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

Microbiologie — Directives générales concernant les méthodes de recherche des Salmonella

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Contents

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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.

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.

International Standard ISO 6579 was prepared by Technical Committee ISO/TC 34, *Agricultural food products.*

This second edition cancels and replaces the first edition (ISO 6579 : 1981), of which it constitutes a technical revision.

Annexes A, B and C form an integral part of this International Standard. Annex D is for information only.

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 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.

1) For meat and meat products, see ISO 3565 : 1975, *Meat and meat products — Detection of* Salmonellæ *(Reference method)*. For milk and milk products, see ISO 6785 : 1985, *Milk and milk products — Detection of* Salmonella.

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

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*.

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

2 Definitions

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

2.1 *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.

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

3 Principle

In general, the detection of *Salmonella* necessitates four successive stages (see also annex A). [1)]

3.1 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 8.2) for 16 h to 20 h.

3.2 Enrichment in selective liquid media

Inoculation of a magnesium chloride malachite green medium and of a selenite cystine medium with the culture obtained in 3.1.

Incubation of the magnesium chloride malachite green medium at 42 °C, and incubation of the selenite cystine medium at the specified temperature (see 8.3.2) for 18 h to 24 h.

3.3 Plating out and recognition

From the cultures obtained in 3.2, 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 4.2.4.2).

Incubation at the specified temperature (see 8.4.5), 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*.

3.4 Confirmation

Subculturing of colonies of presumptive *Salmonella*, plated out as described in 3.3, and confirmation by means of appropriate biochemical and serological tests.

4 Culture media, reagents and sera

4.1 Basic materials

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

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

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

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.

Measurements of pH shall be carried out by means of the pH-meter (5.8), measurements being referred to a temperature of 25 °C.

If the prepared culture media and prepared reagents are not used immediately, they shall, unless otherwise specified, be kept in the dark at a temperature between 0 °C and 5 °C, but for no longer than 1 month, and in conditions that prevent any change in their composition.

4.2 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.

4.2.1 Non-selective pre-enrichment medium : buffered peptone water.

See clause B.1.

4.2.2 First selective enrichment medium : Rappaport-Vassiliadis magnesium chloride malachite green medium (RV medium).

See clause B.2.

4.2.3 Second selective enrichment medium : selenite cystine medium.

See clause B.3.

4.2.4 Selective solid plating-out media.

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

See clause B.4.

This first medium is compulsory unless otherwise stated (see 3.3).

4.2.4.2 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.

4.2.5 Nutrient agar.

See clause B.5.

4.2.6 Triple sugar/iron agar (TSI agar).

See clause B.6.

4.2.7 Urea agar (Christensen).

See clause B.7.

4.2.8 Lysine decarboxylation medium.

See clause B.8.

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

See clause B.9.

4.2.10 Reagents for Voges-Proskauer reaction.

See clause B.10.

4.2.10.1 VP medium.

4.2.10.2 Creatine solution (*N*-**amidinosarcosine**).

4.2.10.3 1-naphthol, ethanolic solution.

4.2.10.4 Potassium hydroxide solution.

4.2.11 Reagents for indole reaction.

See clause B.11.

4.2.11.1 Tryptone/tryptophan medium (by Ljutov).

4.2.11.2 Kovacs reagent.

4.2.12 Semi-solid nutrient agar.

See clause B.12.

4.2.13 Saline solution.

See clause B.13.

4.3 Sera

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

5 Apparatus and glassware

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

Usual microbiological laboratory equipment and, in particular, the following.

5.1 Apparatus for dry sterilization (oven) or **wet sterilization (autoclave),** (autoclave either operating separately or being part of a general apparatus for the preparation and distribution of media).

Sterilize apparatus that will come into contact with the diluent, the culture media or the sample, particularly plastic apparatus, except for apparatus that is supplied sterile, by one of the following methods:

a) in the oven by maintaining it at 170 °C to 175 °C for not less than 1 h;

b) in the autoclave by maintaining it at 121 °C ± 1 °C for not less than 20 min.

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

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

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

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

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

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

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

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

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

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

5.11 Measuring cylinders, for preparation of the complete media.

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

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

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.

7 Preparation of the test sample

See 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.

8 Procedure

8.1 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 4.2.1.

In general, to prepare the initial suspension, add a 25 g test portion to 225 ml of pre-enrichment medium (4.2.1), which is the ratio of test portion to pre-enrichment medium specified in this method. [3]

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).

NOTE — Dried, powdered, food products may need a special rehydration procedure to enhance the recovery of *Salmonella*. Two techniques may be used for this purpose, that of immersion and that of agitation.

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

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

3) 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 0,1 ml (RV medium) and 10 ml (selenite cystine medium) portions of the pre-enrichment broths from the 10 separate test portions (8.3.1) may be composited for enrichment in 0,1 litre and 1 litre respectively of selective enrichment medium.

Refer for this purpose to the International Standard appropriate to the product under examination. If such a standard is not available, it is recommended that the parties concerned come to an agreement on this subject.

8.2 Non-selective pre-enrichment

Incubate the initial suspension at the specified temperature, i.e. at 35 °C or 37 °C [1], for not less than 16 h and not more than 20 h.

8.3 Selective enrichment

8.3.1 Transfer 0,1 ml of the culture obtained in 8.2 to a tube containing 10 ml of the RV medium (4.2.2); transfer another 10 ml of the culture obtained in 8.2 to a flask containing 100 ml of selenite cystine medium (4.2.3) [2].

8.3.2 Incubate the two inoculated media (8.3.1) for 18 h to 24 h as follows :

a) the inoculated RV medium at 42 °C;

b) the inoculated selenite cystine medium at the specified temperature, i.e. at 35 °C or 37 °C [1] [3].

8.4 Plating out and identification

8.4.1 After incubation (see 8.3.2) for 18 h to 24 h, take, by means of a loop (5.7), a streak from the culture in the RV 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 4.2.4.1), 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 (4.2.4.2) 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. Use one loop (5.7) 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 in annex D.

8.4.2 Using the culture in the selenite cystine medium, repeat the procedure described in 8.4.1 with the two selective plating-out media.

8.4.3 Turn the dishes (8.4.1 and 8.4.2) so that the bottom is uppermost, and place them in an oven (5.3) controlled at the specified temperature, i.e. at 35 °C or 37 °C. [1]

8.4.4 After a total incubation period of 48 h (see 8.3.2 and 8.4.3), repeat the procedure described in 8.4.1 to 8.4.3 using the two inoculated enrichment media.

8.4.5 After incubation for 20 h to 24 h, examine the dishes (8.4.4) for the presence of typical colonies of *Salmonella*. Typical colonies of *Salmonella* grown on phenol red/brilliant green agar cause the colour of the medium to change from pink to red.

8.4.6 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 h to 24 h.

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

NOTE — Any typical or suspect colony should be subjected to a confirmation (8.5); 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.

8.5 Confirmation

8.5.1 Selection of colonies for confirmation

For confirmation, take from each plate of each selective medium (see 8.4.5 and 8.4.6), 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 (4.2.5), in a manner which will allow well-isolated colonies to develop.

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

Use pure cultures for biochemical and serological confirmation.

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

2) 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 0,1 ml (RV medium) and 10 ml (selenite cystine medium) portions of the pre-enrichment broths from the 10 separate test portions (8.3.1) may be composited for enrichment in 0,1 litre and 1 litre respectively of selective enrichment medium.

3) For the selenite cystine medium, it may, in some cases, be advantageous to raise the incubation temperature to 42 °C. This modification should be indicated in the test report.

8.5.2 Biochemical confirmation

By means of an inoculating wire, inoculate the media specified in 8.5.2.1 to 8.5.2.6 with each of the cultures obtained from the colonies selected in 8.5.1.

8.5.2.1 TSI agar (4.2.6)

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 sulfide
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 sulfide (blackening of the agar).

When a lactose-positive *Salmonella* is isolated (see 3.3), 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 8.5.3).

8.5.2.2 Urea agar (4.2.7)

Streak the agar slope surface.

Incubate for 24 h at 35 °C or 37 °C1) 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.

8.5.2.3 Lysine decarboxylation medium (4.2.8)

Inoculate just below the surface of the liquid medium.

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

A purple colour after incubation indicates a positive reaction.

A yellow colour indicates a negative reaction.

8.5.2.4 Reagent for detection of β-galactosidase (4.2.9)

Suspend a loopful of the suspected colony in a tube containing 0,25 ml of the saline solution (4.2.13).

Add 1 drop of toluene and shake the tube.

Put the tube in a water-bath controlled 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 controlled at 35 °C or 37 °C 1), leave for 24 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 (4.2.9) are used, follow the manufacturer's instructions.

8.5.2.5 Medium for Voges-Proskauer reaction (4.2.10)

Suspend a loopful of the suspected colony in a sterile tube containing 0,2 ml of the VP medium (4.2.10.1).

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

After incubation, add 2 drops of the creatine solution (4.2.10.2), 3 drops of the ethanolic 1-naphthol solution (4.2.10.3) and then 2 drops of the potassium hydroxide solution (4.2.10.4); shake after the addition of each reagent.

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

8.5.2.6 Medium for indole reaction (4.2.11)

Inoculate a tube containing 5 ml of the tryptone-tryptophan medium (4.2.11.1) with the suspected colony.

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

After incubation, add 1 ml of the Kovacs reagent (4.2.11.2).

The formation of a red ring indicates a positive reaction.

A yellow-brown ring indicates a negative reaction.

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

8.5.2.7 Interpretation of the biochemical tests

Salmonella generally show the following reactions[1] :

	Positive or negative reaction	Percentage of *Salmonella* inoculations showing the reaction[2]
TSI glucose (acid formation) (8.5.2.1)	+	100
TSI glucose (gas formation) (8.5.2.1)	+	91,9[3]
TSI lactose (8.5.2.1)	−	99,2[4]
TSI sucrose (8.5.2.1)	−	99,5
TSI hydrogen sulfide (8.5.2.1)	+	91,6
Urea splitting (8.5.2.2)	−	100
Lysine decarboxylation (8.5.2.3)	+	94,6
β-galactosidase reaction (8.5.2.4)	−	98,5[4]
Voges-Proskauer reaction (8.5.2.5)	−	100
Indole reaction (8.5.2.6)	−	98,9

8.5.3 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 (8.5.1) and after auto-agglutinable strains have been eliminated.

8.5.3.1 Elimination of auto-agglutinable strains

Place 1 drop of the saline solution (4.2.13) on a carefully cleaned glass slide.

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 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 impossible.

8.5.3.2 Examination for O-antigens

Using one pure colony recognized as non-auto-agglutinable, proceed according to 8.5.3.1, using 1 drop of the anti-O serum (4.3) instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

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

8.5.3.3 Examination for Vi-antigens

Proceed according to 8.5.3.1, but using 1 drop of the anti-Vi serum (4.3) instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

8.5.3.4 Examination for H-antigens

Inoculate the semi-solid nutrient agar (4.2.12) with a pure non-auto-agglutinable colony.

Incubate the medium for 18 h to 24 h at 35 °C or 37 °C[5].

Use this culture for examination for the H-antigens, proceeding according to 8.5.3.1, but using 1 drop of the anti-H serum (4.3) instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

8.5.4 Interpretation of biochemical and serological reactions

Table 1 gives the interpretation of the confirmatory tests (8.5.2 and 8.5.3) carried out on the colonies used (8.5.1).

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

2) 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.

3) *Salmonella typhi* is anaerogenic.

4) 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.

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

Table 1

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 8.5.3.1)	
No typical reactions	No	O-, Vi- or H-antigen positive	
No typical reactions	No	All reactions negative	Not considered to be *Salmonella*
NOTE — Identification kits currently available commercially and permitting the identification of *Salmonella* may be used.			

8.5.5 Definitive confirmation

Strains which are considered to be *Salmonella*, or which may be *Salmonella*, (see table 1) 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).

9 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.

10 Test report

The test report shall specify the method used and the results obtained. It shall also mention 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.

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 42 °C.

The test report shall also state whether a positive result was obtained only when using a plating-out medium (4.2.4) not specified in this International Standard.

The test report shall include all information necessary for the complete identification of the sample.

11 Quality assurance

To check the ability to detect *Salmonella* with the methods and media described in this International Standard, reference samples should be introduced into control flasks of the pre-enrichment medium (see 4.2.1). Proceed with the control flasks as for the test cultures.