International Standard ISO 6785

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Milk and milk products — Detection of *Salmonella*

Lait et produits laitiers — Recherche des Salmonella

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Foreword

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

International Standard ISO 6785 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*.

NOTE — The method specified in this International Standard has been developed jointly with the International Dairy Federation (IDF) and the Association of Official Analytical Chemists (AOAC) and will also be published by these organizations.

Users should note that all International Standards undergo revision from time to time and that any reference made herein to any other International Standard implies its latest edition, unless otherwise stated.

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Milk and milk products — Detection of *Salmonella*

1 Scope and field of application

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

2 Reference

ISO 707, *Milk and milk products — Methods of sampling*.

3 Definitions

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

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

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

4 Principle

In general, the detection of *Salmonella* necessitates four successive stages as in 4.1 to 4.4. See also the diagram of procedure in the annex.

4.1 Pre-enrichment in liquid medium

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

4.2 Enrichment in selective liquid media

Inoculation of a tetrathionate medium and of a selenite cystine medium with the culture obtained (4.1) and incubation of the tetrathionate medium at 43 °C and of the selenite cystine medium at 37 °C, for two periods of 18 to 24 h.

4.3 Plating out and identification

From the cultures obtained (4.2), inoculation of two selective solid media (brilliant green/phenol red agar and bismuth sulfite agar). [1)]

Incubation at 37 °C and examination after 20 to 24 h and, if necessary, after 40 to 48 h to check the presence of colonies which, from their characteristics, are considered to be presumptive *Salmonellae*.

4.4 Confirmation

Subculturing of colonies of presumptive *Salmonella* (4.3) and confirmation by means of appropriate biochemical and serological tests.

5 Culture media, reagents and sera

5.1 Basic materials

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

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

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

When agar is specified, the amount used should be varied according to the manufacturer's instructions to give media of suitable firmness.

Measurements of pH shall be made using a pH meter, measurements being referred to a temperature of 25 °C. Adjustments, which may not always be necessary, are made by adding either 1 mol/l hydrochloric acid or 1 mol/l sodium hydroxide solution.

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

1) Bismuth sulfite agar allows the recovery of lactose-fermenting *Salmonella* strains.

NOTE — Commercially available rapid diagnostic systems may be used instead of the diagnostic media listed in 5.2.7, 5.2.8, 5.2.9 and 5.3, but see 9.4.4.

5.2 Culture media

5.2.1 Pre-enrichment medium: Buffered peptone water

Composition

Peptone	10,0 g
Sodium chloride	5,0 g
Disodium hydrogenorthophosphate dodecahydrate ($Na_2HPO_4.12H_2O$)	9,0 g
Potassium dihydrogenorthophosphate (KH_2PO_4)	1,5 g
Water	1 000 ml

Preparation

Dissolve the components in the water by boiling.

Adjust the pH so that after sterilization it is 7,0 ± 0,1.

Transfer the medium in quantities of 225 ml into flasks of 500 ml capacity (or multiples of 225 ml into flasks of suitable capacity).

Sterilize the medium for 15 min at 121 ± 1 °C.

5.2.2 First selective enrichment medium: Tetrathionate medium (Muller-Kauffmann)

5.2.2.1 Base

Composition

Meat extract	5,0 g
Peptone	10,0 g
Sodium chloride	3,0 g
Calcium carbonate	45,0 g
Water	1 000 ml

Preparation

Add the dehydrated base components or the complete dehydrated base to the water and boil until soluble components are completely dissolved.

Adjust the pH so that after sterilization it is 7,0 ± 0,1.

Sterilize the base for 15 min at 121 ± 1 °C.

5.2.2.2 Sodium thiosulfate solution

Composition

Sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$)	50,0 g
Water to a final volume of	100 ml

Preparation

Dissolve the sodium thiosulfate in part of the water.

Dilute to the final volume.

Sterilize the solution for 15 min at 121 ± 1 °C.

5.2.2.3 Iodine solution

Composition

Iodine	20,0 g
Potassium iodide	25,0 g
Water to a final volume of	100 ml

Preparation

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

Shake until dissolution is complete.

Dilute to the final volume.

Store the solution in a tightly closed opaque container.

5.2.2.4 Brilliant green solution

Composition

Brilliant green	0,5 g
Water	100 ml

Preparation

Add the brilliant green to the water.

Store the solution for at least 1 day in the dark to allow autosterilization to occur.

5.2.2.5 Ox bile solution

Composition

Ox bile, desiccated	10,0 g
Water	100 ml

Preparation

Dissolve the desiccated ox bile in the water by boiling.

Sterilize the solution for 15 min at 121 ± 1 °C.

5.2.2.6 Complete medium

Composition

Base (5.2.2.1)	900 ml
Sodium thiosulfate solution (5.2.2.2)	100 ml
Iodine solution (5.2.2.3)	20 ml
Brilliant green solution (5.2.2.4)	2 ml
Ox bile solution (5.2.2.5)	50 ml

Preparation

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

Mix the liquids well after each addition.

Distribute the medium aseptically in quantities of 100 ml into sterile flasks of capacity 500 ml.

Store at 0 to 5 °C in the dark until needed, but use within 1 week of preparation.

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 medium

Composition

Tryptone	5,0 g
Lactose	4,0 g
Disodium hydrogenorthophosphate (Na_2HPO_4)	10,0 g
Sodium hydrogenselenite	4,0 g
Water	1 000 ml

Preparation

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

Adjust the pH to 7,0 ± 0,1.

Do not autoclave.

5.2.3.2 L-Cystine solution

Composition

L-Cystine	0,1 g
Sodium hydroxide, solution, c(NaOH) = 1 mol/l	15 ml

Preparation

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

Do not autoclave.

5.2.3.3 Complete medium

Cool the base medium and add the L-Cystine solution aseptically in the proportion of 0,1 ml per 10 ml of base medium.

Adjust the pH, if necessary, to 7,0 ± 0,1.

Do not autoclave.

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

Use the medium on the day of preparation.

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

5.2.4.1 Base medium

Composition

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

Preparation

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

Adjust the pH so that after sterilization it is 7,0 ± 0,1.

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

Sterilize for 15 min at 121 ± 1 °C.

5.2.4.2 Sugar/phenol red solution

Composition

Lactose	10,0 g
Sucrose	10,0 g
Phenol red	0,09 g
Water to a final volume of	100 ml

Preparation

Dissolve the ingredients in the water.

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

Cool to 55 °C and use immediately.

5.2.4.3 Complete medium

Composition

Base medium (5.2.4.1)	900 ml
Sugar/phenol red solution (5.2.4.2)	100 ml
Brilliant green solution (5.2.2.4)	1 ml

1) According to the manufacturer's instructions.

Preparation

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

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

5.2.4.4 Preparation of plates

Transfer quantities of about 15 ml of the complete medium cooled to 45 °C to sterile Petri dishes (of diameter 90 mm) and allow to solidify.

Immediately before use, dry the plates carefully, preferably with the lids off and the agar surface downwards, in an oven or incubator maintained at 50 ± 5 °C for 30 min.

Prepared plates shall not be stored for longer than 4 h at room temperature or 24 h at 0 to 5 °C.

5.2.5 Second identification medium: Bismuth sulfite agar

Composition

Peptone	10,0 g
Beef extract	5,0 g
Glucose	5,0 g
Disodium hydrogenorthophosphate	4,0 g
Iron(II) sulfate	0,3 g
Ammonium bismuth citrate [1]	1,85 g
Sodium sulfite [1]	6,15 g
Agar	20,0 g
Brilliant green	0,025 g
Water	1 000 ml

Preparation

Dissolve the ingredients in the water by boiling for approximately 1 min.

Adjust the pH to 7,7 ± 0,1.

Cool to 45 to 50 °C, suspending the precipitate with gentle agitation.

Do not sterilize the medium.

Transfer the medium in quantities of 20 ml to sterile Petri dishes (of diameter 90 mm) and allow to solidify.

Immediately before use, dry the plates carefully, preferably with the lids off and the agar surface downwards, in an oven or incubator maintained at 50 ±5 °C for 30 min.

Use dried plates between 24 and 48 h after their preparation. Store them in the dark.

5.2.6 Nutrient agar

Composition

Meat extract	3,0 g
Peptone	5,0 g
Agar	12,0 g
Water	1 000 ml

Preparation

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

Adjust the pH so that after sterilization it is 7,0 ± 0,1.

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

Sterilize the medium for 15 min at 121 ± 1 °C.

Preparation of nutrient agar plates

Transfer about 15 ml of the melted medium to sterile Petri dishes (of diameter 90 mm) and proceed as specified in 5.2.4.4.

5.2.7 Triple sugar/iron agar (TSI agar)

Composition

Meat extract	3,0 g
Yeast extract	3,0 g
Peptone	20,0 g
Sodium chloride	5,0 g
Lactose	10,0 g
Sucrose	10,0 g
Glucose	1,0 g
Iron(III) citrate	0,3 g
Sodium thiosulfate	0,3 g
Phenol red	0,024 g
Agar	12,0 g
Water	1 000 ml

Preparation

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

Adjust the pH so that after sterilization it is 7,4 ± 0,1.

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

Sterilize the medium for 10 min at 121 ± 1 °C.

Allow to set in a sloping position to give a butt of depth 2,5 cm and a slant of 4 to 5 cm.

1) Instead of these components, 8 g of bismuth sulfite [$Bi_2(SO_3)_3$] indicator may be used.

5.2.8 Urea agar (Christensen)

5.2.8.1 Base medium

Composition

Peptone	1,0 g
Glucose	1,0 g
Sodium chloride	5,0 g
Potassium dihydrogenorthophosphate (KH_2PO_4)	2,0 g
Phenol red	0,012 g
Agar	15,0 g
Water	1 000 ml

Preparation

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

Adjust the pH, if necessary, so that after sterilization it is 6,8 ± 0,1.

Sterilize the base for 15 min at 121 ± 1 °C.

5.2.8.2 Urea solution

Composition

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

Preparation

Dissolve the urea in the water.

Sterilize by filtration and check the sterility. (For details of the technique of sterilization by filtration reference may be made to any appropriate textbook on microbiology.)

5.2.8.3 Complete medium

Composition

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

Preparation

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

Transfer the complete medium in quantities of 10 ml to sterile tubes. Allow to set in a sloping position.

5.2.9 Lysine decarboxylation medium

Composition

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

Preparation

Dissolve the components in the water by boiling.

Adjust the pH so that after sterilization it is 6,8 ± 0,1.

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

Sterilize the medium for 10 min at 121 ± 1 °C.

5.3 Reagents

5.3.1 Saline solution

Composition

Sodium chloride	8,5 g
Water	1 000 ml

Preparation

Dissolve the sodium chloride in the water by boiling.

Adjust the pH so that after sterilization it is 7,0 ± 0,1.

Transfer quantities of the solution to flasks or tubes so that they will contain 90 to 100 ml after sterilization.

Sterilize the solution for 15 min at 121 ± 1 °C.

5.3.2 Reagents for β-galactosidase reaction [1)]

5.3.2.1 Toluene

5.3.2.2 Buffer solution

Composition

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

Preparation

Dissolve the sodium dihydrogenorthophosphate in approximately 45 ml of water.

Adjust the pH to 7,0 ± 0,1 with approximately 3 ml of the sodium hydroxide solution.

Add water to a final volume of 50 ml.

1) Commercially available ONPG-impregnated discs may be used.

5.3.2.3 ONPG solution

Composition

2-Nitrophenyl β-D-galactopyranoside (ONPG)	80 mg
Water	15 ml

Preparation

Dissolve the ONPG in the water at 50 °C.

Cool the solution.

5.3.2.4 Complete reagent

Composition

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

Preparation

Add the buffer solution to the ONPG solution.

5.3.3 Reagents for Voges-Proskauer reaction (rapid method by Barry and Feeney)

5.3.3.1 VP medium

Composition

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

Preparation

Dissolve the components in the water.

Adjust the pH so that after sterilization it is 6,9 ± 0,1.

Transfer 3 ml of the medium into each of several tubes.

Sterilize the medium for no longer than 15 min at 121 ± 1 °C.

5.3.3.2 Creatine solution

Composition

Creatine monohydrate (*N*-amidinosarcosine)	0,5 g
Water	100 ml

Preparation

Dissolve the creatine monohydrate in the water.

5.3.3.3 1-Naphthol, ethanolic solution

Composition

1-Naphthol	6 g
Ethanol, 96 % (*V*/*V*)	100 ml

Preparation

Dissolve the 1-naphthol in the ethanol.

5.3.3.4 Potassium hydroxide solution

Composition

Potassium hydroxide	40 g
Water	100 ml

Preparation

Dissolve the potassium hydroxide in the water.

5.3.4 Reagents for indole reaction

5.3.4.1 Tryptone/tryptophan medium (by Ljutov)

Composition

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

Preparation

Dissolve the components in the water by boiling and filter.

Adjust the pH so that after sterilization it is 7,5 ± 0,1.

Transfer 5 ml of the medium into each of several tubes.

Sterilize the medium for 15 min at 121 ± 1 °C.

5.3.4.2 Kovacs reagent

Composition

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

Preparation

Mix the components.

5.3.5 Semi-solid nutrient agar

Composition

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

1) According to the manufacturer's instructions.

Preparation

Dissolve the dehydrated base components in the water by boiling.

Adjust the pH so that after sterilization it is 7,0 ± 0,1.

Transfer the medium to flasks of capacity not more than 500 ml.

Sterilize the medium for 15 min at 121 ± 1 °C.

Preparation of agar plates

Place in sterile Petri dishes (of diameter 90 mm) about 15 ml of the freshly prepared complete medium. The plates shall not be dried.

5.4 Sera

Several anti-*Salmonella* sera 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). For each serum, follow the instructions for use given by the manufacturer.

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

6 Apparatus and glassware

Usual microbiological laboratory equipment, and in particular

6.1 Apparatus

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

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

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

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

An autoclave is also necessary for the sterilization of culture media and reagents. It shall be capable of being maintained at 121 ± 1 °C, and at 115 ± 1 °C.

6.1.2 Drying cabinet, oven, or **incubator,** ventilated by convection (for drying the surface of agar plates), capable of being maintained at 50 ± 5 °C.

6.1.3 Incubator, capable of being maintained at 37 ± 1 °C.

6.1.4 Incubator, capable of being maintained at 43 ± 0,5 °C.

6.1.5 Water-baths, capable of being maintained at 45 ± 1 °C and 37 ± 1 °C.

6.1.6 Blending equipment

One of the following shall be used:

a) **a rotary blender,** operating at a rotational frequency between 8 000 and 45 000 min^{-1}, with glass or metal bowls preferably fitted with lids, resistant to the conditions of sterilization;

b) **a peristaltic-type blender** (stomacher), with sterile plastic bags.

NOTE — The bowls or plastic bags should have sufficient capacity to allow the sample to be properly mixed with the appropriate amount of diluent. In general, the volume of the container should be equal to about twice the volume of the sample plus diluent.

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

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

6.1.9 Refrigerator (for storage of prepared media and reagents), capable of being maintained at 0 to 5 °C.

6.2 Glassware

The glassware shall be resistant to repeated sterilization.

6.2.1 Culture bottles or flasks[1], for sterilization and storage of culture media and incubation of liquid media.

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

6.2.3 Measuring cylinders, for preparation of the complete media.

6.2.4 Graduated pipettes, of nominal capacities 25, 10 and 1 ml, graduated respectively in divisions of 0,5, 0,5 and 0,1 ml.

6.2.5 Petri dishes, as follows:

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

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

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

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

Lid, with a ridge

external diameter, maximum	102 mm

NOTE — Plastic Petri dishes may be used, even if of slightly different dimensions.

7 Sampling

See ISO 707.

Follow the instructions for sampling for microbiological purposes.

8 Preparation of the test sample

8.1 Milk

Agitate the sample thoroughly so that the micro-organisms are distributed as evenly as possible by rapidly inverting the sample container 25 times. Foaming should be avoided or foam allowed to disperse. The interval between mixing and removing the test portion should not exceed 3 min.

8.2 Dried milk, dried whey, dried buttermilk, lactose, casein

Thoroughly mix the contents of the closed container by repeatedly shaking and inverting. If the container is too full to permit thorough mixing, transfer to a larger container. Mix.

8.3 Butter

Melt the sample in a sterile container in a water-bath maintained at 45 ± 1 °C (6.1.5). Agitate during melting and remove the container immediately from the water-bath when the sample has just melted.

8.4 Cheese

Normally, the laboratory sample (taken in accordance with ISO 707) will constitute the test sample. Proceed then as described in 9.1.5.

8.5 Edible ices

Proceed as in the case of butter (8.3) but using a water-bath maintained at no more than 37 °C (6.1.5), as the sample shall not be allowed to exceed this temperature.

8.6 Fermented milks, yoghurts, custards, desserts

Mix the contents of the closed container by repeatedly shaking and inverting or open the container and mix the contents aseptically using a sterile spatula or spoon.

9 Procedure

See the safety precautions in clause 12.

9.1 Test portion and pre-enrichment

Add the test portion to the pre-enrichment medium and follow the operations described in 9.1.1 to 9.1.7.

For a summary of pre-enrichment and enrichment procedures, refer to table 1.

9.1.1 Milk

Pre-enrichment is unnecessary. See 9.2, using 25 ml of test sample and 225 ml of enrichment medium respectively.

9.1.2 Dried milk

Prepare a stoppered flask with 225 ml of sterile distilled water and 1 ml of the brilliant green solution (5.2.2.4). Weigh 25 g of the test sample 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 ± 10 min before incubation. Adjustment of the pH is not necessary. If after 3 h of incubation the dried milk is still not dissolved, mix the contents of the flask by shaking.

9.1.3 Dried whey, dried buttermilk

Weigh 25 g of the test sample aseptically into a stoppered flask containing 225 ml of sterile distilled water. Shake until dissolved and add 1 ml of the brilliant green solution (5.2.2.4).

9.1.4 Lactose

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

9.1.5 Casein, cheese

Weigh 25 g of the test sample aseptically into the sterile container of a high-speed or peristaltic-type blender (6.1.6), and add 225 ml of the pre-enrichment medium (5.2.1) at 45 °C. Blend until the test portion is thoroughly dispersed (1 to 3 min). Ensure that the temperature of the dispersion does not exceed 45 °C.

9.1.6 Butter

Shake the melted test sample and with a pipette, warmed to approximately 45 °C, transfer 25 ml of the test sample into a flask containing 225 ml of the pre-enrichment medium (5.2.1). Mix.

9.1.7 Frozen milk products (including edible ices)

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

Table 1 — Summary of pre-enrichment and enrichment procedures

Product	Sample size	Pre-enrichment medium *	Method of preparation	Enrichment medium
Milk	50 ml (2 × 25 ml)	None	Mix	225 ml tetrathionate 225 ml selenite cystine
Dried milk	25 g	Distilled water plus brilliant green solution	Soak for 60 ± 10 min, do not mix **	100 ml tetrathionate 100 ml selenite cystine
Dried whey, dried buttermilk	25 g	Distilled water plus brilliant green solution	Mix	100 ml tetrathionate 100 ml selenite cystine
Lactose	25 g	225 ml buffered peptone water	Mix	100 ml tetrathionate 100 ml selenite cystine
Casein, cheese	25 g	225 ml buffered peptone water	Blend at max 45 °C	100 ml tetrathionate 100 ml selenite cystine
Butter	25 ml	225 ml buffered peptone water	Mix	100 ml tetrathionate 100 ml selenite cystine
Frozen milk products	25 ml	225 ml buffered peptone water	Mix	100 ml tetrathionate 100 ml selenite cystine
Fermented milks, yoghurt, custard, desserts	25 g	225 ml buffered peptone water	Mix	100 ml tetrathionate 100 ml selenite cystine

* When the pre-enrichment medium is used, after incubation for 20 h at 37 °C, subculture 10 ml of the incubated sample — pre-enrichment medium mixture to each enrichment medium.

** If after 3 h of incubation the dried milk is still not dissolved, mix the contents of the flasks by shaking.

9.1.8 Fermented milks, yoghurt, custards, desserts

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

NOTES

1 To reduce the examination workload when more than one 25 g test portion 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 portions of 25 g are to be examined, combine the 10 units to form a composite test portion of 250 g and dissolve or disperse in 2,25 litres of pre-enrichment medium. Alternatively, the 10 ml portions of the pre-enrichment medium from the 10 separate test portions may be composited for enrichment in 1 litre of selective enrichment medium.

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

9.1.9 Incubation

Incubate the flasks prepared according to 9.1.2 to 9.1.8 at 37 °C for 16 to 20 h.

9.2 Enrichment

9.2.1 Transfer 10 ml of the incubated pre-enrichment medium (9.1) to a flask containing 100 ml of the tetrathionate selective enrichment medium (5.2.2); transfer another 10 ml of the incubated pre-enrichment medium to a flask containing 100 ml of the selenite cystine selective enrichment medium (5.2.3).

In the case of milk, transfer 25 ml of the test sample aseptically into 225 ml of the tetrathionate medium (5.2.2) and a further 25 ml into 225 ml of the selenite cystine medium (5.2.3).

9.2.2 Incubate the inoculated tetrathionate medium for 18 to 24 h at 43 ± 0,5 °C and the inoculated selenite cystine medium for 18 to 24 h at 37 ± 1 °C.

9.3 Plating out and identification

9.3.1 Take a loopful from each flask and streak on to the surface of a brilliant green/phenol red agar plate (5.2.4) so that well isolated colonies will be obtained. In the same way streak a loopful from each flask on to the surface of a bismuth sulfite agar plate (5.2.5). Continue the incubation of the flasks (see 9.3.3).

9.3.2 Incubate the plates (bottom uppermost) at 37 ± 1 °C for 20 to 24 h.

9.3.3 After incubating the flasks for a further 18 to 24 h, repeat the plating out and incubation procedure described in 9.3.1 and 9.3.2.

9.3.4 Examine the plates 9.3.2 and 9.3.3, after incubation, for the presence of typical colonies of *Salmonella*. If growth is slight, and no typical colonies of *Salmonella* are present, reincubate the plates at 37 ± 1 °C for a further 18 to 24 h and reexamine the plates for the presence of typical colonies of *Salmonella*.

9.3.5 Typical colonies of *Salmonella* may be characterized as follows.

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

On bismuth sulfite agar (5.2.5), typical colonies of *Salmonella* are brown or black with a metallic sheen. Some strains produce green colonies.

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 species to species or between batches of media, suspect colonies, as well as typical colonies, should be selected for confirmation.

9.4 Confirmation

9.4.1 Selection of colonies for confirmation

From each plate of each identification medium (9.3.5), select five typical or suspect colonies or, if there are fewer than five such colonies, select all for confirmation.

9.4.2 Incubation

Streak the selected colonies on to the surface of nutrient agar plates (5.2.6) in a manner which will allow well isolated colonies to develop. Incubate the plates at 37 ± 1 °C for 18 to 24 h.

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

9.4.3 Biochemical confirmation

Inoculate the following media with pure colonies by means of an inoculating wire.

9.4.3.1 TSI agar (5.2.7)

Streak the agar slope surface and stab the butt.

Incubate for 24 h at 37 ± 1 °C.

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)

9.4.3.2 Urea agar (5.2.8)

Streak the agar slope surface.

Incubate for 24 h at 37 ± 1 °C.

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.

9.4.3.3 Lysine decarboxylation medium (5.2.9)

Inoculate just below the surface of the liquid medium.

Incubate for 24 h at 37 ± 1 °C.

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

A yellow colour indicates a negative reaction.

9.4.3.4 β-Galactosidase reaction (5.3.2)

Suspend a loopful of the suspected colony in a tube containing 0,25 ml of the saline solution (5.3.1).

Add 1 drop of toluene and shake the tube.

Put the tube in a water-bath at 37 ± 1 °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 ± 1 °C and leave for 24 h.

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

9.4.3.5 Voges-Proskauer reaction (5.3.3)

Suspend a loopful of the suspected colony in two tubes each containing 0,2 ml of the VP medium (5.3.3.1).

Incubate one tube at room temperature and the other at 37 °C for 24 h.

After incubation, add to each tube 2 drops of the creatine solution (5.3.3.2), 3 drops of the ethanolic 1-naphthol 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.

9.4.3.6 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 for 24 h at 37 °C.