TECHNICAL SPECIFICATION

ISO/TS 6579-2

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Microbiology of food and animal feed — Horizontal method for the detection, enumeration and serotyping of *Salmonella* —

Part 2: Enumeration by a miniaturized most probable number technique

Microbiologie des aliments — Méthode horizontale pour la recherche, le dénombrement et le sérotypage des Salmonella —

Partie 2: Dénombrement par une technique miniaturisée du nombre le plus probable

Reference number
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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

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

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

— an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

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

ISO/TS 6579-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

ISO 6579 consists of the following part, under the general title *Microbiology of food and animal feed — Horizontal method for the detection, enumeration and serotyping of* Salmonella:

— *Part 2: Enumeration·by·a·miniaturized·most·probable·number·technique* [Technical Specification]

Additional parts, dealing with a detection method and guidance for serotyping are planned. ISO 6579:2002 is to be withdrawn at a later date.

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Introduction

The procedure described is based on the method reported in Reference [1].

The enumeration procedure described here concerns a miniaturized most probable number (MPN) technique. For this mini-MSRV (modified semi-solid Rappaport–Vassiliadis) MPN technique, the volume of primary dilution tested is less than the volume in the detection method specified in ISO 6579:2002 + Cor.1:2004 + Amd.1:2007.[5] For this reason, the sensitivity of the mini-MSRV technique is lower than in these detection methods (Reference [1]). The detection limit of the mini-MSRV method is approximately 1 cfu/g, but can vary according to *Salmonella* serovar and per matrix. For the previously mentioned detection methods, this is typically 1 cfu/25 g (0,04 cfu/g). For samples with (very) low numbers of *Salmonella* spp. ($<$1 cfu/g), it is possible that the mini-MSRV procedure is not sufficiently sensitive. If a quantitative result is requested for samples likely to contain such low numbers (and tested negative with this mini-MSRV technique, for example), it is advisable to enumerate with a “conventional” MPN technique (not miniaturized). For other samples, the mini-MSRV method can have an advantage over the conventional MPN technique because the performance of the miniaturized MPN technique can take less time and need fewer resources (due to small amounts).

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Microbiology of food and animal feed — Horizontal method for the detection, enumeration and serotyping of *Salmonella* —

Part 2: Enumeration by a miniaturized most probable number technique

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.
Persons using this International Standard should be familiar with normal laboratory practice. This standard does not purport to address all of the safety aspects, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

1 Scope

This part of ISO 6579 gives a method for the enumeration of *Salmonella* spp. present in:

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

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

— animal faeces;

— environmental samples from the primary production stage;

by calculation of the most probable number (MPN).

The method is based on miniaturization of the dilution, pre-enrichment and selective enrichment steps. The selective enrichment medium, modified semi-solid Rappaport–Vassiliadis (MSRV), is intended for the detection of motile salmonellae and is not appropriate for the detection of non-motile salmonellae.

It is possible that the method is less appropriate to enumerate *Salmonella* ser. Typhi and *Salmonella* ser. Paratyphi.

The method is not appropriate for the enumeration of *Salmonella* spp. in (very) low contaminated samples (<1 cfu/g).

NOTE The number of non-motile salmonellae is generally low in most of the matrices relevant for this method. In this note, examples are given for samples from primary production. The non-motile *Salmonella* biovars of *Salmonella* Gallinarum (*Salmonella* Gallinarum biovar gallinarum and *Salmonella* Gallinarum biovar pullorum) do not seem to survive long in environmental samples and are therefore rarely detected in faecal or environmental (such as dust) samples (regardless of the method). The number of other non-motile *Salmonella* serovars in faecal samples seems to be generally low. For example, in Reference [4] in which approximately 1 000 faecal samples of poultry layer flocks and approximately 900 faecal samples of broiler flocks were analysed, less than 1 % of the total number of samples were positive in a selective broth and at the same time negative on MSRV medium (and likely to be non-motile). Similar results were found in a Dutch study with ca 3 200 faecal samples of pigs (unpublished data). On the other hand, in the case of the study reported in Reference [4], up to almost 40 % of positive samples would not have been detected (i.e. false negatives) if only a selective broth (in this case Rappaport–Vassiliadis) had been used instead of a semi-solid medium.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 6887 (all parts), *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

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ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO/TS 11133-1, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory*

ISO/TS 11133-2, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 2: Practical guidelines on performance testing of culture media*

3 Terms and definitions

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

3.1
Salmonella
microorganism which forms typical or less typical colonies on solid selective media and which displays specific biochemical and serological characteristics

NOTE Suitable tests for the specific biochemical and serological characteristics are specified in this part of ISO 6579.

3.2
count of *Salmonella*
number of *Salmonella* spp. found per millilitre or per gram of a test sample or per surface area or on an object (e.g. bootsocks)

4 Principle

4.1 General

The enumeration of *Salmonella* spp. in the MPN format necessitates four successive stages (4.2 to 4.5).

4.2 Pre-enrichment in non-selective liquid medium

Preparation of a 10^{-1} dilution of the sample in buffered peptone water (BPW) (initial suspension).

Addition of the initial suspension to the first (empty) row of three wells of a 12-well microtiter plate.

Inoculation of the second row of three wells containing non-selective pre-enrichment broth (BPW) with a specified quantity, from the first row in a 12-well microtiter plate.

Inoculation of the third, fourth and if necessary more rows of three wells containing BPW.

Incubation of the 12-well microtiter plates at 37 °C for 18 h.

4.3 Enrichment on a selective semi-solid medium

Subculturing of each well obtained in 4.2 in a well containing a semi-solid agar (MSRV).

Incubation at 41,5 °C (MSRV) for 24 h. If MSRV is negative after 24 h, the plate is incubated for a further 24 h.

4.4 Selective plating and identification

From the (suspect) cultures (the highest dilutions) obtained in 4.3, a selective solid medium xylose–lysine–deoxycholate (XLD) agar is inoculated and incubated at 37 °C for 24 h.

4.5 Confirmation

Colonies of presumptive *Salmonella* obtained in 4.4 are confirmed by means of appropriate biochemical and serological tests.

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4.6 Calculation of most probable number

From the number of confirmed positive wells, the MPN of *Salmonella* spp. per millilitre or per gram of the test sample is calculated.

5 Culture media and sera

5.1 General

For current laboratory practice, see ISO 7218.

For the performance testing of media, follow the recommendations of ISO/TS 11133-1, ISO/TS 11133-2 and the information as given in A.8.

All media and reagents needed are described in Annex A. Alternatively, dehydrated complete media or diluents can be used. In the latter case, follow the manufacturer's instructions.

5.2 Culture media

5.2.1 Non-selective pre-enrichment medium: Buffered peptone water (BPW). See A.1.

5.2.2 Semi-solid selective enrichment agar: Modified semi-solid Rappaport–Vassiliadis medium (MSRV). See A.2.

5.2.3 Xylose–lysine–deoxycholate (XLD) agar. See A.3.

5.2.4 Nutrient agar. See A.4.

5.2.5 Triple sugar–iron agar (TSI agar). See A.5.

As an alternative, a double sugar–iron agar (Kligler–Hajna) may be used.

5.2.6 Urea agar (Christensen). See A.6.

5.2.7 L-Lysine decarboxylation medium (LDC). See A.7.

5.2.8 Antisera. Several types of agglutinating sera containing antibodies for one or several O-antigens and for one or several H-antigens are commercially available.

Every attempt should be made to ensure that the anti-sera used are adequate to provide for the detection of all *Salmonella* serotypes.

6 Apparatus and glassware

Disposable supplies are an acceptable alternative to reusable glassware if it has similar specifications.

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

6.1 Apparatus for dry sterilization (oven) or **wet sterilization (autoclave)**. See ISO 7218.

6.2 Drying cabinet or **ventilated oven**, capable of being maintained at between 25 °C and 50 °C or a **laminar air flow cabinet**.

6.3 Incubators, capable of operating at 37 °C ± 1 °C and 41,5 °C ± 1 °C.

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6.4 Water bath, capable of operating at 47 °C to 50 °C.

6.5 Refrigerator (for storage of prepared media), capable of operating at 5 °C ± 3 °C.

6.6 pH-meter, having a resolution of 0,01 pH and accurate to within ± 0,1 pH units at 25 °C. See ISO 7218.

6.7 Sterile test tubes and **flasks**, of appropriate capacity. Flasks or bottles and test tubes with non-toxic metallic or plastic (screw) caps may be used.

6.8 Sterile loops of 1 µl.

6.9 Sterile graduated pipettes or **automatic pipettes**, of nominal capacities 10 ml (with 0,5 ml division), 2 ml (with 0,1 ml division), 0,1 ml (with 0,01 ml division). Multi-channel pipettes of 0,5 ml and 0,02 ml nominal capacity for pipetting three wells at a time.

6.10 Sterile Petri dishes, of approximately 90 mm diameter.

6.11 Sterile 12-well microtiter plates with wells of approximately 25 mm diameter and 20 mm deep (5 ml) with a flat bottom with lid.

6.12 Homogenizer. See ISO 7218.

7 Sampling

Sampling is not part of the method specified in this part of ISO 6579. See the specific International Standard dealing with the product concerned.

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

If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on the subject.

8 Preparation of test sample

Prepare the test sample 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 the subject.

9 Procedure

9.1 Test portion, initial suspension

See ISO 6887 and any specific International Standard appropriate to the product concerned. Prepare an initial suspension by diluting the test portion 10-fold in BPW (5.2.1). For example, add 25 g of sample to 225 ml of BPW and homogenize, e.g. in a stomacher (6.12), for 1 min.

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9.2 Dilution and pre-enrichment in non-selective liquid medium

Take a 12-well microtiter plate (6.11) with empty wells in the first row of three wells and containing 2 ml BPW (5.2.1) in the other wells (second, third and fourth row with each 3 wells; see Annex B).

NOTE 1 As a general case, the described procedure specifies dilutions for one 12-well microtiter plate. When a higher number than 500 cfu/g of *Salmonella* is suspected, it is necessary to proceed with a second 12-well microtitre plate containing 2 ml BPW (5.2.1) in each well. Prepare a sufficient number of plates (dilutions) to ensure that the final well in the 12-well microtiter plate yields a negative result.

Transfer to each well of the first (empty) row of three wells, using a pipette (6.9), 2,5 ml of the initial suspension (9.1).

Transfer 0,5 ml (e.g. by using a multi-channel pipette; 6.9) of each well from the first row into the 2 ml BPW in the successive wells in the second row (first 5^{-1} dilution).

Transfer 0,5 ml (e.g. by using a multi-channel pipette; 6.9, with new tips) of each well from the second row into the 2 ml BPW in the successive wells in the third row (second 5^{-1} dilution).

Before transferring the 0,5 ml of the second row into the third row, mix the suspensions in the wells by repeatedly (carefully) sucking up and blowing out of the suspension in the pipette and in the wells.

Proceed in the same way for the other rows.

Incubate the 12-well microtiter plate at 37 °C (6.3) for 18 h ± 2 h.

NOTE 2 As the contamination level of the tested samples is generally unknown and often low, it can prove worthwhile to check for the presence of *Salmonella* spp. in the sample by also culturing the initial suspension. For this purpose, incubate (6.3) the initial suspension (9.1) at 37 °C for 18 h± 2 h. For the next culture steps, follow the procedures as described in 9.3 to 9.5. In 9.3: inoculate a Petri dish containing MSRV with 1–3 equally spaced spots with a total volume of 0,1 ml of the incubated BPW culture of the initial suspension (9.1).

9.3 Selective enrichment on a semi-solid medium

Allow the MSRV (5.2.2) in the 12-well microtiter plates to equilibrate at room temperature if they were stored at a lower temperature.

Inoculate each well containing 2 ml MSRV with 20 µl of the BPW culture (9.2), e.g. using a multi-channel pipette (6.9). Use new tips for each row of three wells.

Place the 20 µl BPW culture at the margin of the well and on the surface of the medium (see Annex B).

When taking a subculture from BPW, try not to disturb particulate samples. Therefore, move the microtitre plates carefully. Avoid pipetting particulate matter on to the MSRV plates.

Incubate the inoculated MSRV plates at 41,5 °C (6.3) for 24 h ± 3 h.

Do not invert the plates.

Suspect wells show a grey-white, turbid zone extending out from the inoculated drop. The turbid zone is characterized by a white halo with a clearly defined edge.

If the wells are negative after 24 h, reincubate for a further 24 h ± 3 h.

9.4 Selective plating

Allow the XLD agar (5.2.3) plates to equilibrate at room temperature if they were stored at lower temperature. If necessary, dry the surface of the plates before use.

Subculture suspect MSRV wells (9.3) by dipping a 1 µl loop (6.8) just inside the border of the opaque growth and by inoculating this culture material on the surface of one normal size XLD plate, so that well-isolated colonies are obtained.

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Subculture at least the highest dilutions: the ones which still show three suspect MSRV wells as well as the subsequent dilutions showing two or one suspect MSRV wells.

Incubate the inverted XLD plates at 37 °C (6.3) for 24 h ± 3 h.

Return negative MSRV plates to the 41,5 °C incubator and incubate for a further 24 h ± 3 h. Perform the selective plating procedure if after 48 h of incubation additional wells become suspect.

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

NOTE Hydrogen sulfide-negative variants of *Salmonella* spp. grown on XLD agar are pink with a darker pink centre. Lactose-positive variants of *Salmonella* spp. grown on XLD agar are yellow with or without blackening.

9.5 Biochemical and serological confirmation

9.5.1 General

Perform confirmation on at least one well-isolated suspect colony from each XLD plate (9.4). If no well-isolated colony can be obtained, it may be necessary to perform an extra culture step on XLD agar or on a non-selective agar, e.g. nutrient agar (5.2.4), to obtain well-isolated colonies.

If all wells selected fail to confirm as *Salmonella* spp., presumptive MSRV wells not already subcultured (in lower dilutions) should be checked.

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

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

9.5.2 Selection of colonies for confirmation

For confirmation, take from each XLD plate (9.4) at least one colony considered to be typical or suspect.

If well-isolated colonies (of a pure culture) are available on the selective plating media (9.4), the biochemical confirmation can be performed directly on a suspect, well-isolated colony from the selective plating medium. The culture step on the non-selective agar medium, like nutrient agar (5.2.4) can then be performed in parallel with the biochemical tests for purity check of the colony taken from the selective agar medium. Incubate inoculated nutrient agar plates at 37 °C (6.3) for 24 h ± 3 h.

Use pure cultures for biochemical and serological confirmation.

9.5.3 Biochemical confirmation

9.5.3.1 General

By means of an inoculating wire, inoculate the media specified in 9.5.3.2, 9.5.3.3 and 9.5.3.4 with each of the cultures obtained from the colonies selected in 9.5.2.

9.5.3.2 TSI agar (5.2.5)

Streak the agar slant surface and stab the butt. Incubate at 37 °C (6.3) for 24 h ± 3 h.

Interpret the changes in the medium as follows.

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Butt	yellow	glucose positive (glucose used)
	red or unchanged	glucose negative (glucose not used)
	black	formation of hydrogen sulfide
	bubbles or cracks	gas formation from glucose
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 cultures of *Salmonella* spp. show alkaline (red) slant and acid (yellow) butts with gas formation (bubbles) and (in about 90 % of the cases) formation of hydrogen sulfide (blackening of the agar) see Table 1.

A lactose-positive variant of *Salmonella* spp. gives a yellow slant on TSI. 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 (5.2.6)

Streak the agar slant surface. Incubate at 37 °C (6.3) for 24 h ± 3 h and examine at intervals.

If the reaction is positive, decomposition 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.

Typical *Salmonella* cultures do not hydrolyse urea, so that the colour of the urea remains unchanged (yellow; see Table 1).

9.5.3.4 L-Lysine decarboxylation medium (5.2.7)

Inoculate just below the surface of the liquid medium. Incubate at 37 °C (6.3) for 24 h ± 3 h.

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

Most typical *Salmonella* cultures show a positive reaction (see Table 1).

9.5.3.5 Interpretation of the biochemical tests

Salmonella generally show the reactions given in Table 1. If these reactions are found it can be considered that the sample may contain *Salmonella* spp.

Table 1 — Interpretation of biochemical tests; typical reactions of most *Salmonella* serovars (percentages indicated in brackets)

TSI agar			
Butt	yellow	glucose positive (100 %)	
	black	formation of hydrogen sulfide, H_2S (91,6 %)	
	bubbles and/or cracks	gas formation from glucose (91,9 %)	
Slant surface	red or unchanged	lactose and/or sucrose negative (respectively 99,2 % and 99,5 %)	
Urea agar			
—	yellow, no colour changing of the medium		negative (100 %)
LDC			
—	purple colour and turbidity		positive (94,6 %)

9.5.4 Serological confirmation and serotyping

Biochemical test results can indicate whether an isolate belongs to the genus *Salmonella*. For a full typing of *Salmonella* strains, serotyping has also to be performed. Serological confirmation gives additional information

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to which serogroup the isolate belongs. With serotyping it is possible to further type the isolate to serovar level. For further details, see ISO 6579[5] and Reference [3].

10 Expression of results

Count the number of wells giving a positive confirmed reaction for each dilution. Calculate the MPN from the number of positive confirmed wells at each dilution.

If all wells are negative, but the initial suspension (10^{-1} dilution) was found to be positive for *Salmonella* spp. (after confirmation), the result can be reported as: *Salmonella* spp. present in the amount of sample tested (e.g. 25 g), but less than the lower limit of detection of the mini-MPN method (<1 cfu/g).

For the calculation of the MPN, the formulas given in ISO 7218 can be used, as no “standard” MPN tables are known to be available for the dilutions used. A software program for use in Excel[1)] has been written that can handle up to 10 levels of serial dilutions. It is highly recommended that this program be used rather than other programs, since the results for any specific combination of results derived with three dilutions will be the same as those in the tables published in ISO 7218. Details of the calculations are described in Reference [2] and the software is freely available for download from:

http://standards.iso.org/iso/ts/6579/-2/

11 Test report

The test report shall contain at least the following information:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, with reference to this part of ISO 6579 (ISO/TS 6579-2:2012);

d) all operating detail not specified in this part of ISO 6579, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

e) the test result(s) obtained;

f) if repeatability has been checked, the final quoted result obtained.

1) Excel is the trade name of a product supplied by Microsoft. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

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Annex A
(informative)

Composition and preparation of culture media and reagents

IMPORTANT — If media or reagents are prepared from dehydrated complete media or reagents, or if ready-to-use media and reagents are used, follow the manufacturer's instructions regarding preparation, storage conditions, expiry date and use.

The shelf-lives of the media indicated in this annex have been shown in some studies. The user should verify this under their own storage conditions (see ISO/TS 11133-1 and ISO/TS 11133-2).

Performance testing for the quality assurance of the culture media is described in A.8.

A.1 Buffered peptone water (BPW)

A.1.1 Composition

Peptone[a]	10,0 g
Sodium chloride	5,0 g
Disodium hydrogenphosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)	9,0 g
Potassium dihydrogenphosphate (KH_2PO_4)	1,5 g
Water	1 000 ml

[a] For example, enzymatic digest of casein.

A.1.2 Preparation

Dissolve the components in the water, by heating (without boiling) if necessary.

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

Dispense the medium into flasks (6.7) of suitable capacity to obtain the portions necessary for the test.

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

Store airtight, closed flasks in the dark at 5 °C (6.5) for up to 6 months.

Transfer aseptically 2 ml, e.g. by using a multi-channel pipette (6.9), into each well from the 12-well microtitre plates (6.11). Prepare one 12-well microtitre plate with an empty first row of three wells.

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A.2 Modified semi-solid Rappaport–Vassiliadis (MSRV) medium

A.2.1 Base medium

A.2.1.1 Composition

Enzymatic digest of animal and plant tissue	4,6 g
Acid hydrolysate of casein	4,6 g
Sodium chloride (NaCl)	7,3 g
Potassium dihydrogenphosphate (KH_2PO_4)	1,5 g
Magnesium chloride anhydrous ($MgCl_2$)	10,9 g
Malachite green oxalate	0,04 g
Agar	2,7 g
Water	1 000 ml

A.2.1.2 Preparation

Dissolve the components in the water.

Heat to boiling with agitation.

IMPORTANT Do not autoclave.

Do not hold the medium at high temperatures longer than necessary.

Cool the medium to 47 °C to 50 °C.

A.2.2 Novobiocin solution

A.2.2.1 Composition

Novobiocin sodium salt	0,05 g
Water	10 ml

A.2.2.2 Preparation

Dissolve the novobiocin sodium salt in the water.

Sterilize by filtration through a filter with a pore size of 0,22 μm.

The solution may be stored at 5 °C (6.5) for up to 4 weeks, or in small portions (e.g. of 2 ml) at –20 °C for up to 1 year.

A.2.3 Complete medium

A.2.3.1 Composition

Base medium (A.2.1)	1 000 ml
Novobiocin solution (A.2.2)	2 ml

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A.2.3.2 Preparation

Aseptically add 2 ml of the novobiocin solution (A.2.2) to 1 000 ml of base medium (A.2.1) at 47 °C to 50 °C. Mix carefully.

The final concentration of novobiocin is 10 mg/l MSRV medium.

The final pH should be 5,2 (5,1 to 5,4) at 20 °C to 25 °C.

Transfer 2 ml (e.g. by using a multi-channel pipette, 6.9) into each well from the 12-well microtitre plates (6.11).

If necessary, also pour into plates up to a final volume of 15 ml to 20 ml in Petri dishes with a diameter of 90 mm (6.10).

Allow the medium to solidify before moving and handle with care.

Store the plates, with surface upwards, at 5 °C (6.5) in the dark for up to 2 weeks.

Do not invert the plates, as the semi-solid agar is too liquid.

Any plates in which the semi-solid agar has liquefied or fragmented should not be used.

Immediately before use, and only if visible moisture is apparent, dry the surface of the agar plates carefully, e.g. by placing them with the lids off and the agar surface upwards in a laminar air flow cabinet. Take care not to overdry the medium.

A.3 Xylose–lysine–deoxycholate (XLD) agar

A.3.1 Composition

Yeast extract	3,0 g
Sodium chloride (NaCl)	5,0 g
D(+)-Xylose	3,75 g
Lactose	7,5 g
Sucrose (saccharose)	7,5 g
L(+)-lysine hydrochloride	5,0 g
Sodium thiosulfate ($Na_2S_2O_3$)	6,8 g
Iron(III) ammonium citrate	0,8 g
Phenol red	0,08 g
Sodium deoxycholate	1,0 g
Agar	9 g to 18 g[a]
Water	1 000 ml

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

A.3.2 Preparation

Dissolve the components in the water.

Heat with frequent agitation, until the medium starts to boil. Avoid overheating.

IMPORTANT — Do not autoclave.

It is important to avoid preparing large volumes which causes prolonged heating.

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Adjust the pH, if necessary, so that it corresponds to 7,4 ± 0,2 at 25 °C.

Transfer the medium to a water bath (6.4) at 47 °C to 50 °C.

Pour into Petri dishes (6.10) as soon as the medium has cooled. Allow to solidify.

Store the poured plates (upside down) at 5 °C (6.5), in the dark for up to 4 weeks. Protect them from drying.

Immediately before use and only if necessary, dry the surface of the agar plates carefully. Take care not to overdry the medium.

A.4 Nutrient agar

A.4.1 Composition

Meat extract	3,0 g
Peptone[a]	5,0 g
Sodium chloride (NaCl) (optional)	5,0 g
Agar	9 g to 18 g[b]
Water	1 000 ml

[a] For example, enzymatic digest of casein.

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

A.4.2 Preparation

Dissolve the components in the water by heating.

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

Transfer the culture medium into flasks (6.7) of appropriate capacity.

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

Transfer about 15 ml of the melted medium into sterile Petri dishes (6.10). Allow to solidify.

Store the poured plates (upside down) at 5 °C (6.5) in the dark for up to 4 weeks. Protect them from drying.

Alternatively, other appropriate non-selective agar media may be used.

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A.5 Triple sugar–iron agar (TSI agar)

A.5.1 Composition

Meat extract	3,0 g
Yeast extract	3,0 g
Peptone	20,0 g
Sodium chloride (NaCl)	5,0 g
Lactose	10,0 g
Sucrose	10,0 g
Glucose (dextrose)	1,0 g
Iron(III) citrate (ferric citrate)	0,3 g
Sodium thiosulfate ($Na_2S_2O_3$)	0,3 g
Phenol red	0,024 g
Agar	9 g to 18 g[a]
Water	1 000 ml

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

A.5.2 Preparation

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

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

Dispense the medium in quantities of 10 ml into tubes, preferably with screw caps.

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

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

Tubes closed with screw caps can be stored at 5 °C (6.5), in the dark for up to 3 months.

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A.6 Urea agar (UA) Christensen

A.6.1 Base medium

A.6.1.1 Composition

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

[a] For example, enzymatic digest of casein.

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

A.6.1.2 Preparation

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

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

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

Store airtight, closed flasks at 5 °C (6.5), in the dark for up to 3 months.

A.6.2 Urea solution

A.6.2.1 Composition

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

A.6.2.2 Preparation

Dissolve the urea in the water and sterilize by filtration.

Store airtight, closed flasks at 5 °C (6.5), in the dark for up to 3 months.

A.6.3 Complete medium

A.6.3.1 Composition

Base (A.6.1)	950 ml
Urea (A.6.2)	50 ml

A.6.3.2 Preparation

Add, under aseptic conditions, the urea solution to the base, previously melted and then cooled to 47 °C to 50 °C.

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

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Allow to set in a sloping position.

Tubes closed with screw caps can be stored at 5 °C (6.5), in the dark for up to 3 months.

A.7 L-Lysine decarboxylation medium (LDC)

A.7.1 Composition

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

A.7.2 Preparation

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

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

Dispense the medium in quantities of 2-5 ml into sterile narrow culture tubes (6.7), preferably with screw caps.

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

Tubes closed with screw caps can be stored at 5 °C (6.5), in the dark for up to 3 months.

A.8 Performance testing for the quality assurance of the culture media

Information on the performance testing of the culture media indicated in this part of ISO 6579 is given in Table A.1. For the definitions of selectivity and productivity, see ISO/TS 11133-1 and ISO/TS 11133-2.

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Table A.1 — Performance testing of culture media

Medium	Function	Incubation	Control strains[a]	Criterion[c]
BPW	Productivity	18 h at 37 °C	*Salmonella* Typhimurium WDCM 00031[b] *Salmonella* Enteritidis WDCM 00030	Turbidity (1 to 2)
MSRV	Productivity	(2x) 24 h at 41,5 °C	*Salmonella* Typhimurium WDCM 00031[b] *Salmonella* Enteritidis WDCM 00030	Grey-white, turbid zone extending out from inoculated drop(s). After 24 h–48 h, the turbid zone(s) will be (almost) fully migrated over the plate
	Selectivity	(2x) 24 h at 41,5 °C	*Escherichia coli* WDCM 00012[b] *Escherichia coli* WDCM 00013	Possible growth at the place of the inoculated drop(s) without a turbid zone
			Enterococcus faecalis WDCM 00009[b] *Enterococcus faecalis* WDCM 00087	No growth
XLD	Productivity	24 h at 37 °C	*Salmonella* Typhimurium WDCM 00031[b] *Salmonella* Enteritidis WDCM 00030	Good growth (2) of colonies with black centre and a lightly transparent zone of reddish colour due to the colour change of the medium
	Selectivity	24 h at 37 °C	*Escherichia coli* WDCM 00012[b] *Escherichia coli* WDCM 00013	Growth or partial inhibition (0 to 1) of yellow colonies
			Enterococcus faecalis WDCM 00009[b] *Enterococcus faecalis* WDCM 00087	Total inhibition (0)
Nutrient agar	Productivity	24 h at 37 °C	*Salmonella* Typhimurium WDCM 00031	Good growth (2)

[a]For information on culture collection strain numbers and contact details, refer to the reference strain catalogue available on http://refs.wdcm.org/home.htm (WDCM: World Data Centre for Microorganisms).

[b]Strain to be used to be chosen by the end user laboratory (minimum).

[c]Growth is categorized as: 0 — no growth; 1 — weak growth (partial inhibition); 2 — good growth (see ISO/TS 11133-1 and ISO/TS 11133-2).

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Annex B
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Diagram of procedure

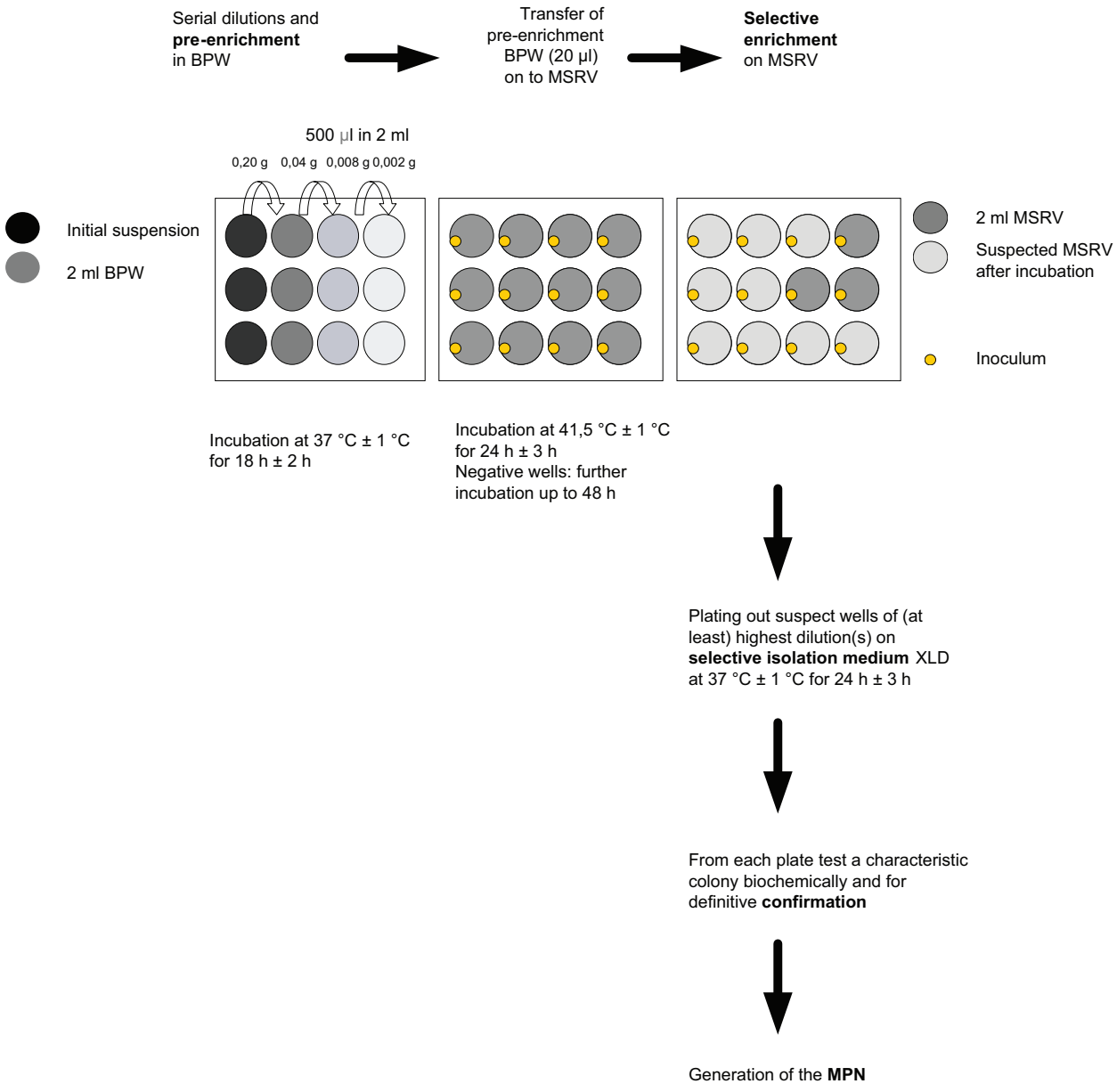

Figure B.1 — Schematic presentation of the mini-MSRV protocol

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Bibliography

[1] FRAVALO, P., HASCOET, Y., LE FELLIC, M., QUEGUINER, S., PETTON, J., SALVAT, G. Convenient method for rapid and quantitative assessment of *Salmonella enterica* contamination: The mini-MSRV MPN technique. *J. Rapid Meth. Automat. Microbiol.* 2003, **11**, pp. 81–88

[2] JARVIS, B., WILRICH, C., WILRICH, P.-T. Reconsideration of the derivation of most probable numbers, their standard deviations, confidence bounds and rarity values. *J. Appl. Microbiol.* 2010, **109**, pp. 1660–1667

[3] GRIMONT, P.A.D., WEILL, F.-X. *Antigenic formulae of the* Salmonella *serovars*, 9th edition, WHO Collaborating Centre for Reference and Research on *Salmonella*. Paris: Institut Pasteur, 2007. 166 p. Available (viewed 2012-10-15) at: http://www.pasteur.fr/ip/portal/action/WebdriveActionEvent/oid/01s-000036-089

[4] VOOGT, N., RAES, M., WANNET, W.J.B., HENKEN, A.M., VAN DE GIESSEN, A.W. Comparison of selective enrichment media for the detection of *Salmonella* in poultry faeces. *Lett. Appl. Microbiol.* 2001, **32**, pp. 89–92

[5] ISO 6579:2002 + Cor.1:2004 + Amd.1:2007, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of* Salmonella *spp.*

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