INTERNATIONAL STANDARD

ISO 6785

IDF 93

Second edition
2001-05-15

Milk and milk products — Detection of *Salmonella* spp.

Lait et produits laitiers — Recherche de Salmonella *spp.*

Reference numbers
ISO 6785:2001(E)
IDF 93:2001(E)

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Foreword

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

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 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.

International Standard ISO 6785 | IDF 93 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

This second edition cancels and replaces the first edition (ISO 6785:1985), which has been technically revised.

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

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Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO and AOAC International in the development of standard methods of analysis and sampling for milk and milk products.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of National Committees casting a vote.

International Standard ISO 6785|IDF 93 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

All work was carried out by the Joint ISO/IDF/AOAC Action Team on *Harmonization*, of the Standing Committee on *Microbial methods of analysis*, under the aegis of its project leader, Mr. H. Becker (DE).

This fourth edition cancels and replaces the third edition (IDF 93:1995).

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Milk and milk products — Detection of *Salmonella* spp.

1 Scope

This International Standard specifies a method for the detection of *Salmonella* spp. in milk and milk products.

2 Normative reference

The following normative document contains 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 edition of the normative document 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 8261 | IDF 122, *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 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*
detection of the presence or absence of these microorganisms, in a particular mass or volume, 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 annex A).

4.2 Pre-enrichment in non-selective liquid medium

Inoculation of the pre-enrichment medium with the test portion, and incubation at 37 °C for 16 h to 20 h.

4.3 Enrichment in selective liquid media

Inoculation of Rappaport-Vassiliadis modified magnesium chloride/malachite green medium and of selenite/cystine medium with the culture obtained in 4.2.

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Incubation of the Rappaport-Vassiliadis modified magnesium chloride/malachite green medium in the water bath or incubator (6.4) set at 41,5 °C for 24 h and then a further 24 h.

Incubation of the selenite/cystine medium in the incubator (6.3) set at 37 °C for 24 h and then a further 24 h.

4.4 Streaking out and recognition

From the cultures obtained (4.3), inoculation of two selective solid media (brilliant green/phenol red agar and any other suitable solid selective medium).

NOTE Suitable media allow the recovery of lactose-fermenting *Salmonella* strains.

Incubation of the brilliant green/phenol red agar in the incubator (6.3) set at 37 °C and examination after 20 h to 24 h and, if necessary, again after 40 h to 48 h to check the presence of colonies which, from their characteristics, are considered to be presumptive *Salmonella*.

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

4.5 Confirmation

Subculturing of colonies of presumptive *Salmonella* (4.4) and confirmation by means of appropriate biochemical and serological tests.

5 Culture media, reagents and sera

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

Use only reagents of recognized analytical grade, unless otherwise specified.

The pH values given refer to a temperature of 25 °C. Adjustments, if necessary, are made by adding either hydrochloric acid [$c\,(\mathrm{HCl}) = 1$ mol/l] or sodium hydroxide solution [$c\,(\mathrm{NaOH}) = 1$ mol/l].

If not used immediately, store the prepared culture media and reagents under conditions that do not produce any change in their composition, in the dark at a temperature between 0 °C and + 5 °C, for no longer than 1 month, unless otherwise stated.

5.1 Water

Use distilled or demineralized water or water of equivalent purity. The water shall be free from substances that might inhibit the growth of microorganisms under the test conditions specified in this International Standard.

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5.2 Culture media

5.2.1 Pre-enrichment medium: Buffered peptone water

5.2.1.1 Composition

Component	Amount
Peptone	10,0 g
Sodium chloride (NaCl)	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

5.2.1.2 Preparation

Dissolve the components in the water by heating. Adjust the pH so that after sterilization it is 7,0 ± 0,1.

Transfer the medium in quantities of 225 ml into flasks (6.9) of capacity 500 ml (or multiples of 225 ml into flasks of suitable capacity). Sterilize in the autoclave (6.1) set at 121 °C for 15 min. Cool to room temperature.

5.2.2 First selective enrichment medium: Rappaport-Vassiliadis modified magnesium chloride/malachite green medium (RVS broth)

5.2.2.1 Solution A

5.2.2.1.1 Composition

Component	Amount
Enzymatic digest of soya	5,0 g
Sodium chloride (NaCl)	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

5.2.2.1.2 Preparation

Dissolve the components in the water by heating to about 70 °C. Prepare solution A on the day of preparation of the complete RVS medium.

5.2.2.2 Solution B

5.2.2.2.1 Composition

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

5.2.2.2.2 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. For instance, 250 g of $MgCl_2 \cdot 6H_2O$ is added to 625 ml of water, giving a solution of total volume of 795 ml and a concentration of about 0,3 g/ml of $MgCl_2 \cdot 6H_2O$.

Solution B can be stored in an airtight brown glass bottle at room temperature for at least 2 years.

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5.2.2.3 Solution C

5.2.2.3.1 Composition

Malachite green oxalate	0,4 g
Water	100 ml

5.2.2.3.2 Preparation

Dissolve the malachite green oxalate in the water.

Solution C can be stored in a brown glass bottle at room temperature for at least 8 months.

5.2.2.4 Complete medium

5.2.2.4.1 Composition

Solution A (5.2.2.1)	1 000 ml
Solution B (5.2.2.2)	100 ml
Solution C (5.2.2.3)	10 ml

5.2.2.4.2 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 ± 0,1. Dispense 10 ml quantities of the thus-obtained solution into test tubes (6.9) or into sterile flasks (6.8) of suitable capacity to obtain the portions necessary for the test. Sterilize in the autoclave (6.1) set at 115 °C for 15 min.

Store the prepared medium in the refrigerator at 3 °C ± 2 °C.

5.2.3 Second selective enrichment medium: Selenite/cystine medium

WARNING — Extreme care should be taken with the laboratory use of selenite solutions because of their potentially toxic effect. Do not pipette by mouth under any circumstances.

5.2.3.1 Base

5.2.3.1.1 Composition

Tryptone	5,0 g
Lactose	4,0 g
Disodium hydrogen phosphate dodecahydrate	
($Na_2HPO_4 \cdot 12H_2O$)	10,0 g
Sodium hydrogen selenite	4,0 g
Water	1 000 ml

5.2.3.1.2 Preparation

Dissolve the first three basic components in the water by boiling for 5 min. After cooling, add the sodium hydrogen selenite. Adjust the pH, if necessary, to 7,0 ± 0,1. Do not sterilize.

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5.2.3.2 L-Cystine solution

5.2.3.2.1 Composition

L-Cystine	0,1 g
Sodium hydroxide solution, $c\,(\text{NaOH}) = 1$ mol/l	15 ml
Sterile water	approx. 85 ml

5.2.3.2.2 Preparation

Add the components to a sterile 100 ml one-mark volumetric flask. Dilute to the mark with sterile water. Do not sterilize.

5.2.3.3 Complete medium

5.2.3.3.1 Composition

Base (5.2.3.1)	1 000 ml
L-Cystine solution (5.2.3.2)	10 ml

5.2.3.3.2 Preparation

Add the L-cystine solution aseptically to the base. Adjust the pH, if necessary, to 7,0 ± 0,1. Dispense the medium aseptically into sterile flasks of suitable capacity to obtain the portions necessary for the test.

The medium may be used until a red precipitate occurs.

5.2.4 First selective solid medium: Brilliant green/phenol red agar (Edel and Kampelmacher)

5.2.4.1 Base

5.2.4.1.1 Composition

Meat extract powder	5,0 g
Peptone	10,0 g
Yeast extract powder	3,0 g
Disodium hydrogen phosphate (Na_2HPO_4)	1,0 g
Sodium dihydrogen phosphate (NaH_2PO_4)	0,6 g
Agar	12 g to 18 g [a]
Water	900 ml

[a] Depending on the gel strength of the agar.

5.2.4.1.2 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 7,0 ± 0,1. Transfer the base to tubes (6.9) or flasks (6.8) of appropriate capacity. Sterilize in the autoclave (6.1) set at 121 °C for 15 min.

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5.2.4.2 Sugar/phenol red solution

5.2.4.2.1 Composition

Lactose	10,0 g
Sucrose	10,0 g
Phenol red	0,09 g
Water	approx. 80 ml

5.2.4.2.2 Preparation

Dissolve the components in approximately 50 ml of water in a 100 ml one-mark volumetric flask. Dilute to the mark with the water. Heat the solution in a water bath (6.5) set at 70 °C for 20 min. Cool in another water bath (6.5) set at 55 °C. Use the solution immediately after cooling.

5.2.4.3 Brilliant green solution

5.2.4.3.1 Composition

Brilliant green (see specification in annex B)	about 0,5 g
Water	100 ml

5.2.4.3.2 Preparation

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

5.2.4.4 Complete medium

5.2.4.4.1 Composition

Base (5.2.4.1)	900 ml
Sugar/phenol red solution (5.2.4.2)	100 ml
Brilliant green solution (5.2.4.3)	1 ml

5.2.4.4.2 Preparation

Add the brilliant green solution (5.2.4.3) aseptically to the sugar/phenol red solution (5.2.4.2) cooled in a water bath (6.5) to 55 °C. Add this to the base, preheated in the water bath to 55 °C, and mix. The temperature of the water bath should be kept between 50 °C and 55 °C while mixing.

5.2.4.4.3 Preparation of the agar plates

Place in each of an appropriate number of large Petri dishes (6.12) about 40 ml of the freshly prepared complete medium (5.2.4.4). If large dishes are not available, place about 15 ml of the medium in small Petri dishes (6.12). Allow to solidify.

If prepared in advance, store the agar plates for no longer than 4 h at room temperature or no longer than 1 week between 0 °C and + 5 °C.

Immediately before use, dry the agar plates carefully (preferably with the lids off and the agar surface downwards) in the oven (6.2) set at 50 °C or in the laminar airflow cabinet (6.2) until the surface of the agar is dry.

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5.2.5 Second selective solid medium

The choice of the second medium is left to the discretion of the testing laboratory.

5.2.6 Nutrient agar

5.2.6.1 Composition

Meat extract	3,0 g
Peptone	5,0 g
Agar	12 g to 18 g [b]
Water	1 000 ml

[b] Depending on the gel strength of the agar.

5.2.6.2 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 7,0 ± 0,1. Transfer the culture medium into tubes (6.9) or bottles (6.8) of appropriate capacity. Sterilize in the autoclave (6.1) set at 121 °C for 15 min.

5.2.6.3 Preparation of agar plates

Transfer about 15 ml of the melted medium to sterile small Petri dishes (6.12) and proceed as in 5.2.4.4.3.

5.2.7 Triple sugar/iron agar (TSI agar)

5.2.7.1 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 thiosulfate	0,3 g
Phenol red	0,024 g
Agar	12 g to 18 g [c]
Water	1 000 ml

[c] Depending on the gel strength of the agar.

5.2.7.2 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 ± 0,1. Dispense the medium in quantities of 10 ml into test tubes (6.9) of diameter 17 mm to 18 mm. Sterilize in the autoclave (6.1) set at 121 °C for 15 min. Allow to set in a sloping position to give a butt of depth 2,5 cm and a slant of 4 cm to 5 cm.

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5.2.8 Urea agar (Christensen)

5.2.8.1 Base

5.2.8.1.1 Composition

Peptone	1,0 g
Glucose	1,0 g
Sodium chloride	5,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	2,0 g
Phenol red	0,012 g
Agar	12 g to 18 g [d]
Water	1 000 ml
[d] Depending on the gel strength of the agar.	

5.2.8.1.2 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 ± 0,1. Sterilize in the autoclave (6.1) set at 121 °C for 15 min.

5.2.8.2 Urea solution

5.2.8.2.1 Composition

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

5.2.8.2.2 Preparation

Dissolve the urea in the water. Sterilize by filtration and check the sterility. (For details of the technique of sterilization by filtration, refer to any appropriate textbook on microbiology.)

5.2.8.3 Complete medium

5.2.8.3.1 Composition

Base (5.2.8.1)	950 ml
Urea solution (5.2.8.2)	50 ml

5.2.8.3.2 Preparation

Add the urea solution aseptically to the base, previously melted and then cooled in the water bath (6.5) to 45 °C. Dispense the complete medium in quantities of 10 ml into sterile tubes (6.9). Allow to set in a sloping position.

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5.2.9 L-Lystine decarboxylation medium

5.2.9.1 Composition

L-Lystine monohydrochloride	5,0 g
Yeast extract	3,0 g
Glucose	1,0 g
Bromocresol purple	0,015 g
Water	1 000 ml

5.2.9.2 Preparation

Dissolve the components in the water, by heating if necessary. Adjust the pH, if necessary, so that after sterilization it is 6,8 ± 0,1. Transfer the medium in quantities of 5 ml into narrow culture tubes (6.9). Sterilize in the autoclave (6.1) set at 121 °C for 15 min.

5.3 Reagents

5.3.1 Saline solution

5.3.1.1 Composition

Sodium chloride	8,5 g
Water	1 000 ml

5.3.1.2 Preparation

Dissolve the sodium chloride in the water, by heating if necessary. Adjust the pH so that after sterilization it is 7,0 ± 0,1. Transfer quantities of the solution to flasks (6.8) or tubes (6.9) so that they will contain 90 ml to 100 ml after sterilization. Sterilize in the autoclave (6.1) set at 121 °C for 15 min.

5.3.2 Reagents for β-galactosidase reaction

5.3.2.1 Toluene

5.3.2.2 Buffer solution

5.3.2.2.1 Composition

Sodium dihydrogen phosphate (NaH_2PO_4)	6,9 g
Sodium hydroxide, 10 mol/l solution	approx. 3 ml [e]
Water	approx. 50 ml

[e] Depending on the volume necessary to adjust the pH.

5.3.2.2.2 Preparation

Dissolve the sodium dihydrogen phosphate in approximately 45 ml of water in a 50 ml one-mark volumetric flask. Adjust the pH to 7,0 ± 0,1 with the sodium hydroxide solution. Dilute to the mark with water.

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5.3.2.3 ONPG solution

5.3.2.3.1 Composition

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

5.3.2.3.2 Preparation

Dissolve the ONPG in the water preheated to 50 °C ± 1 °C. Cool the solution to room temperature.

5.3.2.4 Complete reagent

5.3.2.4.1 Composition

Buffer solution (5.3.2.2)	5 ml
ONPG solution (5.3.2.3)	15 ml

5.3.2.4.2 Preparation

Add the buffer solution to the ONPG solution.

5.3.3 Reagents for Voges-Proskauer (VP) reaction

5.3.3.1 VP medium

5.3.3.1.1 Composition

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

5.3.3.1.2 Preparation

Dissolve the components in the water, by heating if necessary. Adjust the pH, if necessary, so that after sterilization it is 6,9 ± 0,1. Transfer 3 ml of the thus-obtained VP medium into each of several tubes (6.9). Sterilize in the autoclave (6.1) set at 115 °C for 20 min.

5.3.3.2 Creatine solution (*N*-amidinosarcosine)

5.3.3.2.1 Composition

Creatine monohydrate	0,5 g
Water	100 ml

5.3.3.2.2 Preparation

Dissolve the creatine monohydrate in the water.

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5.3.3.3 1-Naphthol, ethanolic solution

5.3.3.3.1 Composition

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

5.3.3.3.2 Preparation

Dissolve the 1-naphthol in the ethanol.

5.3.3.4 Potassium hydroxide solution

5.3.3.4.1 Composition

Potassium hydroxide	40 g
Water	100 ml

5.3.3.4.2 Preparation

Dissolve the potassium hydroxide in the water.

5.3.4 Reagents for indole reaction

5.3.4.1 Tryptone/tryptophan medium

5.3.4.1.1 Composition

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

5.3.4.1.2 Preparation

Dissolve the components in the water at 100 °C in the boiling water bath (6.5). Adjust the pH, if necessary, so that after sterilization it is 7,5 ± 0,1. Dispense 5 ml of the thus-obtained medium into each of several tubes (6.9). Sterilize in the autoclave (6.1) set at 121 °C for 15 min.

5.3.4.2 Kovac's reagent

5.3.4.2.1 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

5.3.4.2.2 Preparation

Mix the above-mentioned components.

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5.3.5 Semi-solid nutrient agar

5.3.5.1 Composition

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

[f] Depending on the gel strength of the agar.

5.3.5.2 Preparation

Dissolve the components in the water, by heating if necessary. Adjust the pH, if necessary, so that after sterilization it is 7,0 ± 0,1. Transfer the medium to flasks (6.8) of appropriate capacity. Sterilize in the autoclave (6.1) set at 121 °C for 15 min.

5.3.5.3 Preparation of agar plates

Place in small sterile Petri dishes (6.12) about 15 ml of the freshly prepared medium. Do not dry the agar plates.

5.4 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* serovars. Assistance towards this objective may be obtained by using 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 and, in particular, the following.

6.1 Apparatus for wet sterilization (autoclave), capable of operating at 121 °C ± 1 °C and 115 °C ± 1 °C.

6.2 Oven, ventilated by convection, capable of operating at 50 °C ± 5 °C, or laminar airflow cabinet.

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

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

6.5 Water baths, capable of operating at 37 °C ± 1 °C, 45 °C ± 1 °C, 55 °C ± 1 °C, 70 °C ± 1 °C, respectively, and boiling.

6.6 Loops, made of platinum/iridium or nickel/chromium, of diameter approximately 3 mm.

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

6.8 Culture bottles or **flasks**, with non-toxic metallic or plastic screw-caps.

6.9 Culture tubes, of diameter 8 mm and of length 160 mm, or other appropriate sizes.

6.10 Measuring cylinders.

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6.11 Graduated pipettes or **automated pipettors**, of nominal capacities 10 ml and 1 ml, graduated in 0,5 ml and 0,1 ml divisions, respectively.

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

7 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707.

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

8 Preparation of test sample

Prepare the test sample in accordance with ISO 8261/IDF 122.

9 Procedure

9.1 Safety precautions

See clause 12.

9.2 Test portion and pre-enrichment

9.2.1 General

To prepare the primary dilution, add 25 g of the test sample (clause 8) to 225 ml of pre-enrichment medium (5.2.1), which is the ratio of test sample to pre-enrichment medium specified in this method. 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).

9.2.2 Raw milk, heat-treated milk and liquid milk products

Pipette 25 ml of the test sample (clause 8) into a flask (6.8) containing 225 ml of the pre-enrichment medium (5.2.1) and mix.

9.2.3 Dried milk products

Prepare a stoppered flask (6.8) with 225 ml of the pre-enrichment medium (5.2.1).

Weigh 25 g of the test sample (clause 8) aseptically and pour it over the surface of the liquid in the flask. Stopper the flask, but do not shake. Allow to stand undisturbed at room temperature for 60 min $\pm$ 10 min before incubation. Adjustment of the pH is not necessary. If after 1 h of incubation the dried milk is still not dissolved, mix the contents of the flask by shaking manually or stirring with a sterile spatula.

9.2.4 Lactose

Weigh 25 g of the test sample (clause 8) aseptically into a stoppered flask (6.8) containing 225 ml of the pre-enrichment medium (5.2.1) and shake to dissolve.

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9.2.5 Casein, caseinates, cheese

Weigh 25 g of the test sample (clause 8) aseptically into the sterile container of a high-speed or peristaltic-type blender. Add 225 ml of the pre-enrichment medium (5.2.1) preheated to 45 °C. Blend until the test sample is thoroughly dispersed (1 min to 3 min). Ensure that the temperature of the dispersion does not exceed 45 °C.

9.2.6 Butter

Shake the melted test sample (clause 8). Transfer with a pipette, preheated to approximately 45 °C, 25 ml of the test sample to a flask (6.8) containing 225 ml of the pre-enrichment medium (5.2.1) and mix.

9.2.7 Frozen milk products (including edible ices)

Pipette 25 ml of the melted test sample (clause 8), preheated to no more than 37 °C, into a flask (6.8) containing 225 ml of the pre-enrichment medium (5.2.1) and mix.

9.2.8 Fermented milks, yoghurt, custards, desserts

Weigh 25 g of the test sample (clause 8) aseptically into a stoppered flask (6.8) containing glass beads and 225 ml of the pre-enrichment medium (5.2.1) and shake to disperse.

To reduce the examination workload when more than one 25 g test sample from a specified lot of milk or milk product has to be examined, and when evidence is available that compositing (pooling the test portions) does not affect the result for the milk or milk product, the test portions may be composited. For example, if 10 test samples of 25 g are to be examined, combine the 10 samples to form a composite test sample of 250 g and dissolve or disperse in 2,25 l of pre-enrichment medium.

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

Unless otherwise stated, check the pH of the suspension and adjust, if necessary, to 6,8 ± 0,1.

9.3 Enrichment

9.3.1 Non-selective pre-enrichment

Incubate the flasks prepared according to 9.2.2 to 9.2.8 in the incubator (6.3) set at 37 °C for 16 h to 20 h.

9.3.2 Selective enrichment

9.3.2.1 Transfer 0,1 ml of the culture obtained in 9.3.1 to a culture tube (6.9) containing 10 ml of the RVS medium (5.2.2). Transfer 10 ml of the culture obtained in 9.3.1 to a flask (6.8) containing 100 ml of selenite/cystine medium (5.2.3).

9.3.2.2 Incubate the inoculated RVS medium (9.3.2.1) in the water bath or incubator (6.4) set at 41,5 °C for 18 h to 24 h. Incubate the inoculated selenite/cystine medium (9.3.2.1) in the incubator (6.3) set at 37 °C for 18 h to 24 h.

9.4 Streaking out and recognition

9.4.1 Using the culture obtained in the RVS medium (9.3.2.2) (after incubation for 18 h to 24 h) inoculate, by means of a loop (6.6), the surface of one large Petri dish (6.12) containing the brilliant green/phenol red agar (5.2.4) 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.

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The following method of streaking is recommended when brilliant green/phenol red agar is used. Use one loop (6.6) for two dishes. Take a droplet from the edge of the surface of the fluid. Inoculate both dishes according to Figure C.1 [a) and b)]. 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 in Figure C.1 a).

Proceed in the same way with the second selective solid medium (5.2.5) using a new loop and Petri dishes of appropriate size.

9.4.2 Using the culture obtained in the selenite/cystine medium (9.3.2.2) after incubation for 18 h to 24 h, repeat the procedure described in 9.4.1 with the two selective solid media.

9.4.3 Incubate the plates (bottom uppermost) in the incubator (6.3) set at 37 °C for 20 h to 24 h.

9.4.4 After incubating the RVS medium and the selenite/cystine medium for a further 18 h to 24 h, repeat the streaking and incubation procedure described in 9.4.1 to 9.4.3.

9.4.5 After each incubation (9.4.3 and 9.4.4) examine the plates for the presence of typical colonies of *Salmonella*. If growth is slight, and no typical colonies of *Salmonella* are present, re-incubate the plates in the incubator (6.3) set at 37 °C for a further 18 h to 24 h and re-examine the plates for the presence of typical colonies of *Salmonella*.

9.4.6 On brilliant green/phenol red agar (5.2.4), typical colonies of *Salmonella* are pink with bright red surrounding medium.

NOTE Since the recognition of colonies of *Salmonella* is to a large extent a matter of experience, and since their appearance on identification media may vary from serovar to serovar or between batches of media, suspect colonies, as well as typical colonies, should be selected for confirmation.

9.5 Confirmation

9.5.1 Selection of colonies for confirmation

From each plate of each selective solid medium (9.4.1), select five typical or suspect colonies or, if there are fewer than five such colonies, select all for confirmation.

9.5.2 Incubation

Streak the selected colonies onto the surface of nutrient agar plates (5.2.6) in a manner which will allow well-isolated colonies to develop. Incubate the plates in the incubator (6.3) set at 37 °C for 18 h to 24 h.

After incubation, select pure, well-isolated colonies for biochemical and serological confirmation.

9.5.3 Biochemical confirmation

9.5.3.1 General

Inoculate the media specified in 9.5.3.2 to 9.5.3.7 with pure colonies (9.5.2) by means of an inoculating wire.

9.5.3.2 Triple sugar/iron agar (5.2.7)

Streak the agar slope surface and stab the butt. Incubate in the incubator (6.3) set at 37 °C for 24 h. Interpret the changes in the medium as follows:

a) Butt

yellow: glucose positive (fermentation of glucose)

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red or unchanged:	glucose negative (no fermentation of glucose)
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 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, the TSI slant is yellow. Thus, preliminary confirmation of *Salmonella* cultures shall not be based on the results of the TSI agar test only.

9.5.3.3 Urea agar (5.2.8)

Streak the agar slope surface. Incubate in the incubator (6.3) set at 37 °C for 24 h. 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 (5.2.9)

Inoculate just below the surface of the liquid medium. Incubate in the incubator (6.3) set at 37 °C for 24 h. A purple colour, after growth has occurred, indicates a positive reaction. A yellow colour indicates a negative reaction.

9.5.3.5 β-Galactosidase reaction (5.3.2)

Suspend a loopful of the suspected colony in a tube (6.9) containing 0,25 ml of the saline solution (5.3.1). Add 1 drop of toluene (5.3.2.1) and shake the tube. Put the tube in a water bath (6.5) set at 37 °C and leave for several minutes. Add 0,25 ml of the β-galactosidase reagent and mix. Replace the tube in the water bath at 37 °C and leave for 24 h.

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

9.5.3.6 Voges-Proskauer reaction (5.3.3)

Suspend a loopful of the suspected colony in two tubes (6.9) each containing 0,2 ml of the VP medium (5.3.3.1). Incubate one tube at room temperature and the other in the incubator (6.3) set at 37 °C for 24 h. After incubation, add in the following order only to each tube 2 drops of the creatine solution (5.3.3.2), 3 drops of the 1-naphthol ethanolic solution (5.3.3.3) and then 2 drops of the potassium hydroxide solution (5.3.3.4); shake after the addition of each reagent. The formation of a pink to bright red colour within 15 min indicates a positive reaction.

The above-mentioned order should be followed strictly. If, for instant, potassium hydroxide is added before 1-naphthol ethanolic solution and peptone is present, the result might be a solution with a pinkish colour which can mask a possible positive reaction.

9.5.3.7 Indole reaction (5.3.4)

Inoculate a tube containing 5 ml of the tryptone/tryptophan medium (5.3.4.1) with the suspected colony. Incubate in the incubator (6.3) set a 37 °C for 24 h. After incubation, add 1 ml of Kovac's reagent (5.3.4.2).

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

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9.5.3.8 Interpretation of the biochemical tests

Interpret the results according to Table 1.

Table 1 — Interpretation of results

Confirmatory test	Positive or negative reaction	Percent of *Salmonella* strains showing the reaction
TSI glucose (acid formation) (9.5.3.2)	+	100
TSI glucose (gas formation) (9.5.3.2)	+	91,9
TSI lactose (9.5.3.2)	–	99,2 [a]
TSI sucrose (9.5.3.2)	–	99,5
TSI hydrogen sulfide (9.5.3.2)	+	91,6
Urea splitting (9.5.3.3)	–	100
L-Lysine decarboxylation (9.5.3.4)	+	94,6
β-Galactosidase reaction (9.5.3.5)	–	98,5
Voges-Proskauer reaction (9.5.3.6)	–	100
Indole reaction (9.5.3.7)	–	98,9

[a] The *Salmonella enterica* subsp. *arizonae* and *diarizonae* 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.

9.5.4 Commercial diagnostic systems

Identification kits currently available commercially and permitting the identification of *Salmonella* may be used.

9.5.5 Serological confirmation

9.5.5.1 General

The detection of the presence of *Salmonella* O-, Vi- and H-antigens is carried out by slide agglutination with the appropriate sera on pure colonies (see 9.5.2) after auto-agglutinable strains have been eliminated (9.5.5.2).

9.5.5.2 Elimination of auto-agglutinable strains

Place one drop of the saline solution (5.3.1) on a carefully cleaned glass slide. Disperse in this drop part of the colony (9.5.2) 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 strains are considered auto-agglutinable. The serological confirmation of these auto-agglutinable strains by the procedures specified in 9.5.5.3, 9.5.5.4 and 9.5.5.5 is impossible.

9.5.5.3 Examination for O-antigens

Use pure non-auto-agglutinable (see 9.5.5.2) strains. Proceed according to 9.5.5.2, using one drop of anti-O serum (5.4) instead of the saline solution (5.3.1). Use the monovalent or polyvalent sera one after the other.

9.5.5.4 Examination for Vi-antigens

Proceed according to 9.5.5.3, using one drop of anti-Vi serum (5.4) instead of the saline solution (5.3.1).

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9.5.5.5 Examination for H-antigens

Inoculate the semi-solid nutrient agar (5.3.5) with a pure non-auto-agglutinable colony (9.5.5.2). Incubate the medium in the incubator (6.3) set at 37 °C for 18 h to 24 h. Use this culture for examination of the H-antigens, proceeding according to 9.5.5.3, but using one drop of anti-H serum (5.4) instead of the saline solution (5.3.1).

9.5.5.6 Interpretation of serological reactions

If agglutination occurs, the reactions are considered positive.

9.5.6 Interpretation of biochemical and serological reactions

Table 2 gives the interpretation of the confirmatory tests (9.5.3 and 9.5.5) carried out on the colonies used (9.5.2).

Table 2 — Interpretation of the confirmatory test

<table>
<tr><th>Biochemical reactions</th><th>Auto-agglutination</th><th>Serological reactions</th><th>Interpretation</th></tr>
<tr><td>Typical reactions</td><td>No</td><td>O-, Vi- or H- antigen positive</td><td>Strains considered to be Salmonella</td></tr>
<tr><td>Typical reactions</td><td>No</td><td>All reactions negative</td><td rowspan="3">May be Salmonella</td></tr>
<tr><td>Typical reactions</td><td>Yes</td><td>Not tested (see 9.5.3.2)</td></tr>
<tr><td>No typical reactions</td><td>No</td><td>O-, Vi- or H- antigen positive</td></tr>
<tr><td>No typical reactions</td><td>No</td><td>All reactions negative</td><td>Not considered to be Salmonella</td></tr>
</table>

9.5.7 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).

10 Control cultures

In order to check the ability of the enrichment and identification media to support the growth of *Salmonella*, a reference culture of recently isolated *Salmonella* or of a *Salmonella* strain from a recognized culture collection centre should be introduced into control flasks of both enrichment media (see 9.3.2). Proceed with the control flasks as for the test cultures to demonstrate that the positive control culture is recovered.

11 Expression of results

In accordance with the results of the interpretation, report the presence or absence of *Salmonella* in the test sample, specifying the mass, in grams, or the volume, in millilitres, of sample tested.

12 Safety precautions

12.1 The procedure specified in this International Standard shall only be carried out in laboratories with suitable facilities and under the control of a qualified microbiologist.

12.2 These procedures shall not be performed in quality control laboratories, or in food manufacturing or processing premises, where there is a risk of contamination of the environment.

12.3 Full bacteriological precautions shall be taken at all times whilst carrying out the procedure specified in this International Standard. Particular attention shall be given to the sterilization of used equipment and media after testing suspect samples and prior to disposal or reuse.

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12.4 Special care should be taken with the laboratory use of selenite solutions because of their potentially toxic effect. Do not pipette by mouth under any circumstances.

NOTE For further and more detailed safety precautions reference is made to ISO 7218, in general and clauses 3, 4 and 7 in particular.

13 Test report

The test report shall specify:

- all information necessary for the complete identification of the sample;
- the sampling method used, if known;
- the test method used, with reference to this International Standard;
- all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the result(s);
- the test result(s) obtained or, if the repeatability has been checked, the final quoted result(s) obtained.

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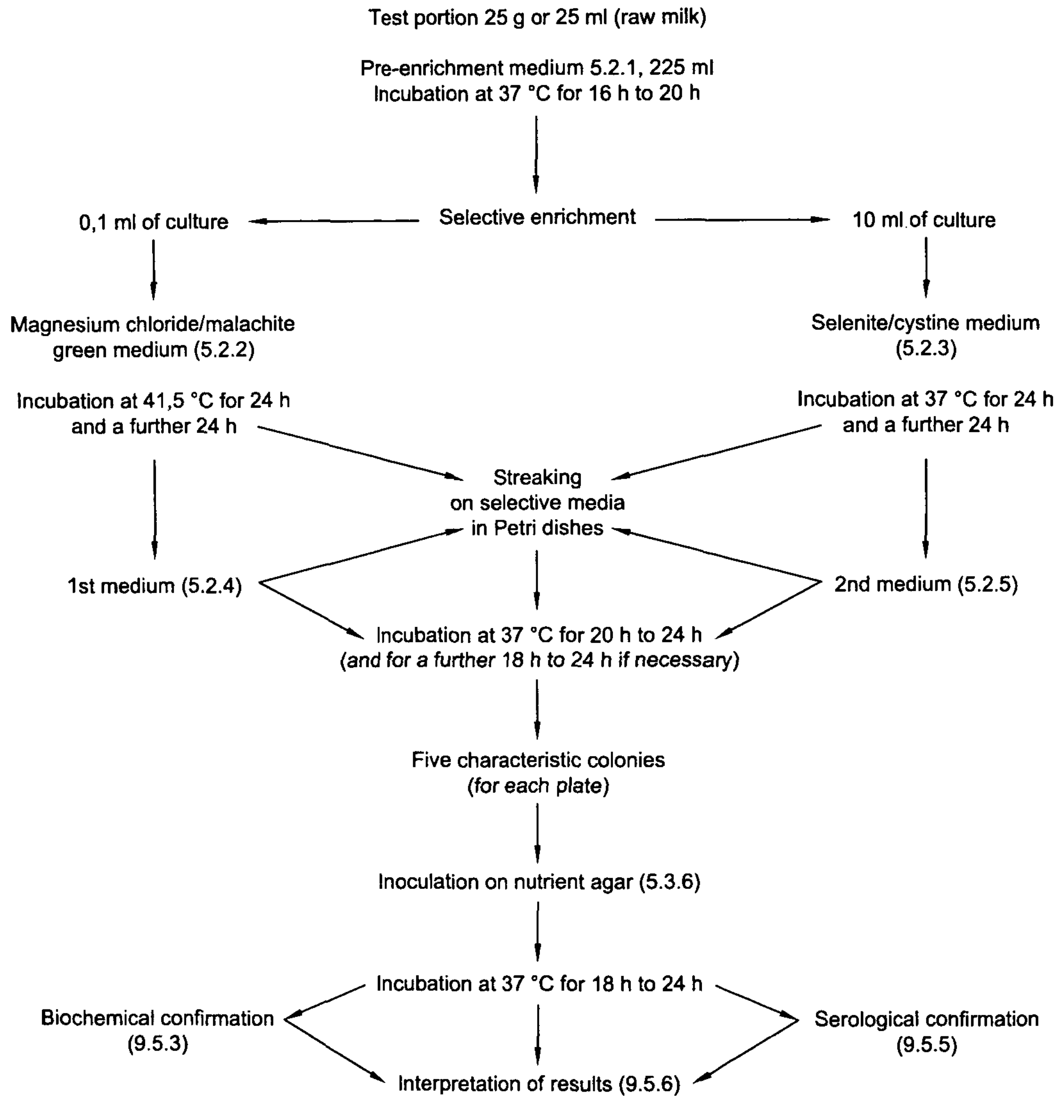
Annex A
(normative)

Diagram of procedure



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Annex B
(normative)

Specification for brilliant green

B.1 Bacteriological performance

It shall suppress the spreading of *Proteus* on phenol red/brilliant green agar (5.2.4) while not inhibiting the growth of *Salmonella*.

B.2 Test method

B.2.1 Medium

Prepare the brilliant green/phenol red agar plates according to 5.2.4 but with various concentrations of brilliant green within the range 4,5 mg/l to 6 mg/l.

B.2.2 Procedure

Inoculate one set of agar plates having different concentrations of brilliant green with a pure culture of a swarming *Proteus*, and another similar set with a pure culture of *Salmonella*, and incubate these at 37 °C for no longer than 24 h.

A satisfactory concentration of brilliant green should allow growth of *Salmonella* with typical pink colonies, 1 mm to 2 mm in diameter, and limited growth of *Proteus*; that is, no spreading. The concentration of brilliant green which shows this pattern should be used for the preparation of the brilliant green solution (see 5.2.4.3).

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Annex C
(informative)

Standard method for streaking agar plates

Key
1 Main streak

a) 1st plate

b) 2nd plate

Figure C.1

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