INTERNATIONAL STANDARD

ISO 6340

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Water quality — Detection of *Salmonella* species

Qualité de l'eau — Recherche de Salmonella

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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 6340 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

Annexes A and B of this International Standard are for information only.

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Introduction

Salmonella species are bacteria which are widely distributed all over the world. They are usually classified as pathogens, although their virulence and pathogenesis vary widely. The natural hosts of *Salmonella* species include humans, agricultural and domestic livestock and wild animals including birds. Humans and animals may excrete these bacteria while carrying them asymptomatically, as well as during disease. It is therefore impossible to eliminate them from the environment. Due to the severe diseases which can follow the infection of humans, the transmission of *Salmonella* species via different vehicles has to be minimized.

Since water is one of the vehicles, the presence or absence of *Salmonella* species should be monitored in water. *Salmonella* species may be present in all types of domestic and agricultural sewage, fresh waters, including ground and drinking waters, and also sea water.

The detection of *Salmonella* in water usually requires a concentration step. Since cells of *Salmonella* species may be injured in the aqueous environment, their detection in water usually requires a pre-enrichment step. The procedure described in this International Standard consists of regular enrichment(s), selection and confirmation steps.

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Water quality — Detection of *Salmonella* species

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

1 Scope

This International Standard specifies a method for the detection of *Salmonella* species in water samples for monitoring purposes. In special epidemiological situations, other media may also be required.

The method can be applied to all kinds of water, except raw sewage.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods.*

ISO 5667-1:1980, *Water quality — Sampling — Part 1: Guidance on the design of sampling programmes.*

ISO 5667-2:1991, *Water quality — Sampling — Part 2: Guidance on sampling techniques.*

ISO 5667-3:1994, *Water quality — Sampling — Part 3: Guidance on the preservation and handling of samples.*

ISO 6579:1993, *Microbiology — General guidance on methods for the detection of Salmonella.*

3 Definitions

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

3.1 *Salmonella* species: Gram-negative, oxidase-negative, facultatively anaerobic, non-sporeforming, rod-shaped bacteria which generally form colonies of 2 mm to 4 mm in diameter on solid selective media. They form typical colonies on solid selective media and display the biochemical and serological characteristics described when tests are carried out in accordance with this International Standard.

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

4 Principle

The detection of *Salmonella* species requires four successive stages.

4.1 Pre-enrichment

Pre-enrichment is necessary to enable injured cells to grow. If necessary, samples can be concentrated using membrane filtration. The membrane filter with cells, or a known volume of sample or its dilution, is transferred to non-selective broth (buffered peptone water) for incubation at the optimal temperature for mesophilic bacteria.

4.2 Enrichment in selective liquid medium

A selective enrichment step is necessary to increase the proportion of *Salmonella* species in relation to background flora. For this purpose, inoculum from pre-enrichment broth is transferred to malachite green/magnesium chloride (modified Rappaport-Vassiliadis) medium which is incubated at an elevated temperature to increase its selectivity.

NOTE 1 For the detection of *Salmonella typhi*, which is usually not important for water quality monitoring but may be required under special circumstances, selenite cystine medium (also available as dehydrated complete medium from different manufacturers) can be used for incubating the cultures at 36 °C ± 2 °C for up to 24 h. In certain special epidemiological situations, addition of other media may be necessary.

4.3 Selection on agar media

Solid selective media are used after the liquid enrichment steps for the detection and isolation of *Salmonella* species. In order to increase the probability of detecting *Salmonella* organisms, at least two different media are inoculated from selective enrichment cultures:

— brilliant green/phenol red lactose agar;

— xylose lysine deoxycholate agar;

— bismuth sulfite agar (optional).

4.4 Confirmation

The occurrence of typical colonies of *Salmonella* species on selective agar media is not sufficient evidence for the presence of *Salmonella* species. Therefore, it is necessary to subculture presumptive *Salmonella* colonies on different media for biochemical and serological confirmation (see table 1).

NOTE 2 Commercially available identification kits suitable for the identification of *Salmonella* species can be used instead of the tests listed in table 1, provided that they are used according to the manufacturer's instructions and on condition that they can be considered at least as reliable as the tests listed in table 1.

5 Culture media and confirmation media

Use reagents of analytical quality for the preparation of culture media, unless otherwise specified. Prepare media using glass-distilled water, or water of equivalent quality, complying with grade 3 of ISO 3696.

If commercially available dehydrated media are used, prepare them according to the manufacturer's instructions and add selective agents as supplements to give the specified concentrations.

All pH values given in this International Standard are for media after sterilization; for pH correction, use sodium hydroxide or hydrochloric acid at concentrations of 1 mol/l each.

5.1 Culture media

5.1.1 Pre-enrichment medium: buffered peptone water

5.1.1.1 Composition

	Single strength	Double strength
Peptone	10 g	20 g
Sodium chloride (NaCl)	5 g	10 g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)	9 g	18 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,5 g	3 g
Water	to 1 000 ml	to 1 000 ml

5.1.1.2 Preparation

Dissolve all the constituents in water by heating gently, but do not boil the solution.

Adjust the pH to 7,2 ± 0,1, with sodium hydroxide solution or hydrochloric acid.

Dispense the medium into culture bottles/tubes.

Sterilize the medium in the autoclave (6.1.2) at 121 °C ± 1 °C for 15 min.

Store in a refrigerator for up to 3 months.

5.1.2 Enrichment medium: malachite green/magnesium chloride (modified Rappaport-Vassiliadis medium)

5.1.2.1 Composition

Basic medium	
Peptone, enzymatic digest of animal tissue	4 g
Peptone, from soybeans	1 g
Sodium chloride (NaCl)	8 g
Dipotassium hydrogen phosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$)	0,4 g
Potassium dihydrogen phosphate (KH_2PO_4)	0,6 g
Water	to 1 000 ml

Supplement 1 [1]	
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	31,7 g
Water	to 100 ml

1) As this salt is very hygroscopic, it is advisable either to store it in a dessicator or to dissolve the entire contents of a newly opened container of magnesium chloride in such a way that the mass concentration of magnesium chloride hexahydrate is 28,6 g/l in the final medium. The magnesium chloride solution can be stored for a long time in a sealed container.

Supplement 2	
Malachite green oxalate	0,4 g
Water	to 100 ml

5.1.2.2 Preparation

Dissolve all the constituents of the basic medium in water by heating gently, but do not boil the solution.

Add the prepared magnesium chloride solution (supplement 1) and 10 ml of the malachite green solution (supplement 2) to the basic medium.

Adjust the pH to 5,2 ± 0,1, with sodium hydroxide solution or hydrochloric acid.

Dispense about 10 ml of the medium into each culture tube.

Sterilize the medium in the autoclave (6.1.2) at 115 °C ± 1 °C for 15 min.

5.1.3 Optional enrichment medium: selenite cystine

5.1.3.1 Composition

Casein-peptone	5 g
L-Cystine	0,01 g
Lactose	4 g
Disodium hydrogen phosphate (Na_2HPO_4)	10 g
Sodium hydrogen selenite ($NaHSeO_3$)	4 g
Water	to 1 000 ml

5.1.3.2 Preparation

Dissolve all the constituents in water by heating gently, but do not boil the solution.

CAUTION — Do not sterilize the medium in the autoclave. Apply sterile filtration instead, and do not use the medium if red sediments appear.

Adjust the pH to 7,0 ± 0,2.

WARNING — Inhalation of sodium hydrogen selenite dust and direct contact with the skin is very dangerous. The dust irritates the eyes, skin and mucous membranes and can penetrate skin both as a powder and as a solution. It has long-term health effects and may be carcinogenic. Reaction with acids liberates gaseous hydrogen selenide, which is very dangerous if inhaled and irritate the eyes and mucous membranes. Sodium hydrogen selenite and its solution must be handled under a hood using gloves and, if required, a respirator mask should be used. Contact with acids must be avoided. Store in tightly closed containers in a well-ventilated area that is dry and separated from acids.

5.1.4 Selective solid media

5.1.4.1 Brilliant green/phenol red lactose agar (according to Edel and Kampelmacher)

5.1.4.1.1 Composition

Basic medium	
Meat extract powder	5 g
Peptone, enzymatic digest of animal tissue	5 g
Disodium hydrogen phosphate (Na_2HPO_4)	1 g
Sodium dihydrogen phosphate (NaH_2PO_4)	0,6 g
Agar	about 15 g
Water	to 900 ml

Supplement 1	
Lactose	10 g
Sucrose	10 g
Phenol red	0,09 g
Water	to 100 ml

Supplement 2	
Brilliant green	0,5 g
Water	to 100 ml

5.1.4.1.2 Preparation

Dissolve all the constituents of the basic medium in water, and sterilize in the autoclave (6.1.2) at 121 °C ± 1 °C for 15 min.

Prepare supplement 1 by dissolving the constituents in sterile water. Heat the solution in a water bath (6.2) at 70 °C for 20 min. Cool it to 55 °C ± 1 °C and use it immediately.

Prepare supplement 2 by dissolving the brilliant green in the water. Store the solution for at least 1 day in the dark to allow autosterilization to occur.

Add the prepared sugar/phenol red solution (supplement 1) and 1 ml of the brilliant green solution (supplement 2) to the agar before distribution into Petri dishes (6.7); ensure that the final pH is 7,0 ± 0,1. Immediately before use, dry the agar plates until the surface of the agar is dry. Use freshly prepared plates.

5.1.4.2 Xylose lysine deoxycholate agar

5.1.4.2.1 Composition

Basic medium	
D(+)-Xylose	3,5 g
L(+)-Lysine	5 g
Sodium deoxycholate	2,5 g
Yeast extract	3 g
Saccharose	7,5 g
Lactose	7,5 g
Sodium chloride (NaCl)	5 g
Sodium thiosulfate ($Na_2S_2O_3$)	6,8 g
Iron(III) ammonium citrate	0,8 g
Agar	about 13 g
Water	to 1 000 ml

Supplement	
Phenol red	0,4 g
Water	to 100 ml

5.1.4.2.2 Preparation

Dissolve all the constituents, including 20 ml of the phenol red solution (supplement), by heating to bring to the boil.

Adjust the pH to 7,4 ± 0,1.

CAUTION — To avoid overheating, do not prepare portions larger than 1 litre at a time. Do not sterilize the medium in the autoclave. After preparation, transfer to a water bath at 50 °C and pour into Petri dishes (6.7) as soon as the medium has cooled.

5.1.4.3 Bismuth sulfite agar (according to Wilson and Blair)

5.1.4.3.1 Composition

Basic medium	
Meat extract	5 g
Peptone, enzymatic digest of animal tissue	10 g
D(+)-Glucose	5 g
Disodium hydrogen phosphate (Na_2HPO_4)	4 g
Iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)	0,3 g
Bismuth sulfite [$Bi_2(SO_3)_3$]	8g
Agar	about 15 g
Water	to 1 000 ml

Supplement	
Brilliant green	0,5 g
Water	to 100 ml

5.1.4.3.2 Preparation

Dissolve all the constituents, including 5 ml of the brilliant green solution (supplement), by heating. Do not sterilize the medium in the autoclave.

Adjust the pH to 7,6 ± 0,1 and pour about 20 ml of the dissolved, but cloudy medium into each Petri dish (6.7). Ensure that the agar is pale brownish or reddish yellow (fallow) or greenish. If the colour is brown, do not use the medium.

5.2 Confirmation media

5.2.1 Nutrient agar

5.2.1.1 Composition

Meat extract	3 g
Peptone, enzymatic digest of animal tissue	5 g
Agar	about 15 g
Water	to 1 000 ml
Sodium chloride (NaCl) (optional)	5 g

5.2.1.2 Preparation

Dissolve all the constituents by boiling.

Adjust the pH to 7,0 ± 0,1.

Sterilize the medium in the autoclave (6.1.2) at 121 °C ± 1 °C for 15 min.

Dispense into Petri dishes (6.7).

5.2.2 Iron/two-sugar agar (according to Kligler)

5.2.2.1 Composition

Basic medium	
Meat extract	3 g
Yeast extract	3 g
Peptone, enzymatic digest of animal tissue	20 g
Lactose	10 g
D(+)-Glucose	1 g
Iron(III) citrate	0,5 g
Sodium chloride (NaCl)	5 g
Sodium thiosulfate ($Na_2S_2O_3$)	0,3 g
Agar	about 12 g
Water	to 1 000 ml

Supplement	
Phenol red	0,4 g
Water	to 100 ml

5.2.2.2 Preparation

Dissolve all the constituents, including 6 ml of the phenol red solution (supplement), by heating.

Adjust the pH to a final value of 7,4 ± 0,1.

Sterilize the medium in the autoclave (6.1.2) at 121 °C ± 1 °C for 15 min.

Pour about 6 ml of the medium into each tube.

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

5.2.3 Urea agar (according to Christensen)

5.2.3.1 Composition

Basic medium	
Peptone, enzymatic digest of animal tissue	1 g
D(+)-Glucose	1 g
Sodium chloride (NaCl)	5 g
Potassium dihydrogen phosphate (KH_2PO_4)	2 g
Agar	about 15 g
Water	to 1 000 ml

Supplement 1	
Phenol red	0,4 g
Water	to 100 ml

Supplement 2	
Urea	40 g
Water	to 100 ml

5.2.3.2 Preparation

Dissolve all the constituents of the basic medium and 3 ml of the phenol red solution (supplement 1), by heating if necessary.

Sterilize the medium in the autoclave (6.1.2) at 121 °C ± 1 °C for 15 min.

Allow the agar to cool to about 50 °C.

Sterilize the urea solution (supplement 2) by filtration and add 50 ml of this solution under aseptic conditions to 950 ml of the agar (basic medium and supplement 1).

Dispense about 6 ml of the urea agar medium into each tube.

Ensure that the final pH is 6,8 ± 0,1.

Allow to set in a sloping position.