface of agar plates), capable of being controlled at 50 ± 1 °C.

6.1.3 Incubator, capable of being controlled at 35 ± 1 °C or 37 ± 1 °C, depending on the temperature adopted[1] (for maintaining the inoculated media, plates and tubes within one of these temperature ranges).

6.1.4 Water bath, capable of being controlled at 43 ± 0,1 °C, or **incubator,** capable of being controlled at 42,5 ± 0,5 °C (for maintaining inoculated liquid media within one of these temperature ranges).

6.1.5 Water baths (for heating and cooling solutions and culture media to the appropriate temperatures), capable of being controlled at 45 ± 1 °C, 55 ± 1 °C and 70 ± 1 °C.

6.1.6 Water bath, capable of being controlled at 35 ± 1 °C or 37 ± 1 °C, depending on the temperature adopted.[1]

6.1.7 Loops, of platinum-iridium or nickel-chromium, of diameter approximately 3 mm.

6.1.8 pH-meter (for measuring the pH of prepared media and reagents), having an accuracy of calibration of ± 0,1 pH unit at 25 °C.

6.1.9 Refrigerator (for storage of prepared media and reagents), capable of being controlled at 4 ± 2 °C.

6.2 Glassware

The glassware shall be resistant to repeated sterilization.

6.2.1 Culture bottles or flasks[2], for sterilization and storage of culture media and incubation of liquid media.

6.2.2 Culture tubes, 8 mm in diameter and 160 mm in length, for the lysine decarboxylation medium.

6.2.3 Measuring cylinders, for preparation of the complete media.

6.2.4 Graduated pipettes, of nominal capacities 10 ml and 1 ml, graduated respectively in 0,5 ml and 0,1 ml.

6.2.5 Petri dishes, as follows :

6.2.5.1 Large-size dish.

Dish

external diameter	140 ± 2 mm
external height	30 ± 2 mm
glass thickness	1,5 ± 0,5 mm

The rim shall be ground in a plane parallel to the base.

The bottom of the dish shall be flat and parallel to the base.

Lid, with a ridge

external diameter	150 ± 2 mm
external height	15 ± 2 mm
glass thickness	1,5 ± 0,5 mm

1) The temperature should be agreed between the parties concerned and recorded in the test report.

2) Bottles or flasks with metal screw-caps may be used.

6.2.5.2 Small-size dish.

Dish

internal diameter	90 ± 2 mm
external height, minimum	18 mm

The rim shall be ground in a plane parallel to the base.

The bottom of the dish shall be flat and parallel to the base.

Lid, with a ridge

external diameter, maximum	102 mm

NOTE — Alternatively, plastic Petri dishes may be used, even if of slightly different dimensions from the glass dishes described in 6.2.5.1 and 6.2.5.2.

7 Culture media, reagents and sera

7.1 Basic materials

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

The chemicals used for preparing the culture media and the reagents shall be of recognized analytical quality.

The water used shall be distilled or deionized water, and shall be free from substances that might inhibit growth of micro-organisms under the test conditions.

The pH measurements shall be carried out by means of the pH-meter (6.1.8).

If the prepared media and prepared reagents are not used immediately, they shall, unless otherwise specified, be kept in the dark at a temperature of about 4 °C, but for no longer than 1 month, and in conditions that prevent any change in their composition.

7.2 Culture media and reagents

NOTE — Because of the large number of culture media and reagents, it has been considered preferable, for the clarity of the text, to give their composition and preparation in annex B.

7.2.1 Pre-enrichment medium : buffered peptone water.

See B.1.

7.2.2 First enrichment medium : tetrathionate medium (Muller-Kauffmann).

See B.2.

7.2.3 Second enrichment medium : selenite cystine medium.

See B.3.

7.2.4 Selective solid plating-out media.

7.2.4.1 First medium : phenol red/brilliant green agar (Edel and Kampelmacher).

See B.4.

This first medium is compulsory unless otherwise stated (see 4.3).

7.2.4.2 Second medium.

The choice of the second medium is left to the discretion of the testing laboratory, unless there is a specific International Standard, relating to the product to be examined, which specifies the composition of this second medium.

7.2.5 Nutrient agar.

See B.5.

7.2.6 Triple sugar/iron agar (TSI agar).

See B.6.

7.2.7 Urea agar (Christensen).

See B.7.

7.2.8 Lysine decarboxylation medium.

See B.8.

7.2.9 Reagent for detection of β**-galactosidase** (or **prepared paper discs,** used in accordance with the manufacturer's instructions).

See B.9.

7.2.10 Reagents for Voges-Proskauer reaction.

See B.10.

7.2.10.1 VP medium.

7.2.10.2 Creatine solution (*N*-amidinosarcosine).

7.2.10.3 1-naphthol, ethanolic solution.

7.2.10.4 Potassium hydroxide solution.

7.2.11 Reagents for indole reaction.

See B.11.

7.2.11.1 Tryptone/tryptophan medium (by Ljutov).

7.2.11.2 Kovacs reagent.

7.2.12 Semi-solid nutrient agar.

See B.12.

7.2.13 Saline solution.

See B.13.

7.3 Sera

Several types for agglutinant sera containing antibodies for one or several O-antigens, are available commercially, i.e. anti-sera containing one or more "O" groups (called monovalent or polyvalent anti-O sera), anti-Vi sera, and anti-sera containing antibodies for one or several H-factors (called monovalent or polyvalent anti-H sera).

Every attempt should be made to ensure that the anti-sera used are adequate to provide for the detection of all *Salmonella* serotypes. Assistance toward this objective may be obtained by using anti-sera prepared by a supplier recognized as competent (for example, an appropriate government agency).

8 Preparation of test sample

Refer to the International Standard appropriate to the product under examination. If an International Standard is not available, it is recommended that agreement be reached on this subject by the parties concerned.

9 Procedure

9.1 Test portion and initial suspension

Refer to the International Standard appropriate to the product under examination.

For preparation of the initial suspension, use as diluent the pre-enrichment medium specified in 7.2.1.

In general, to prepare the initial suspension, add a 25 g test portion to 225 ml of pre-enrichment medium (7.2.1), which is the ratio of test portion to pre-enrichment medium specified in this method.[1)]

If the prescribed test portion is other than 25 g, use the necessary quantity of pre-enrichment medium to yield approximately a 1 : 10 dilution (mass to volume).

9.2 Non-selective pre-enrichment

Incubate the initial suspension at 35 °C or 37 °C[2)] for not less than 16 h and not more than 20 h.

9.3 Selective enrichment

9.3.1 Transfer 10 ml of the culture obtained in 9.2 to a flask containing 100 ml of the tetrathionate medium (7.2.2); transfer another 10 ml of the culture obtained in 9.2 to a flask containing 100 ml of selenite cystine medium (7.2.3)[1)].

9.3.2 Incubate the two inoculated media (9.3.1) as follows :

a) the inoculated tetrathionate medium at 43 °C;

b) the inoculated selenite cystine medium at the specified temperature, i.e. at 35 °C or 37 °C.[2)]

9.4 Plating out and identification

9.4.1 After incubation (see 9.3.2) for 18 to 24 h, take, by means of a loop (6.1.7), a streak from the culture in the tetrathionate medium, and inoculate the surface of one large-size Petri dish containing the first selective plating-out medium (generally the phenol red/brilliant green agar, see 7.2.4.1), so that well-isolated colonies will be obtained.

In the absence of large dishes, use two small dishes, one after the other, using the same loop (see the note).

Proceed in the same way with the second selective plating-out medium (7.2.4.2) using a new loop and Petri dishes of appropriate size.

NOTE — The following method of streaking is recommended when phenol red/brilliant green agar is used. Take one loopful (6.1.7) for two dishes. Take a droplet from the edge of the surface of the fluid. Inoculate both dishes according to the two diagrams in annex D. Use the whole dish; loop streaks should be spaced about 0,5 cm apart. (Do not flame the loop or recharge it after making the first streak, nor when passing to the second dish.) When only one large dish is used, the method of streaking should be as indicated for the first dish.

9.4.2 Using the culture in the selenite cystine medium, repeat the procedure described in 9.4.1 with the two selective plating-out media.

1) To reduce the examination workload when more than one 25 g test portion from a specified lot of food has to be examined, and when evidence is available that compositing (pooling the test portions) does not affect the result for that particular food, the test portions may be composited. For example, if 10 test portions of 25 g are to be examined, combine the 10 units to form a composite test portion of 250 g and add 2,25 litres of pre-enrichment broth. Alternatively, the 10 ml portions of the pre-enrichment broths from the 10 separate test portions (9.3.1) may be composited for enrichment in 1 litre of selective enrichment medium.

2) The temperature should be agreed between the parties concerned and recorded in the test report.

For the selenite cystine medium, it may, in some cases, be advantageous to raise the incubation temperature to 43 °C. This modification should be indicated in the test report.

9.4.3 Turn the dishes (9.4.1 and 9.4.2) so that the bottom is uppermost, and place them in an incubator (6.1.3) at the specified temperature, i.e. at 35 °C or 37 °C.[1)]

9.4.4 After a total incubation period of 48 h (see 9.3.2), repeat the procedure described in 9.4.1 to 9.4.3 using the two inoculated enrichment media.

9.4.5 After incubation for 20 to 24 h, examine the dishes (9.4.3 and 9.4.4) for the presence of typical colonies of *Salmonella*. Typical colonies of *Salmonella* grown on brilliant green/phenol red agar cause the colour of the medium to change from pink to red.

9.4.6 If growth is slight or if no typical colonies of *Salmonella* are present, reincubate at 35 °C or at 37 °C[1)] for a further 18 to 24 h.

Re-examine the plates for the presence of typical colonies of *Salmonella*.

NOTE — Subject any typical or suspect colony to a confirmation (9.5); the recognition of colonies of *Salmonella* is to a large extent a matter of experience, and their appearance may vary somewhat, not only from species to species, but also from batch to batch of medium. In this respect, agglutination, at this stage, of colonies with polyvalent *Salmonella* anti-serum may facilitate recognition of suspected colonies.

9.5 Confirmation

9.5.1 Selection of colonies for confirmation

For confirmation, take from each plate of each selective medium (see 9.4.3 and 9.4.4), five colonies considered to be typical or suspect.

If on one plate there are fewer than five typical or suspect colonies, take for confirmation all the typical or suspect colonies.

Streak the selected colonies onto the surface of pre-dried nutrient agar plates (7.2.6), in a manner which will allow well-isolated colonies to develop.

Incubate the inoculated plates at 35 °C or 37 °C[1)] for 18 to 24 h.

Use pure colonies for biochemical and serological confirmation.

9.5.2 Biochemical confirmation

By means of an inoculating wire, inoculate the media indicated in 9.5.2.1 to 9.5.2.6 with each of the cultures obtained from the colonies selected in 9.5.1.

9.5.2.1 TSI agar (7.2.6)

Streak the agar slope surface and stab the butt.

Incubate for 24 h at 35 °C or 37 °C.[1)]

Interpret the changes in the medium as follows :

Butt

yellow	:	glucose positive (fermentation of glucose)
red or unchanged	:	glucose negative (no fermentation of glucose)
black	:	formation of hydrogen sulphide
bubbles or cracks	:	gas formation from glucose

Slant surface

yellow	:	lactose and/or sucrose positive (lactose and/or sucrose used)
red or unchanged	:	lactose and sucrose negative (neither lactose nor sucrose used)

Typical *Salmonella* cultures show alkaline (red) slants with gas formation and acid (yellow) butts, with (in about 90 % of the cases) formation of hydrogen sulphide (blackening of the agar).

When a lactose-positive *Salmonella* is isolated (see 4.3), the TSI slant is yellow. Thus, preliminary confirmation of *Salmonella* cultures shall not be based on the results of the TSI agar-test only (see 9.5.3).

9.5.2.2 Urea agar (7.2.7)

Streak the agar slope surface.

Incubate for 24 h at 35 °C or 37 °C[1)] and examine at intervals.

If the reaction is positive, splitting of urea liberates ammonia, which changes the colour of phenol red to rose-pink and later to deep cerise. The reaction is often apparent after 2 to 24 h.

9.5.2.3 Lysine decarboxylation medium (7.2.8)

Inoculate just below the surface of the liquid medium.

Incubate for 24 h at 35 °C or 37 °C.[1)]

1) The temperature should be agreed between the parties concerned and recorded in the test report.

A purple colour after growth has occurred indicates a positive reaction.

A yellow colour indicates a negative reaction.

9.5.2.4 Reagent for detection of β-galactosidase (7.2.9)

Suspend a loopful of the suspected colony in a tube containing 0,25 ml of the saline solution (7.3).

Add 1 drop of toluene and shake the tube.

Put the tube in a water bath at 35 °C or 37 °C[1] and leave for several minutes.

Add 0,25 ml of the reagent for detection of β-galactosidase and mix.

Replace the tube in the water bath at 35 °C or 37 °C[1], leave for 24 h and examine at intervals.

A yellow colour indicates a positive reaction. The reaction is often apparent after 20 min.

9.5.2.5 Medium for Voges-Proskauer reaction (7.2.10)

Suspend a loopful of the suspected colony in a sterile tube containing 0,2 ml of the VP medium (7.2.10.1).

Incubate for 24 h at 35 °C or 37 °C.[1]

After incubation, add 2 drops of the creatine solution (7.2.10.2), 3 drops of the ethanolic 1-naphthol solution (7.2.10.3) and then 2 drops of the potassium hydroxide solution (7.2.10.4); shake after the addition of each reagent.

The formation of a pink to bright red colour within 15 min indicates a positive reaction.

9.5.2.6 Medium for indole reaction (7.2.11)

Inoculate a tube containing 5 ml of the tryptone-tryptophan medium (7.2.11.1) with the suspect colony.

Incubate for 24 h at 35 °C or 37 °C.[1]

After incubation, add 1 ml of the Kovacs reagent (7.2.11.2).

The formation of a red ring indicates a positive reaction.

A yellow-brown ring indicates a negative reaction.

9.5.2.7 Interpretation of the biochemical tests

Salmonella generally show the following reactions[2] :

	Positive or negative reaction	Percentage of *Salmonella* strains showing the reaction[3]
TSI glucose (acid formation) (9.5.2.1)	+	100
TSI glucose (gas formation) (9.5.2.1)	+	91,9[4]
TSI lactose (9.5.2.1)	–	99,2[5]
TSI sucrose (9.5.2.1)	–	99,5
TSI hydrogen sulphide (9.5.2.1)	+	91,6
Urea splitting (9.5.2.2)	–	100
Lysine decarboxylation (9.5.2.3)	+	94,6
β-galactosidase reaction (9.5.2.4)	–	98,5 [5]
Voges-Proskauer reaction (9.5.2.5)	–	100
Indole reaction (9.5.2.6)	–	98,9

9.5.3 Serological confirmation

The detection of the presence of *Salmonella* O-, Vi- and H-antigens is tested by slide agglutination with the appropriate sera, from pure colonies (9.5.1) and after auto-agglutinable strains have been eliminated.

9.5.3.1 Elimination of auto-agglutinable strains

Place one drop of the saline solution (7.2.13) on a carefully cleaned glass slide.

1) The temperature should be agreed between the parties concerned and recorded in the test report.

2) W. H. Ewing and M. M. Ball. *The biochemical reactions of members of the genus* Salmonella (1966). National Communicable Disease Center, Atlanta, Georgia, USA.

3) These percentages indicate only that not all strains of *Salmonella* show the reactions marked + or – . These percentages may vary from country to country and from food product to food product.

4) *Salmonella typhi* is anaerogenic.

5) The *Salmonella* subgenus III (Arizona) gives positive or negative lactose reactions but is always β-galactosidase-positive. The *Salmonella* subgenus II gives a negative lactose reaction, but may give a positive β-galactosidase reaction. For the study of strains, it may be useful to carry out complementary biochemical tests.

Disperse in this drop part of the colony to be tested, so as to obtain a homogeneous and turbid suspension.

Rock the slide gently for 30 to 60 s.

Observe the result against a dark background, preferably with the aid of a magnifying glass.

If the bacteria have clumped into more or less distinct units, the strain is considered auto-agglutinable, and shall not be submitted to the following tests as the detection of the antigens is impossible.

9.5.3.2 Examination for O-antigens

Using one pure colony recognized as non-auto-agglutinable, proceed according to 9.5.3.1, using one drop of the anti-O serum (7.3) instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

Use the poly- and monovalent sera one after the other.

9.5.3.3 Examination for Vi-antigens

Proceed according to 9.5.3.1, but using a drop of the anti-Vi serum (7.3) instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

9.5.3.4 Examination for H-antigens

Inoculate the semi-solid nutrient agar (7.2.12) with a pure non-auto-agglutinable colony.

Incubate the medium for 18 to 24 h at 35 °C or 37 °C.[1].

Use this culture for examination for the H-antigens, proceeding according to 9.5.3.1, but using a drop of the anti-H serum (7.3) instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

9.5.4 Interpretation of biochemical and serological reactions

The table gives the interpretation of the confirmatory tests (9.5.2 and 9.5.3) carried out on the colonies used (9.5.1).

Table

Biochemical reactions	Auto-agglutination	Serological reactions	Interpretation
Typical	No	O-, Vi- or H-antigen positive	Strains considered to be *Salmonella*
Typical	No	All reactions negative	May be *Salmonella*
Typical	Yes	Not tested (see 9.5.3.1)	
No typical reactions	No	O-, Vi- or H-antigen positive	
No typical reactions	No	All reactions negative	Not considered to be *Salmonella*

9.5.5 Definitive confirmation

Strains which are considered to be *Salmonella*, or which may be *Salmonella*, (see the table) shall be sent to a recognized *Salmonella* reference centre for definitive typing.

This dispatch shall be accompanied by all possible information concerning the strain(s).

10 Expression of results

In accordance with the results of the interpretation, indicate the presence or absence of *Salmonella* in a test portion of x g of product.

11 Test report

The test report shall show the method used and the results obtained. It shall also mention all operating conditions not specified in this International Standard or regarded as optional, as well as any circumstances that may have influenced the result.

Report, in particular, the incubation temperature used, i.e. 35 °C or 37 °C, and in the case of the selenite cystine medium, whether the temperature was raised to 43 °C.

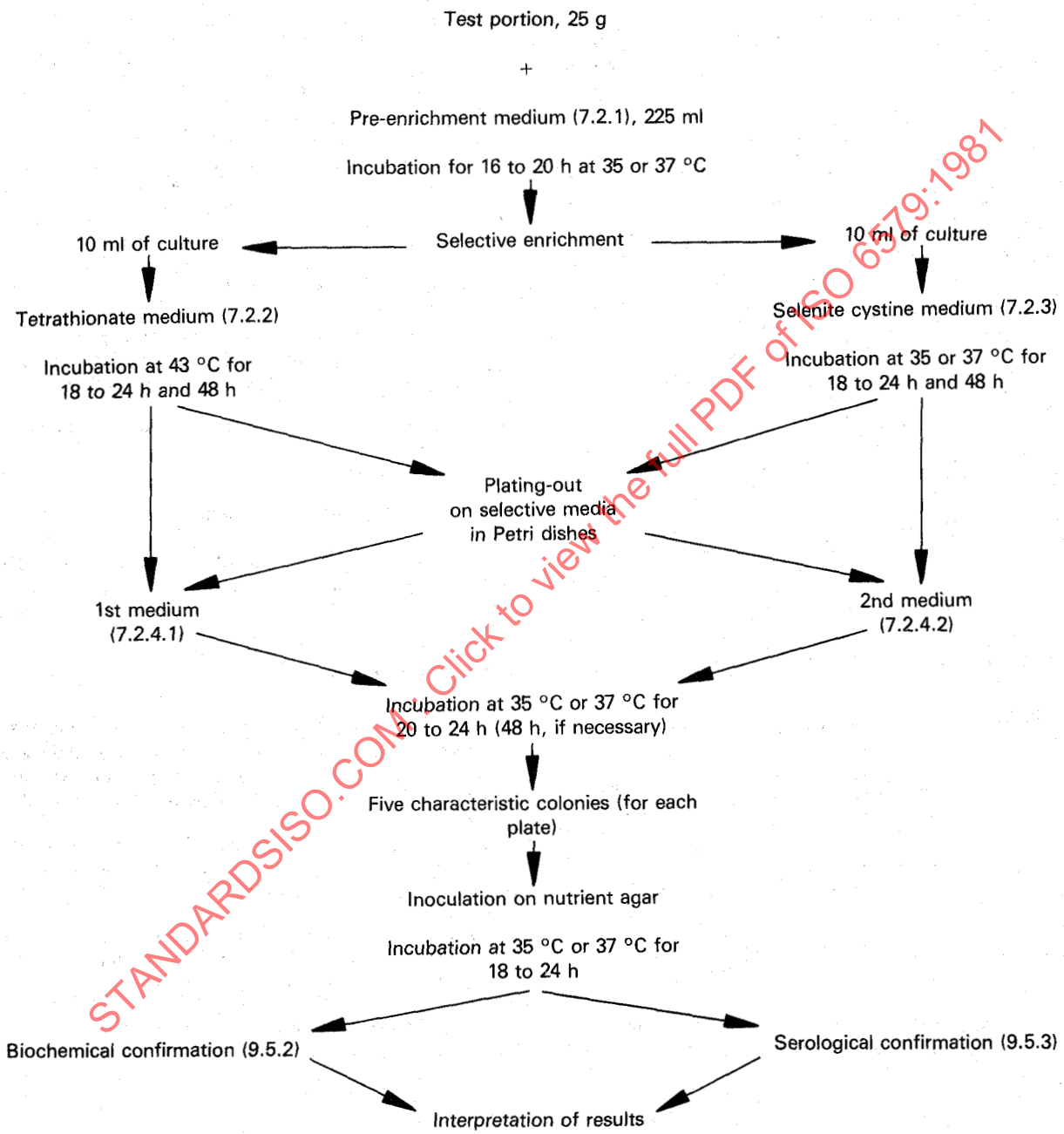
The test report shall also state whether a positive result was obtained only when using a plating-out medium (7.2.4) not specified in this International Standard.

This report shall include all details required for complete identification of the sample.

1) The temperature should be agreed between the parties concerned and recorded in the test report.

Annex A

Diagram of procedure



Annex B

Composition and preparation of culture media and reagents

B.1 Buffered peptone water

Composition

peptone	10,0 g
sodium chloride	5,0 g
disodium hydrogenorthophosphate dodecahydrate ($Na_2HPO_4.12H_2O$)	9,0 g
potassium dihydrogenorthophosphate (KH_2PO_4)	1,5 g
water	1 000 ml

Preparation

Dissolve the components in the water by boiling.

Adjust the pH, if necessary, so that after sterilization it is 7,0 at 25 °C.

Sterilize the medium by heating in an autoclave (see 6.1.1) for 20 min at 121 °C.

Distribute the medium aseptically into sterile flasks of suitable capacity to obtain the portions necessary for the test.

B.2 Tetrathionate medium (Muller-Kauffmann)

B.2.1 Base

Composition

meat extract	5,0 g
peptone	10,0 g
sodium chloride	3,0 g
calcium carbonate	45 g
water	1 000 ml

Preparation

Add the dehydrated base components or the dehydrated complete base to the water and boil until complete dissolution of the soluble components.

Sterilize the base by heating in an autoclave (see 6.1.1) for 20 min at 121 °C.

B.2.2 Sodium thiosulphate solution

Composition

sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$)	50,0 g
water to a final volume of	100 ml

Preparation

Dissolve the sodium thiosulphate in a part of the water.

Dilute to the final volume.

Sterilize the solution by heating in an autoclave (see 6.1.1) for 20 min at 121 °C.

B.2.3 Iodine solution

Composition

iodine	20,0 g
potassium iodide	25,0 g
water to a final volume of	100 ml

Preparation

Dissolve the potassium iodide in a minimal volume of water and add the iodine.

Shake until dissolution is complete.

Dilute to the final volume.

Store the solution in a tightly closed opaque container.

B.2.4 Brilliant green solution

Composition

brilliant green (see specifications in annex C)	about 0,5 g
water	100 ml

Preparation

Add the brilliant green to the water.

Store the solution for at least one day in the dark to allow auto-sterilization to occur.

B.2.5 Ox bile solution

Composition

ox bile, desiccated	10,0 g
water	100 ml

Preparation

Dissolve the desiccated ox bile in the water by boiling.

Sterilize the solution by heating in an autoclave (see 6.1.1) for 20 min at 121 °C.

B.2.6 Complete medium

Composition

base	900 ml
sodium thiosulphate solution	100 ml
iodine solution	20 ml
brilliant green solution	2 ml
ox bile solution	50 ml

Preparation

Add to the base, under aseptic conditions, the other ingredients, in the above-mentioned order.

Mix the liquids well after each addition.

Distribute the medium aseptically into sterile flasks of suitable capacity to obtain the portions necessary for the test.

Use within one week of preparation.

B.3 Selenite cystine medium

B.3.1 Base

Composition

tryptone[1]	5,0 g
lactose	4,0 g
disodium hydrogenorthophosphate dodecahydrate ($Na_2HPO_4.12H_2O$)	10,0 g
sodium hydrogenselenite	4,0 g
water	1 000 ml

Preparation

Dissolve the first three ingredients in the water by boiling for 5 min. After cooling, add the sodium hydrogenselenite.

Adjust the pH, if necessary so that it is 7,0 at 25 °C.

B.3.2 L-Cystine solution

Composition

L-cystine	0,1 g
sodium hydroxide solution, $c(NaOH) = 1$ mol/l[2]	15 ml

Preparation

Dilute to 100 ml with sterile water in a sterile flask.

Do not autoclave.

B.3.3 Complete medium

Composition

base	1 000 ml
L-cystine solution	10 ml

Preparation

Cool the base and add the L-cystine solution aseptically.

Adjust the pH, if necessary, so that it is 7,0 at 25 °C.

Distribute the medium aseptically into sterile flasks of suitable capacity to obtain the portions necessary for the test.

Use the medium on the day of preparation.

B.4 Phenol red/brilliant green agar (Edel and Kampelmacher)

B.4.1 Base

Composition

meat extract powder	5,0 g
peptone	10,0 g
yeast extract powder	3,0 g
disodium hydrogenorthophosphate (Na_2HPO_4)	1,0 g
sodium dihydrogenorthophosphate (NaH_2PO_4)	0,6 g
agar	12,0 to 18 g[3]
water	900 ml

Preparation

Dissolve the dehydrated base components or the dehydrated complete base in the water by boiling.

Adjust the pH, if necessary, so that after sterilization it is 7,0 at 25 °C.

Transfer the base to tubes or flasks of not more than 500 ml capacity.

Sterilize the base by heating in an autoclave (see 6.1.1) for 15 min at 121 °C.

B.4.2 Sugar/phenol red solution

Composition

lactose	10,0 g
sucrose	10,0 g
phenol red	0,09 g
water to a final volume of	100 ml

1) This term is used at present only by certain producers of media. Any other casein digest giving comparable results may be used.

2) Hitherto expressed as "1 N solution".

3) In accordance with the manufacturer's instructions.

Preparation

Dissolve the ingredients in the water.

Heat in a water bath for 20 min at 70 °C.

Cool to 55 °C and use immediately.

B.4.3 Brilliant green solution

For composition and preparation of this solution, see B.2.4.

B.4.4 Complete medium

Composition

base	900 ml
sugar/phenol red solution	100 ml
brilliant green solution	1 ml

Preparation

Add, under aseptic conditions, the brilliant green solution to the sugar/phenol red solution cooled to approximately 55 °C.

Add to the base at 50 to 55 °C and mix.

B.4.5 Preparation of the agar plates

Place in large Petri dishes (6.2.5.1) about 40 ml of the freshly prepared complete medium (B.4.4). [If large dishes are not available, transfer about 15 ml to small Petri dishes (6.2.5.2).] Allow to solidify.

Immediately before use, dry the agar plates carefully (preferably with the lids off and the agar surface downwards) in an oven or incubator controlled at 50 °C (6.1) for 30 min, or until the surface of the agar is dry.

If prepared in advance, the undried agar plates shall be kept for no longer than 4 h at room temperature or one day in the refrigerator (6.1.9).

B.5 Nutrient agar

Composition

meat extract	3,0 g
peptone	5,0 g
agar	9 to 18 g[1]
water	1 000 ml

Preparation

Dissolve the dehydrated medium components or the dehydrated complete medium in the water by boiling.

Adjust the pH, if necessary, so that after sterilization it is 7,0 at 25 °C.

Transfer the culture medium into tubes or bottles of not more than 500 ml capacity.

Sterilize the medium by heating in an autoclave (see 6.1.1) for 20 min at 121 °C.

Preparation of nutrient agar plates

Transfer about 15 ml of the melted medium to sterile small Petri dishes (6.2.5.2) and proceed as in B.4.5.

B.6 Triple sugar/iron agar (TSI agar)

Composition

meat extract	3,0 g
yeast extract	3,0 g
peptone	20,0 g
sodium chloride	5,0 g
lactose	10,0 g
sucrose	10,0 g
glucose	1,0 g
iron(III) citrate	0,3 g
sodium thiosulphate	0,3 g
phenol red	0,024 g
agar	12 to 18 g[1]
water	1 000 ml

Preparation

Dissolve the dehydrated medium components or the dehydrated complete medium in the water by boiling.

Adjust the pH, if necessary, so that after sterilization it is 7,4 at 25 °C.

Transfer the medium in quantities of 10 ml to tubes of diameter 17 to 18 mm.

Sterilize the medium by heating in an autoclave (see 6.1.1) for 10 min at 121 °C.

Allow to set in a sloping position to give a butt of depth 2,5 cm.

B.7 Urea agar (Christensen)

B.7.1 Base

Composition

peptone	1,0 g
glucose	1,0 g
sodium chloride	5,0 g
potassium dihydrogenorthophosphate (KH_2PO_4)	2,0 g

1) In accordance with the manufacturer's instructions.

phenol red	0,012 g
agar	12 to 18 g[1]
water	1 000 ml

Preparation

Dissolve the dehydrated base components or the dehydrated complete base in the water by boiling.

Adjust the pH, if necessary, so that after sterilization it is 6,8 at 25 °C.

Sterilize the base by heating in an autoclave (see 6.1.1) for 20 min at 121 °C.

B.7.2 Urea solution

Composition

urea	400 g
water to a final volume of	1 000 ml

Preparation

Dissolve the urea in the water.

Sterilize by filtration and check the sterility.

(For details of the technique of sterilization by filtration, reference should be made to any appropriate textbook on microbiology.)

B.7.3 Complete medium

Composition

base (B.7.1)	950 ml
urea solution (B.7.2)	50 ml

Preparation

Add, under aseptic conditions, the urea solution to the base, previously melted and then cooled to 45 °C.

Transfer the complete medium in quantities of 10 ml to sterile tubes.

Allow to set in a sloping position.

B.8 Lysine decarboxylation medium

Composition

L-lysine monohydrochloride	5,0 g
yeast extract	3,0 g
glucose	1,0 g
bromocresol purple	0,015 g
water	1 000 ml

Preparation

Dissolve the components in the water by boiling.

Adjust the pH, if necessary, so that after sterilization it is 6,8 at 25 °C.

Transfer the medium in quantities of 5 ml to narrow culture tubes approximately 8 mm in diameter and 160 mm in length.

Sterilize the medium by heating in an autoclave (see 6.1.1) for 10 min at 121 °C.

B.9 β-galactosidase reagent

B.9.1 Buffer solution

Composition

sodium dihydrogenorthophosphate (NaH_2PO_4)	6,9 g
sodium hydroxide, approximately 0,1 mol/l solution	3 ml (approximately)
water to a final volume of	50 ml

Preparation

Dissolve the sodium dihydrogenorthophosphate in approximately 45 ml of water.

Adjust the pH to 7,0 with the sodium hydroxide solution.

Add water to a final volume of 50 ml.

Store in the refrigerator (6.1.9).

B.9.2 ONPG solution

Composition

2-nitrophenyl β-D-galactopyranoside (ONPG)	80 mg
water	15 ml

Preparation

Dissolve the ONPG in the water at 50 °C.

Cool the solution.

B.9.3 Complete reagent

Composition

buffer solution (B.9.1)	5 ml
ONPG solution (B.9.2)	15 ml

Preparation

Add the buffer solution to the ONPG solution.

1) In accordance with the manufacturer's instructions.