
**Microbiology of food and animal feeding
stuffs — Horizontal methods for the
detection and enumeration of
Enterobacteriaceae —**

Part 1:
**Detection and enumeration by MPN
technique with pre-enrichment**

*Microbiologie des aliments — Méthodes horizontales pour la recherche
et le dénombrement des Enterobacteriaceae —*

*Partie 1. Recherche et dénombrement à l'aide de la technique NPP
avec préenrichissement*



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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.

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.

This first edition of ISO 21528-1, together with ISO 21528-2, cancels and replaces the following standards:

- ISO 5552:1997, *Meat and meat products — Detection and enumeration of Enterobacteriaceae without resuscitation — MPN technique and colony-count technique*;
- ISO 7402:1993, *Microbiology — General guidance for the enumeration of Enterobacteriaceae without resuscitation — MPN technique and colony-count technique*;
- ISO 8523:1991, *Microbiology — General guidance for the detection of Enterobacteriaceae with pre-enrichment*.

ISO 21528-1 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

ISO 21528 consists of the following parts, under the general title *Microbiology of food and animal feeding stuffs — Horizontal methods for the detection and enumeration of Enterobacteriaceae*:

- *Part 1: Detection and enumeration by MPN technique with pre-enrichment*
- *Part 2: Colony-count method*

Introduction

This part of ISO 21528 is intended to provide general guidance for the examination of products not dealt with by existing International Standards and to be taken into account by organizations preparing microbiological test methods for application to foods or animal feeding stuffs. Because of the large variety of products within this field of application, these guidelines may not be appropriate in every detail for certain products, and for some other products it may be necessary to use different methods. Nevertheless, it is hoped that in all cases every attempt will be made to apply the guidelines provided as far as possible and that deviations from them will only be made if absolutely necessary for technical reasons.

When this part of ISO 21528 is next reviewed, account will be taken of all information then available regarding the extent to which the guidelines have been followed and the reasons for deviation from them in the case of particular products.

The harmonization of test methods cannot be immediate, and for certain groups of products International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed they will be changed to comply with this part of ISO 21528 so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

Microbiology of food and animal feeding stuffs — Horizontal methods for the detection and enumeration of Enterobacteriaceae —

Part 1: Detection and enumeration by MPN technique with pre-enrichment

1 Scope

This part of ISO 21528 specifies a method, with pre-enrichment, for the detection of Enterobacteriaceae. It is applicable to

- products intended for human consumption and the feeding of animals, and
- environmental samples in the area of food production and food handling.

Enumeration is carried out by calculation of the most probable number (MPN) after incubation at 37 °C (or 30 °C)¹⁾ in liquid medium.

This method is applicable

- when the microorganisms sought are expected to need resuscitation before enrichment, and
- when the number sought is expected to be in the range 1 to 100 per millilitre or per gram of test sample.

A limitation on the applicability of this part of ISO 21528 is imposed by the susceptibility of the method to a large degree of variability (see Clause 11).

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 6887-1:1999, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*

1) The temperature of 37 °C is generally used when the enumeration of Enterobacteriaceae is for a hygienic indicator. Alternatively, a temperature of 30 °C can be chosen when the enumeration of Enterobacteriaceae is conducted for technological purposes and includes psychrotrophic Enterobacteriaceae.

ISO 6887-2, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 2: Specific rules for the preparation of meat and meat products*

ISO 6887-3, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products*

ISO 6887-4, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products*

ISO 7218:1996, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations*

ISO 8261, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

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 11133-2:2003, *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 Enterobacteriaceae
microorganisms that form characteristic colonies on violet red bile glucose agar and that ferment glucose and show a negative oxidase reaction when the tests are carried out in accordance with the methods specified in this part of ISO 21528

3.2 detection of Enterobacteriaceae
determination of the presence or absence of these bacteria, in a particular quantity of product, when tests are carried out in accordance with this part of ISO 21528

3.3 enumeration of Enterobacteriaceae
most probable number of Enterobacteriaceae found per millilitre or per gram of the test sample when the test is carried out according to the method specified in this part of ISO 21528

4 Principle

4.1 Detection of Enterobacteriaceae (see Annex A)

4.1.1 Pre-enrichment in non-selective medium

Buffered peptone water (BPW) is inoculated with the test portion, then incubated at 37 °C (or 30 °C)¹⁾ for 18 h ± 2 h.

4.1.2 Enrichment in selective liquid medium

The enrichment broth [buffered brilliant green bile glucose broth (EE broth)] is inoculated with the culture obtained after pre-enrichment, then incubated at 37 °C (or 30 °C)¹ for 24 h ± 2 h.

4.1.3 Isolation and selection for confirmation

A selective solid medium (violet red bile glucose agar) is inoculated with the culture obtained after enrichment in EE broth, then incubated at 37 °C (or 30 °C)¹. It is examined after 24 h ± 2 h to check for the presence of colonies presumed by their characteristics to be Enterobacteriaceae.

4.1.4 Confirmation

Colonies of presumptive Enterobacteriaceae are subcultured onto non-selective medium, and confirmed by means of tests for the fermentation of glucose and the presence of oxidase.

4.2 Enumeration by the MPN technique (see Annex B)

4.2.1 Pre-enrichment in non-selective medium

A test portion of x g is added to $9x$ ml of buffered peptone water (BPW) and homogenized. One or more 10-fold dilutions (according to the expected level of contamination) are prepared in BPW. Aliquots (10 ml) of this initial dilution are transferred to three tubes. Then 3×1 ml of the initial dilution are added to 9 ml of BPW and 3×1 ml of each further dilution are added to 9 ml of BPW. These tubes are incubated 37 °C (or 30 °C)¹ for 18 h ± 2 h.

4.2.2 Enrichment in selective liquid medium

Tubes of liquid enrichment broth (EE broth) are inoculated with each tube of culture obtained after pre-enrichment (at least 3×3). The tubes are incubated at 37 °C (or 30 °C)¹ for 24 h ± 2 h.

4.2.3 Isolation and selection for confirmation

A selective solid medium (violet red bile glucose agar) is inoculated with a loop from each of the incubated cultures obtained after enrichment in EE broth, then incubated at 37 °C (or 30 °C)¹. It is examined after 24 h ± 2 h to check for the presence of colonies presumed by their characteristics to be Enterobacteriaceae.

4.2.4 Confirmation

Colonies of presumptive Enterobacteriaceae are subcultured on non-selective medium, then confirmed by means of tests for the fermentation of glucose and the presence of oxidase.

4.2.5 Calculation

The most probable number of Enterobacteriaceae per millilitre or per gram of the test sample is calculated from the number of confirmed positive tubes using the MPN table (see ISO 7218).

5 Diluent, culture media and reagent

For current laboratory practice, see ISO 7218 and ISO/TS 11133-1 and ISO/TS 11133-2.

5.1 Diluent: buffered peptone water (BPW)

See ISO 6887-1:1999, 5.2.2.

BPW is used as the non-selective pre-enrichment medium for the enumeration method.

5.2 Culture media

5.2.1 Enrichment medium: Buffered brilliant green bile glucose broth (EE broth)

5.2.1.1 Composition

Enzymatic digest of animal tissues	10,0 g
Glucose	5,0 g
Disodium hydrogen phosphate (Na ₂ HPO ₄)	6,45 g
Potassium dihydrogen phosphate (KH ₂ PO ₄)	2,0 g
Beef bile for bacteriological use	20,0 g
Brilliant green (bacteriological quality)	0,0135 g
Water	1 000 ml

5.2.1.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by boiling. Do not heat the medium for longer than 30 min. Cool the medium rapidly.

Adjust the pH, if necessary, so that after boiling it is $7,2 \pm 0,2$ at 25 °C.

Dispense the medium in 10 ml amounts into sterile tubes of appropriate capacity (6.5).

Do not sterilize the medium.

The medium may be stored for up to 1 month at $5\text{ °C} \pm 3\text{ °C}$.

5.2.1.3 Performance testing for the quality assurance of the culture medium

For the definition of selectivity and productivity, refer to ISO/TS 11133-1. For the performance criteria, refer to ISO/TS 11133-2:2003, Table B.3.

5.2.2 Violet red bile glucose (VRBG) agar

5.2.2.1 Components

Enzymatic digest of animal tissues	7,0 g
Yeast extract	3,0 g
Bile salts No. 3	1,5 g
Glucose	10,0 g
Sodium chloride	5,0 g
Neutral red	0,03 g
Crystal violet	0,002 g
Agar	9 g to 18 g ^{a)}
Water	1 000 ml

^{a)} Depending on the gel strength of the agar.

5.2.2.2 Preparation

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

Adjust the pH, if necessary, so that after boiling it is $7,4 \pm 0,2$ at 25 °C.

Dispense the culture medium into sterile tubes or flasks (6.5) of appropriate capacity.

Do not sterilize the medium.

Use the molten medium within 4 h of its preparation.

5.2.2.3 Preparation of agar plates

Immediately transfer approximately 15 ml of the culture medium, cooled to between 44 °C and 47 °C (6.4), to Petri dishes (6.7) and allow to solidify.

Just before use, dry the plates, preferably with the lids off and the agar surface downwards, in a drying cabinet (6.3) until the agar is dry.

If prepared in advance, the undried plates may be stored in conditions that do not change their composition for up to 2 weeks at $5 \text{ °C} \pm 3 \text{ °C}$.

5.2.2.4 Performance testing for the quality assurance of the culture medium

For the definition of selectivity and productivity, refer to ISO/TS 11133-1. For the performance criteria, refer to ISO/TS 11133-2:2003, Table B.1.

5.2.3 Nutrient agar**5.2.3.1 Composition**

Meat extract	3,0 g
Enzymatic digest of animal tissues	5,0 g
Sodium chloride	5,0 g
Agar	9 g to 18 g ^{a)}
Water	1 000 ml
^{a)} Depending on the gel strength of the agar.	

5.2.3.2 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 $7,3 \pm 0,2$ at 25 °C.

Dispense the culture medium into sterile tubes or flasks (6.5) of appropriate capacity.

Sterilize for 15 min in an autoclave (6.1) set at 121 °C.

5.2.3.3 Preparation of agar plates

Transfer portions of about 15 ml of the culture medium, melted and cooled to approximately 47 °C, to Petri dishes (6.7) and allow to solidify.

Just before use, dry the plates, preferably with the lids off and the agar surface downwards, in a drying cabinet (6.3) until the agar is dry.

If prepared in advance, the undried plates may be stored in conditions that do not change their composition for up to 2 weeks at 5 °C ± 3 °C.

5.2.3.4 Performance testing for the quality assurance of the culture medium

For the definition of selectivity and productivity, refer to ISO/TS 11133-1. For the performance criteria, refer to ISO/TS 11133-2:2003, Table B.6.

5.2.4 Glucose agar

5.2.4.1 Composition

Enzymatic digest of casein	10,0 g
Yeast extract	1,5 g
Glucose	10,0 g
Sodium chloride	5,0 g
Bromocresol purple	0,015 g
Agar	9 g to 18 g ^{a)}
Water	1 000 ml

^{a)} Depending on the gel strength of the agar.

5.2.4.2 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 ± 0,2 at 25 °C.

Dispense the culture medium in tubes (6.6) of appropriate capacity (e.g. 10 ml of culture medium for tubes of 16 mm × 160 mm).

Sterilize for 15 min in an autoclave (6.1) set at 121 °C. Leave the tubes in a vertical position.

The medium may be stored for up to 1 week at 5 °C ± 3 °C.

In order to remove oxygen, just before use, heat the medium in boiling water or flowing steam for 15 min, then cool rapidly to the incubation temperature.

5.3 Oxidase reagent

5.3.1 Composition

<i>N,N,N',N'</i> -Tetramethyl- <i>p</i> -phenylenediamine dihydrochloride	1,0 g
Water	100 ml

5.3.2 Preparation

Dissolve the component in the cold water.

Prepare the reagent just before use.

Commercially available disks or sticks may be used. In this case, follow the manufacturer's recommendations.

6 Apparatus and glassware

Usual microbiological laboratory apparatus and, in particular, the following (see ISO 7218).

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

See ISO 7218.

6.2 Incubator, capable of operating at $37\text{ °C} \pm 1\text{ °C}$.

6.3 Drying cabinet (ventilated by convection) or incubator, capable of operating between 37 °C and 55 °C .

6.4 Water bath, or similar apparatus, capable of being maintained at between 44 °C and 47 °C .

6.5 Containers (e.g. bottles, tubes, flasks), suitable for the sterilization and storage of culture media.

6.6 Test tubes, of dimensions $16\text{ mm} \times 160\text{ mm}$ and $20\text{ mm} \times 200\text{ mm}$, and flasks or bottles of capacity between 150 ml and 500 ml .

6.7 Petri dishes, made of glass or plastics, of diameter 90 mm to 100 mm .

6.8 Loops (of diameter approximately 3 mm) and wires, made of platinum/iridium or nickel/chromium, or glass rods, or equivalent sterile disposable loops or inoculating needles.

6.9 Graduated pipettes, of 1 ml nominal capacity, graduated with $0,1\text{ ml}$ divisions, and with an outflow opening of appropriate diameter.

6.10 pH-meter, accurate to within $\pm 0,1\text{ pH}$ unit at 25 °C .

6.11 Homogenizer.

See ISO 7218

7 Sampling

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

Sampling should be carried out in accordance with the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that agreement be reached on this subject by the parties concerned.

8 Preparation of test sample

Prepare the test sample in accordance with ISO 6887-1, ISO 6887-2, ISO 6887-3, ISO 6887-4 or ISO 8261, and/or the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that agreement be reached on this subject by the parties concerned.

9 Procedure

9.1 General

See ISO 7218.

The culture media are usually incubated at 37 °C. Alternatively, an incubation temperature of 30 °C may be used.¹⁾

9.2 Detection method

9.2.1 Test portion and initial suspension

Take a test portion (x g or x ml) according to the sensitivity required and homogenize in $9x$ ml of buffered peptone water (5.1). Transfer the appropriate volume to a sterile tube or container (6.5) according to the limit of detection required.

9.2.2 Non-selective pre-enrichment

Incubate the initial suspension (9.2.1) at 37 °C for 18 h \pm 2 h.

9.2.3 Selective enrichment

Transfer 1 ml of the culture obtained in 9.2.2 to a tube containing 10 ml of the enrichment medium (5.2.1).

Incubate the inoculated medium at 37 °C for 24 h \pm 2 h.

Continue the procedure with isolation and selection for confirmation (9.4).

9.3 Enumeration method (MPN method)

9.3.1 Test portion, initial suspension and further dilutions

Depending on the desired accuracy of the results, inoculate an appropriate number of flasks or tubes with the same dilution (e.g. three, five or ten flasks or tubes). As a general rule, the techniques specified require three flasks or tubes per dilution.

For preparation of the initial suspensions and further dilutions, use the diluent (5.1).

To prepare the initial suspension, take a test portion of x g or x ml and homogenize in $9x$ ml of buffered peptone water (BPW) (5.1). A 10^{-1} dilution is thus obtained.

NOTE 10 ml of the 10^{-1} dilution contain the equivalent of 1 g or 1 ml of sample.

Prepare further dilutions by taking 1 ml of the 10^{-1} dilution and add to 9 ml of BPW. Repeat to make further necessary decimal dilutions.

Transfer three 10 ml volumes of the 10^{-1} suspension to tubes (6.5). Transfer three 1 ml volumes of each subsequent dilution to tubes containing 9 ml of BPW (see Annex B).

9.3.2 Non-selective pre-enrichment

Incubate the initial suspensions and dilutions (total 9 tubes) (see 9.3.1) at 37 °C for 18 h \pm 2 h.

9.3.3 Selective enrichment

Transfer 1 ml of the cultures obtained in 9.3.2 to tubes containing 10 ml of the enrichment medium (5.2.1).

Incubate the inoculated media at 37 °C for 24 h \pm 2 h.

9.4 Isolation and selection for confirmation

9.4.1 Isolation

Using a loop (6.8), streak from the incubated enrichment medium (see 9.2.3) or from each of the incubated tubes (see 9.3.3) the surface of a plate containing the selective medium (5.2.2) and incubate the plate at 37 °C for 24 h ± 2 h.

9.4.2 Selection of colonies for confirmation

Characteristic colonies are pink to red or purple (with or without precipitation haloes).

From each of the incubated plates (see 9.4.1) on which characteristic colonies have developed, choose a well-isolated characteristic colony for subculturing (see 9.5) for the biochemical confirmation tests (see 9.6).

If more than one morphology is present in the colonies, select one colony of each morphology for subculture.

Certain Enterobacteriaceae may cause decoloration of their colonies or of the medium. Therefore, when no characteristic colonies are present, choose whitish colonies for confirmation.

9.5 Subculturing selected colonies

Streak onto nutrient agar plates (5.2.3) each of the colonies selected for confirmation (see 9.4.2).

Incubate these plates at 37 °C for 24 h ± 2 h.

Select a well-isolated colony from each of the incubated plates for the biochemical confirmation tests (see 9.6).

9.6 Biochemical confirmation tests

9.6.1 Oxidase reaction

Using a platinum/iridium loop, wire or glass rod (6.8), take a portion of each well-isolated colony (see 9.5) and streak onto a filter paper moistened with the oxidase reagent (5.3) or onto a commercially available disc. A nickel/chromium loop or wire shall not be used.

Consider the test to be negative if the colour of the filter paper does not turn dark within 10 s.

Consult the manufacturer's instructions for ready-to-use discs.

9.6.2 Fermentation test

Using a wire (6.8), stab the same colonies selected in 9.5 that gave a negative oxidase test into tubes containing glucose agar (5.2.4).

Incubate these tubes at 37 °C for 24 h ± 2 h.

If a yellow colour develops throughout the contents of the tube, regard the reaction as being positive.

10 Expression of results

10.1 Confirmed positive tubes

If any of the selected typical colonies (see 9.4.2) from a subculture (see 9.4.1) is oxidase-negative and glucose-positive, the tube from which the subculture was derived shall be regarded as being positive for Enterobacteriaceae.

10.2 Detection method

In accordance with the results of the interpretation (see 10.1), indicate the presence or absence of Enterobacteriaceae in a test portion of x g or x ml of product (see ISO 7218).

10.3 Enumeration method: Calculation of the most probable number (MPN)

Calculate the most probable number from the number of positive tubes at each dilution. See ISO 7218:1996, 9.4.

11 Precision

It is known that wide variations in results can occur with the MPN technique. Results obtained by this method should therefore be used with caution. Confidence limits are given in ISO 7218:1996, Annex A.

12 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 part of ISO 21528;
- the incubation temperature used;
- all operating details not specified in this part of ISO 21528, or regarded as optional, together with details of any incidents which may have influenced the test results;
- the test results obtained.