INTERNATIONAL STANDARD

ISO 6579

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Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Salmonella* spp.

Microbiologie des aliments — Méthode horizontale pour la recherche des Salmonella *spp.*

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 6579 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

This fourth edition cancels and replaces the third edition (ISO 6579:1993), which has been technically revised.

Annexes A and B form a normative part of this Interntional Standard. Annex C is for information only.

Introduction

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods, which are specific to these products, may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this horizontal method as far as possible.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from this method 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 this horizontal method. 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 this horizontal method will be those necessary for well-established technical reasons.

Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Salmonella* spp.

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Salmonella*, and especially *Salmonella* Typhi and *Salmonella* Paratyphi, 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 specifies a horizontal method for the detection of *Salmonella*, including *Salmonella* Typhi and *Salmonella* Paratyphi.

Subject to the limitations discussed in the Introduction, this International Standard is applicable to

— products intended for human consumption and the feeding of animals;

— environmental samples in the area of food production and food handling.

WARNING — The method may not recover all *Salmonella* Typhi and Paratyphi.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 6887-1, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*

ISO 7218:1996, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations*

ISO 8261, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

3 Terms and definitions

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

3.1
Salmonella
microorganisms which form typical or less 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 *Salmonella* (3.1), in a particular mass or volume of product, when tests are carried out in accordance with this International Standard

4 Principle

4.1 General

The detection of *Salmonella* necessitates four successive stages (see also annex A).

NOTE The *Salmonella* may be present in small numbers and are often accompanied by considerably larger numbers of other *Enterobacteriaceæ* or other families. Furthermore, pre-enrichment is necessary to permit the detection of low numbers of *Salmonella* or injured *Salmonella*.

4.2 Pre-enrichment in non-selective liquid medium

Buffered peptone water is inoculated at ambient temperature with the test portion, then incubated at 37 °C ± 1 °C for 18 h ± 2 h.

For certain foodstuffs the use of other pre-enrichment procedures is necessary. See 9.1.2.

For large quantities, the buffered peptone water should be heated to 37 °C ± 1 °C before inoculation with the test portion.

4.3 Enrichment in selective liquid media

Rappaport-Vassiliadis medium with soya (RVS broth) and Muller-Kauffmann tetrathionate/novobiocin broth (MKTTn broth) are inoculated with the culture obtained in 4.2.

The RVS broth is incubated at 41,5 °C ± 1 °C for 24 h ± 3 h, and the MKTTn broth at 37 °C ± 1 °C for 24 h ± 3 h.

4.4 Plating out and identification

From the cultures obtained in 4.3, two selective solid media are inoculated:

— xylose lysine deoxycholate agar (XLD agar);

— any other solid selective medium complementary to XLD agar and especially appropriate for the isolation of lactose-positive *Salmonella* and *Salmonella* Typhi and *Salmonella* Paratyphi strains; the laboratory may choose which medium to use.

The XLD agar is incubated at 37 °C ± 1 °C and examined after 24 h ± 3 h. The second selective agar is incubated according to the manufacturer's recommendations.

NOTE For information, Brilliant green agar (BGA), bismuth sulfite agar, etc., could be used as the second plating-out medium.

4.5 Confirmation of identity

Colonies of presumptive *Salmonella* are subcultured, then plated out as described in 4.4, and their identity is confirmed by means of appropriate biochemical and serological tests.

5 Culture media, reagents and sera

5.1 General

For current laboratory practice, see ISO 7218.

5.2 Culture media and reagents

NOTE Because of the large number of culture media and reagents, it is considered preferable, for clarity, to give their compositions and preparations in annex B.

5.2.1 Non-selective pre-enrichment medium: Buffered peptone water

See B.1.

5.2.2 First selective enrichment medium: Rappaport-Vassiliadis medium with soya (RVS broth)

See B.2.

5.2.3 Second selective enrichment medium: Muller-Kauffmann tetrathionate novobiocin broth (MKTTn broth)

See B.3.

5.2.4 Solid selective plating-out media

5.2.4.1 First medium: Xylose lysine deoxycholate agar (XLD agar)

See B.4.

5.2.4.2 Second medium

The choice of the second appropriate medium is left to the discretion of the testing laboratory. The manufacturer's instructions should be precisely followed regarding its preparation for use.

5.2.5 Nutrient agar

See B.5.

5.2.6 Triple sugar/iron agar (TSI agar)

See B.6.

5.2.7 Urea agar (Christensen)

See B.7.

5.2.8 L-Lysine decarboxylation medium

See B.8.

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

See B.9.

5.2.10 Reagents for Voges-Proskauer (VP) reaction

See B.10.

5.2.11 Reagents for indole reaction

See B.11.

5.2.12 Semi-solid nutrient agar

See B.12.

5.2.13 Physiological saline solution

See B.13.

5.3 Sera

Several types of agglutinating 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 towards this objective may be obtained by using only anti-sera prepared by a supplier recognized as competent (for example, by an appropriate government agency).

6 Apparatus and glassware

Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

6.1 Apparatus for dry sterilization (oven) or **wet sterilization (autoclave)**

See ISO 7218.

6.2 Drying cabinet or **oven**, ventilated by convection, capable of operating between 37 °C and 55 °C.

6.3 Incubator, capable of operating at 37 °C ± 1 °C.

6.4 Water bath, capable of operating at 41,5 °C ± 1 °C, or **incubator**, capable of operating at 41,5 °C ± 1 °C.

6.5 Water baths, capable of operating at 44 °C to 47 °C.

6.6 Water bath, capable of operating at 37 °C ± 1 °C.

It is recommended to use a water bath (6.4, 6.5 and 6.6) containing an antibacterial agent because of the low infective dose of *Salmonella*.

6.7 Sterile loops, of diameter approximately 3 mm or 10 µl, or **sterile pipettes**.

6.8 pH-meter, having an accuracy of calibration of ± 0,1 pH unit at 20 °C to 25 °C.

6.9 Test tubes or **flasks,** of appropriate capacity.

Bottles or flasks with non-toxic metallic or plastic screw-caps may be used.

6.10 Graduated pipettes or **automatic pipettes**, of nominal capacities 10 ml and 1 ml, graduated respectively in 0,5 ml and 0,1 ml divisions.

6.11 Petri dishes, of small size (diameter 90 mm to 100 mm) and/or large size (diameter 140 mm).

7 Sampling

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

Sampling is not part of the method specified in this International Standard. See the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure (see diagram in annex A)

9.1 Test portion and initial suspension

9.1.1 General

See ISO 6887-1 and the specific International Standard dealing with the product concerned. See ISO 8261 for milk and milk products.

For preparation of the initial suspension, in the general case use as diluent the pre-enrichment medium specified in 5.2.1 and 4.2 (buffered peptone water).

If the specified mass of test portion is other than 25 g, use the necessary quantity of pre-enrichment medium to yield a 1/10 dilution.

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 l of pre-enrichment broth. Alternatively, the 0,1 ml (in 10 ml of RVS broth) and 1 ml (in 10 ml of MKTTn broth) portions of the pre-enrichment broth from the 10 separate test portions (see 9.3.1) may be composited for enrichment in 100 ml of selective enrichment media.

9.1.2 Specific preparations of the initial suspension for certain foodstuffs

NOTE The following specific preparations concern only the case of *Salmonella*. Specific preparations applicable for the determination of any microorganisms are described in ISO 6887-2, ISO 6887-3, ISO 6887-4 and ISO 8261.

9.1.2.1 **Cocoa and cocoa-containing products** (e.g. more than 20 %)

Add to the buffered peptone water (5.2.1) preferably 50 g/l of casein (avoid the use of acid casein), or 100 g/l of sterile skim milk powder and add, after about 2 h incubation, 0,018 g/l of Brilliant green if the foodstuff is likely to be highly contaminated with Gram-positive flora.

9.1.2.2 Acidic and acidifying foodstuffs

Ensure that the pH does not fall to below 4,5 during pre-enrichment.

NOTE The pH of acidic and acidifying foodstuffs is more stable if double-strength buffered peptone water is used.

9.2 Non-selective pre-enrichment

Incubate the initial suspension (9.1) at 37 °C ± 1 °C for 18 h ± 2 h.

9.3 Selective enrichment

9.3.1 Transfer 0,1 ml of the culture obtained in 9.2 to a tube containing 10 ml of the RVS broth (5.2.2); transfer 1 ml of the culture obtained in 9.2 to a tube containing 10 ml of MKTTn broth (5.2.3).

9.3.2 Incubate the inoculated RVS broth (9.3.1) at 41,5 °C ± 1 °C for 24 h ± 3 h and the inoculated MKTTn broth at 37 °C ± 1 °C for 24 h ± 3 h. Care should be taken that the maximum allowed incubation temperature (42,5 °C) is not exceeded.

9.4 Plating out and identification

9.4.1 After incubation for 24 h ± 3 h, using the culture obtained in the RVS broth (9.3.2), inoculate by means of a loop (6.7) the surface of one large-size Petri dish (6.11) containing the first selective plating-out medium (XLD agar, see 5.2.4.1), 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.

Proceed in the same way with the second selective plating-out medium (5.2.4.2) using a sterile loop and Petri dishes as above.

9.4.2 After incubation for 24 h ± 3 h, using the culture obtained in the MKTTn broth (9.3.2), repeat the procedure described in 9.4.1 with the two selective plating-out media.

9.4.3 Invert the dishes (9.4.1 and 9.4.2) so that the bottom is uppermost, and place them in the incubator (6.3) set at 37 °C for the first plating-out medium (5.2.4.1). The manufacturer's instructions shall be followed for the second plating-out medium (5.2.4.2).

9.4.4 After incubation for 24 h ± 3 h, examine the plates (9.4.3) for the presence of typical colonies of *Salmonella* and atypical colonies that may be *Salmonella* (see Note). Mark their position on the bottom of the dish.

Typical colonies of *Salmonella* grown on XLD agar have a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator.

NOTE *Salmonella* H_2S negative variants (e.g. *S*. Paratyphi A) grown on XLD agar are pink with a darker pink centre. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening.

Incubate the second selective solid medium at the appropriate temperature and examine after the appropriate time to check for the presence of colonies which, from their characteristics, are considered to be presumptive *Salmonella*.

9.5 Confirmation

9.5.1 General

If shown to be reliable, commercially available identification kits for the biochemical examination of *Salmonella* may be used. The use of identification kits concerns the biochemical confirmation of colonies. These kits should be used following the manufacturer's instructions.

NOTE The recognition of colonies of *Salmonella* is to a large extent a matter of experience, and their appearance may vary somewhat, not only from serovar to serovar, but also from batch to batch of the selective culture medium used.

9.5.2 Selection of colonies for confirmation

For confirmation, take from each dish (two small-sized dishes or one large-sized dish) of each selective medium (see 9.4) at least one colony considered to be typical or suspect and a further four colonies if the first is negative.

It is recommended that at least five colonies be identified in the case of epidemiological studies. If on one dish 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 (5.2.5), in a manner which will allow well-isolated colonies to develop. Incubate the inoculated plates (9.4.3) at 37 °C ± 1 °C for 24 h ± 3 h.

Use pure cultures for biochemical and serological confirmation.

9.5.3 Biochemical confirmation

9.5.3.1 General

By means of an inoculating wire, inoculate the media specified in 9.5.3.2 to 9.5.3.7 with each of the cultures obtained from the colonies selected in 9.5.2.

9.5.3.2 **TSI agar** (5.2.6)

Streak the agar slant surface and stab the butt. Incubate at 37 °C ± 1 °C for 24 h ± 3 h.

Interpret the changes in the medium as follows.

a) Butt

- yellow — glucose positive (glucose used)
- red or unchanged — glucose negative (glucose not used)
- black — formation of hydrogen sulfide
- bubbles or cracks — gas formation from glucose

b) 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 and acid (yellow) butts with gas formation (bubbles) and (in about 90 % of the cases) formation of hydrogen sulfide (blackening of the agar) (9.5.3.8).

When a lactose-positive *Salmonella* is isolated (see 4.4), 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).

9.5.3.3 **Urea agar** (5.2.7)

Streak the agar slant surface. Incubate at 37 °C ± 1 °C for 24 h ± 3 h 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 h to 4 h.

9.5.3.4 L-Lysine decarboxylation medium (5.2.8)

Inoculate just below the surface of the liquid medium. Incubate at 37 °C ± 1 °C for 24 h ± 3 h.

Turbidity and a purple colour after incubation indicates a positive reaction. A yellow colour indicates a negative reaction.

9.5.3.5 Detection of β-galactosidase (5.2.9)

Suspend a loopful of the suspected colony in a tube containing 0,25 ml of the saline solution (5.2.13).

Add 1 drop of toluene and shake the tube. Put the tube in a water bath (6.6) set at 37 °C and leave for several minutes (approximately 5 min). Add 0,25 ml of the reagent for detection of β-galactosidase and mix.

Replace the tube in the water bath set at 37 °C and leave for 24 h ± 3 h, examining the tube at intervals.

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

If prepared paper discs (5.2.9) are used, follow the manufacturer's instructions.

9.5.3.6 Medium for Voges-Proskauer (VP) reaction (5.2.10)

Suspend a loopful of the suspected colony in a sterile tube containing 3 ml of the VP medium.

Incubate at 37 °C ± 1 °C for 24 h ± 3 h.

After incubation, add two drops of the creatine solution, three drops of the ethanolic solution of 1-naphthol and then two drops of the potassium hydroxide solution; shake after the addition of each reagent.

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

9.5.3.7 Medium for indole reaction (5.2.11)

Inoculate a tube containing 5 ml of the tryptone/tryptophan medium with the suspected colony.

Incubate at 37 °C ± 1 °C for 24 h ± 3 h. After incubation, add 1 ml of the Kovacs reagent.

The formation of a red ring indicates a positive reaction. A yellow-brown ring indicates a negative reaction.

9.5.3.8 Interpretation of the biochemical tests

Salmonella generally show the reactions given in Table 1.

9.5.4 Serological confirmation and serotyping

9.5.4.1 General

The detection of the presence of *Salmonella* O-, Vi- and H-antigens is tested by slide agglutination with the appropriate sera, from pure colonies (9.5.2) and after auto-agglutinable strains have been eliminated. Use the antisera according to the producer's instructions if different from the description below.

9.5.4.2 Elimination of auto-agglutinable strains

Place one drop of the saline solution (5.2.13) onto a carefully cleaned glass slide. Disperse in the drop, by means of a loop (6.7), part of the colony to be tested, in order to obtain a homogeneous and turbid suspension.

NOTE It is also possible to disperse part of the colony to be tested in a drop of water, and then to mix this solution with one drop of saline solution (5.2.13).

Rock the slide gently for 30 s 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 not feasible.

Table 1 — Interpretation of biochemical tests

Test [a] (9.5.3.2 to 9.5.3.7)	***Salmonella* strain**									
	***S.* Typhi**		***S.* Paratyphi A**		***S.* Paratyphi B**		***S.* Paratyphi C**		**Other strains**	
	Reaction	% [b]	Reaction	% [b]	Reaction	% [c]	Reaction	% [c]	Reaction	% [b]
TSI acid from glucose	+	100	+	100	+		+		+	100
TSI gas from glucose	– [d]	0	+	100	+		+		+	92
TSI acid from lactose	–	2	–	100	–		–		–	1
TSI acid from sucrose	–	0	–	0	–		–		–	1
TSI hydrogen sulfide produced	+	97	–	10	+		+		+	92
Urea hydrolysis	–	0	–	0	–		–		–	1
Lysine decarboxylation	+	98	–	0	+		+		+	95
β-Galactosidase reaction	–	0	–	0	–		–		–	2 [e]
Voges-Proskauer reaction	–	0	–	0	–		–		–	0
Production of indole	–	0	–	0	–		–		–	1

[a] From reference [5].

[b] These percentages indicate that not all isolates of *Salmonella* serotype show the reactions marked + or –. These percentages may vary between and within serotypes of food poisoning serotypes from different locations.

[c] The percentages are not known from available literature.

[d] *Salmonella* Typhi is anaerogenic.

[e] The *Salmonella enterica* subspecies *arizonæ* gives a positive or negative lactose reaction but is always β-galactosidase positive. For the study of these strains it may be useful to carry out complementary tests.

9.5.4.3 Examination for O-antigens

Using one non-autoagglutinating pure colony, proceed according to 9.5.4.2, using one drop of the anti-O serum (5.3) instead of the saline solution (5.2.13).

If agglutination occurs, the reaction is considered positive.

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

9.5.4.4 Examination for Vi-antigens

Proceed according to 9.5.4.2, but using one drop of the anti-Vi serum (5.3) instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

9.5.4.5 Examination for H-antigens

Inoculate the semi-solid nutrient agar (5.2.12) with a pure non-auto-agglutinable colony. Incubate the medium at 37 °C ± 1 °C for 24 h ± 3 h.

Use this culture for examination for the H-antigens, proceeding according to 9.5.4.2, but using one drop of the anti-H serum (5.3) instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

9.5.5 Interpretation of biochemical and serological reactions

Table 2 gives the interpretation of the confirmatory tests (9.5.3 and 9.5.4) carried out on the colonies used (9.5.2).

Table 2 — Interpretation of confirmatory tests

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 9.5.4.2)	
No typical reactions	No/Yes	O-, Vi- or H-antigen positive	
No typical reactions	No/Yes	All reactions negative	Not considered to be *Salmonella*

9.5.6 Definitive confirmation

Strains which are considered to be *Salmonella*, or which may be *Salmonella* (see Table 2), 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) and whether it is an outbreak or in food.

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 or x ml of product (see ISO 7218).

See annex C for the precision data obtained from the interlaboratory trial.

11 Test report

The test report shall specify:

— the sampling method used, if known;

— any deviation in the enrichment media or the incubation conditions used;

— all operating conditions not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the results;

— the results obtained.

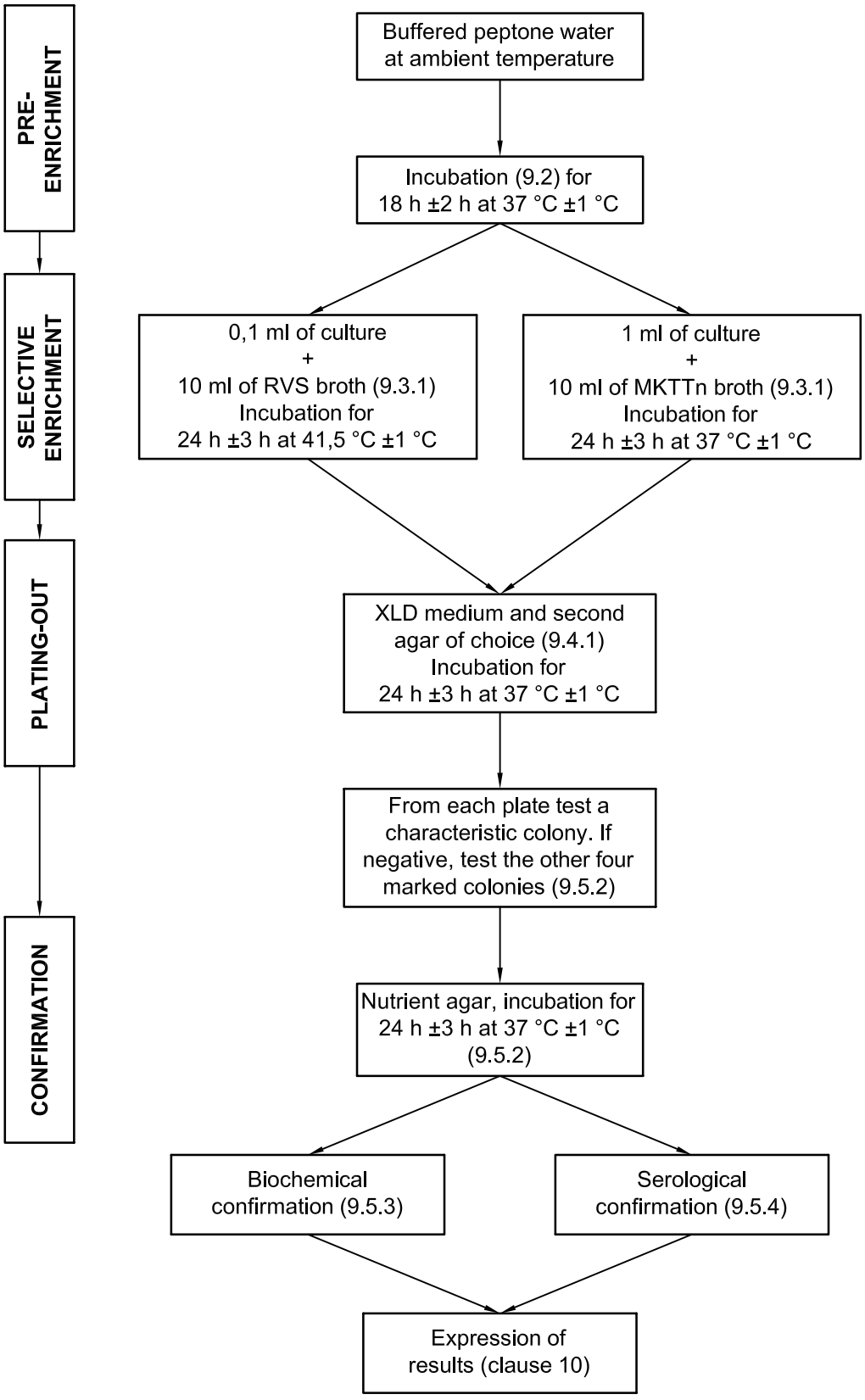
The test report shall also state whether a positive result was obtained only when using a plating-out medium (5.2.4) not specified in this International Standard.

12 Quality assurance

To check the ability of the laboratory to detect *Salmonella* with the methods and media described in this International Standard, introduce reference samples into control flasks of the pre-enrichment medium (see 5.2.1). Proceed with the control flasks as for the test cultures.

Annex A
(normative)

Diagram of procedure



Annex B
(normative)

Composition and preparation of culture media and reagents

B.1 Buffered peptone water

B.1.1 Composition

Enzymatic digest of casein	10,0 g
Sodium chloride	5,0 g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)	9,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,5 g
Water	1 000 ml

B.1.2 Preparation

Dissolve the components in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Dispense the medium into flasks (6.9) of suitable capacity to obtain the portions necessary for the test.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

B.2 Rappaport-Vassiliadis medium with soya (RVS broth)

B.2.1 Solution A

B.2.1.1 Composition

Enzymatic digest of soya	5,0 g
Sodium chloride	8,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,4 g
Dipotassium hydrogen phosphate (K_2HPO_4)	0,2 g
Water	1 000 ml

B.2.1.2 Preparation

Dissolve the components in the water by heating to about 70 °C if necessary.

The solution shall be prepared on the day of preparation of the complete RVS medium.

B.2.2 Solution B

B.2.2.1 Composition

Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	400,0 g
Water	1 000 ml

B.2.2.2 Preparation

Dissolve the magnesium chloride in the water.

As this salt is very hygroscopic, it is advisable to dissolve the entire contents of $MgCl_2 \cdot 6H_2O$ from a newly opened container, according to the formula. For instance, 250 g of $MgCl_2 \cdot 6H_2O$ is added to 625 ml of water, giving a solution of total volume of 788 ml and a mass concentration of about 31,7 g per 100 ml of $MgCl_2 \cdot 6H_2O$.

The solution may be kept in a dark glass bottle with tight stopper at room temperature for at least 2 years.

B.2.3 Solution C

B.2.3.1 Composition

Malachite green oxalate	0,4 g
Water	100 ml

B.2.3.2 Preparation

Dissolve the malachite green oxalate in the water.

The solution may be kept in a brown glass bottle at room temperature for at least 8 months.

B.2.4 Complete medium

B.2.4.1 Composition

Solution A (B.2.1)	1 000 ml
Solution B (B.2.2)	100 ml
Solution C (B.2.3)	10 ml

B.2.4.2 Preparation

Add to 1 000 ml of solution A, 100 ml of solution B and 10 ml of solution C.

Adjust the pH, if necessary, so that after sterilization it is 5,2 ± 0,2.

Before use, dispense into test tubes (6.9) in 10 ml quantities.

Sterilize for 15 min in the autoclave (6.1) set at 115 °C.

Store the prepared medium at 3°C ± 2°C. Use the medium the day of its preparation.

NOTE The final medium composition is: enzymatic digest of soya, 4,5 g/l; sodium chloride, 7,2 g/l; potassium dihydrogen phosphate ($KH_2PO_4 + K_2HPO_4$), 1,44 g/l; anhydrous magnesium chloride ($MgCl_2$), 13,4 g/l or magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), 28,6 g/l; malachite green oxalate, 0,036 g/l.

B.3 Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn) [7]

B.3.1 Base medium

B.3.1.1 Composition

Meat extract	4,3 g
Enzymatic digest of casein	8,6 g
Sodium chloride (NaCl)	2,6 g
Calcium carbonate ($CaCO_3$)	38,7 g
Sodium thiosulfate pentahydrate ($Na_2S_2O_3 \cdot 5H_2O$)	47,8 g
Ox bile for bacteriological use	4,78 g
Brilliant green	9,6 mg
Water	1 000 ml

B.3.1.2 Preparation

Dissolve the dehydrated basic components or the dehydrated complete medium in the water by boiling for 5 min.

Adjust the pH, if necessary, so that it is 8,2 $\pm$ 0,2 at 25 °C.

Thoroughly mix the medium.

The base medium may be stored for 4 weeks at 3 °C $\pm$ 2 °C.

B.3.2 Iodine-iodide solution

B.3.2.1 Composition

Iodine	20,0 g
Potassium iodide (KI)	25,0 g
Water	100 ml

B.3.2.2 Preparation

Completely dissolve the potassium iodide in 10 ml of water, then add the iodine and dilute to 100 ml with sterile water. Do not heat.

Store the prepared solution in the dark at ambient temperature in a tightly closed container.

B.3.3 Novobiocin solution

B.3.3.1 Composition

Novobiocin sodium salt	0,04 g
Water	5 ml

B.3.3.2 Preparation

Dissolve the novobiocin sodium salt in the water and sterilize by filtration.

Store for up to 4 weeks at 3 °C $\pm$ 2 °C.

B.3.4 Complete medium

B.3.4.1 Composition

Base medium (B.3.1)	1 000 ml
Iodine-iodide solution (B.3.2)	20 ml
Novobiocin solution (B.3.3)	5 ml

B.3.4.2 Preparation

Aseptically add 5 ml of the novobiocin solution (B.3.3) to 1 000 ml of base medium (B.3.1). Mix, then add 20 ml of the iodine-iodide solution (B.3.2). Mix well.

Dispense the medium aseptically into sterile flasks (6.9) of suitable capacity to obtain the portions necessary for the test.

The complete medium shall be used the day of its preparation.

B.4 Xylose lysine deoxycholate agar (XLD agar) [7]

B.4.1 Base medium

B.4.1.1 Composition

Yeast extract powder	3,0 g
Sodium chloride (NaCl)	5,0 g
Xylose	3,75 g
Lactose	7,5 g
Sucrose	7,5 g
L-Lysine hydrochloride	5,0 g
Sodium thiosulfate	6,8 g
Iron(III) ammonium citrate	0,8 g
Phenol red	0,08 g
Sodium deoxycholate	1,0 g
Agar	9 g to 18 g [1)]
Water	1 000 ml

B.4.1.2 Preparation

Dissolve the dehydrated base components or the dehydrated complete base in the water by heating, with frequent agitation, until the medium starts to boil. Avoid overheating.

Adjust the pH, if necessary, so that after sterilization it is 7,4 ± 0,2 at 25 °C.

Pour the base to tubes or flasks (6.9) of appropriate capacity.

Heat with frequent agitation until the medium boils and the agar dissolves. Do not overheat.

1) Depending on the gel strength of the agar.

B.4.2 Preparation of the agar plates

Transfer immediately to a water bath (6.5) at 44 °C to 47 °C, agitate and pour into plates. Allow to solidify.

Immediately before use, dry the agar plates carefully (preferably with the lids off and the agar surface downwards) in the oven (6.2) set between 37 °C and 55 °C until the surface of the agar is dry.

Store the poured plates for up to 5 days at 3 °C ± 2 °C.

B.5 Nutrient agar

B.5.1 Composition

Meat extract	3,0 g
Peptone	5,0 g
Agar	9 g to 18 g [1)]
Water	1 000 ml

B.5.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Transfer the culture medium into tubes or bottles (6.9) of appropriate capacity.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

B.5.3 Preparation of nutrient agar plates

Transfer about 15 ml of the melted medium to sterile small Petri dishes (6.11) and proceed as in B.4.2.

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

B.6.1 Composition

Meat extract	3,0 g
Yeast extract	3,0 g
Peptone	20,0 g
Sodium chloride (NaCl)	5,0 g
Lactose	10,0 g
Sucrose	10,0 g
Glucose	1,0 g
Iron(III) citrate	0,3 g
Sodium thiosulfate	0,3 g
Phenol red	0,024 g
Agar	9 g to 18 g [1)]
Water	1 000 ml

B.6.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,4 ± 0,2 at 25 °C.

Dispense the medium into test tubes or dishes in quantities of 10 ml.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

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

B.7 Urea agar (Christensen)

B.7.1 Base medium

B.7.1.1 Composition

Peptone	1,0 g
Glucose	1,0 g
Sodium chloride (NaCl)	5,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	2,0 g
Phenol red	0,012 g
Agar	9 g to 18 g [1)]
Water	1 000 ml

B.7.1.2 Preparation

Dissolve the components or the dehydrated complete base in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 6,8 ± 0,2 at 25 °C.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

B.7.2 Urea solution

B.7.2.1 Composition

Urea	400 g
Water, to a final volume of	1 000 ml

B.7.2.2 Preparation

Dissolve the urea in the water. Sterilize by filtration and check the sterility.

See ISO 7218:1996, 7.3.2.

B.7.3 Complete medium

B.7.3.1 Composition

Base (B.7.1)	950 ml
Urea solution (B.7.2)	50 ml

B.7.3.2 Preparation

Add, under aseptic conditions, the urea solution to the base, previously melted and then cooled to 44 °C to 47 °C.

Dispense the complete medium into sterile tubes (6.9) in quantities of 10 ml.

Allow to set in a sloping position.

B.8 L-Lysine decarboxylation medium

B.8.1 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

B.8.2 Preparation

Dissolve the components in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 6,8 ± 0,2 at 25 °C.

Transfer the medium in quantities of 2 ml to 5 ml to narrow culture tubes (6.9) with screw caps.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

B.9 β-Galactosidase reagent

B.9.1 Buffer solution

B.9.1.1 Composition

Sodium dihydrogen phosphate (NaH_2PO_4)	6,9 g
Sodium hydroxide, 10 mol/l solution	about 3 ml
Water, to a final volume of	50 ml

B.9.1.2 Preparation

Dissolve the sodium dihydrogen phosphate in approximately 45 ml of water in a volumetric flask.

Adjust the pH to 7,0 ± 0,2 at 25 °C with the sodium hydroxide solution.

Add water to a final volume of 50 ml.

B.9.2 ONPG solution

B.9.2.1 Composition

o-Nitrophenyl β-D-galactopyranoside (ONPG)	0,08 g
Water	15 ml

B.9.2.2 Preparation

Dissolve the ONPG in the water at approximately 50 °C.

Cool the solution.

B.9.3 Complete reagent

B.9.3.1 Composition

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

B.9.3.2 Preparation

Add the buffer solution to the ONPG solution.

B.10 Reagents for Voges-Proskauer (VP) reaction

B.10.1 VP medium

B.10.1.1 Composition

Peptone	7,0 g
Glucose	5,0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	5,0 g
Water	1 000 ml

B.10.1.2 Preparation

Dissolve the components in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 6,9 $\pm$ 0,2 at 25 °C.

Transfer the medium to tubes (6.9) in quantities of 3 ml.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

B.10.2 Creatine solution (*N*-amidinosarcosine)

B.10.2.1 Composition

Creatine monohydrate	0,5 g
Water	100 ml

B.10.2.2 Preparation

Dissolve the creatine monohydrate in the water.

B.10.3 1-Naphthol, ethanolic solution

B.10.3.1 Composition

1-Naphthol	6 g
Ethanol, 96 % (volume fraction)	100 ml

B.10.3.2 Preparation

Dissolve the 1-naphthol in the ethanol.

B.10.4 Potassium hydroxide solution

B.10.4.1 Composition

Potassium hydroxide	40 g
Water	100 ml

B.10.4.2 Preparation

Dissolve the potassium hydroxide in the water.

B.11 Reagents for indole reaction

B.11.1 Tryptone/tryptophan medium

B.11.1.1 Composition

Tryptone	10 g
Sodium chloride (NaCl)	5 g
DL-Tryptophan	1 g
Water	1 000 ml

B.11.1.2 Preparation

Dissolve the components in the boiling water.

Adjust the pH, if necessary, so that after sterilization it is 7,5 $\pm$ 0,2 at 25 °C.

Dispense 5 ml of the medium into each of several tubes (6.9).

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

B.11.2 Kovacs reagent

B.11.2.1 Composition

4-Dimethylaminobenzaldehyde	5 g
Hydrochloric acid, ρ = 1,18 g/ml to 1,19 g/ml	25 ml
2-Methylbutan-2-ol	75 ml

B.11.2.2 Preparation

Mix the components.

B.12 Semi-solid nutrient agar

B.12.1 Composition

Meat extract	3,0 g
Peptone	5,0 g
Agar	4 g to 9 g [1)]
Water	1 000 ml

B.12.2 Preparation

Dissolve the components in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Transfer the medium to flasks (6.9) of appropriate capacity.

Sterilize for 15 min in the autoclave (6.1) at 121 °C.

B.12.3 Preparation of agar plates

Pour into small sterile Petri dishes (6.11), about 15 ml of the freshly prepared medium. Do not allow the agar plates to dry.

B.13 Physiological saline solution

B.13.1 Composition

Sodium chloride (NaCl)	8,5 g
Water	1 000 ml

B.13.2 Preparation

Dissolve the sodium chloride in the water.

Adjust the pH, if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Dispense quantities of the solution into flasks or tubes (6.9) so that they will contain 90 ml to 100 ml after sterilization.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

Annex C
(informative)

Results of interlaboratory trial

An international collaborative test was organized in 2000 by AFSSA Ploufragan in Europe, and BioControl Systems in the USA, in the frame of the European project SMT CT 96 2098 [6]. This test involved 11 laboratories in 9 countries in Europe and 10 laboratories in the USA, and was carried out on fresh cheese curd, dried egg powder, raw poultry meat and a reference material. The food samples were each tested at two different levels of contamination, plus a negative control.

The values of the performance characteristics derived from this collaborative test are shown per type of sample in Tables C.1 to C.4. Data obtained by some laboratories have been excluded from the calculations only on the basis of clearly identified technical reasons (deviations to the protocol).

Table C.1 — Results of data analysis obtained with fresh cheese curd samples

	Fresh cheese curd (blank)	**Fresh cheese curd** (low level contamination)	**Fresh cheese curd** (high level contamination)
Number of laboratories having returned results	23	23	23
Number of samples per laboratory	5	5	5
Number of excluded laboratories	2	2	2
Number of laboratories retained after exclusion	21	21	21
Number of accepted samples	105	105	105
Accuracy (specificity), %	100	—	—
Accuracy (sensitivity), %	—	74,3	83,8
Accordance, %	100	83,8	95,2
Concordance, %	100	60,5	71,7

Table C.2 — Results of data analysis obtained with dried egg powder samples

	Dried egg powder (blank)	**Dried egg powder** (low level contamination)	**Dried egg powder** (high level contamination)
Number of laboratories having returned results	26	26	26
Number of samples per laboratory	5	5	5
Number of excluded laboratories	5	5	5
Number of laboratories retained after exclusion	21	21	21
Number of accepted samples	105	105	104
Accuracy (specificity), %	100	—	—
Accuracy (sensitivity), %	—	98,1	99
Accordance, %	100	96,2	98,1
Concordance, %	100	96,2	98,1

Table C.3 — Results of data analysis obtained with raw poultry meat samples

	Raw poultry meat (blank)	**Raw poultry meat** (low level contamination)	**Raw poultry meat** (high level contamination)
Number of laboratories having returned results	25	25	25
Number of samples per laboratory	5	5	5
Number of excluded laboratories	5	5	5
Number of laboratories retained after exclusion	20	20	20
Number of accepted samples	100	99	100
Accuracy (specificity), %	100	—	—
Accuracy (sensitivity), %	—	98	100
Accordance, %	100	96,9	100
Concordance, %	100	96	100

Table C.4 — Results of data analysis obtained with reference materials

	Reference material (capsules containing about 5 cfu of *S*. Typhimurium)
Number of laboratories having returned results	26
Number of samples per laboratory	5
Number of excluded laboratories	1
Number of laboratories retained after exclusion	25
Number of accepted samples	125
Accuracy (specificity), %	—
Accuracy (sensitivity), %	94,4
Accordance, %	88,8
Concordance, %	89,1

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