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**Microbiology of the food chain —  
Horizontal method for the detection  
of *Cronobacter* spp.**

*Microbiologie de la chaîne alimentaire — Méthode horizontale pour  
la recherche de Cronobacter spp.*

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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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see [www.iso.org/directives](http://www.iso.org/directives)).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see [www.iso.org/patents](http://www.iso.org/patents)).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: [www.iso.org/iso/foreword.html](http://www.iso.org/iso/foreword.html).

This document was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/275, *Food Analysis — Horizontal methods*, in collaboration with ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in accordance with the agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This first edition cancels and replaces ISO/TS 22964:2006, which has been technically revised with the following changes:

- the scope has been extended to *Cronobacter* spp. detection in food products for humans and feeding animals and environmental samples and the title changed accordingly;
- the enrichment broth, modified lauryl sulfate tryptose broth (mLST), has been replaced by *Cronobacter* selective broth (CSB);
- the isolation agar, *Enterobacter sakazakii* isolation agar (ESIA) has been replaced by chromogenic *Cronobacter* isolation (CCI) agar;
- several confirmation tests have been replaced by other tests according to [Table 1](#) of this document.

## Introduction

This document describes a horizontal method for the detection of *Cronobacter* spp. in food, in animal feed and in environmental samples. The main changes, listed in the foreword, introduced in this document compared to ISO/TS 22964:2006 are considered as major (see ISO 17468[2]).

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods, which are specific to these products, may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this horizontal method as far as possible.

When this document is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from this method 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 document so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

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# Microbiology of the food chain — Horizontal method for the detection of *Cronobacter* spp.

## 1 Scope

This document specifies a horizontal method for the detection of *Cronobacter* spp.

Subject to the limitations discussed in the introduction, this document is applicable to

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

## 2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

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

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

## 3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- IEC Electropedia: available at <http://www.electropedia.org/>
- ISO Online browsing platform: available at <http://www.iso.org/obp>

### 3.1

#### ***Cronobacter* spp.**

microorganisms which form typical colonies on chromogenic *Cronobacter* isolation (CCI) agar<sup>[10]</sup> and which display the biochemical characteristics described, when tests are carried out in accordance with this document

### 3.2

#### **detection of *Cronobacter* spp.**

determination of *Cronobacter* spp. (3.1) in a particular mass or volume of product or surface area when tests are carried out in accordance with this document

## 4 Abbreviated terms

For the purposes of this document, the following abbreviations apply.

BPW	buffered peptone water
CCI	chromogenic <i>Cronobacter</i> isolation
CSB	<i>Cronobacter</i> selective broth
TSA	tryptone soya agar

## 5 Principle

### 5.1 Non-selective pre-enrichment in BPW

A test portion is inoculated into BPW, then incubated between 34 °C and 38 °C for 18 h ± 2 h.

NOTE *Cronobacter* spp. can be present in small numbers accompanied by other *Enterobacteriaceae* such as *E. cloacae* that could interfere with their detection.

### 5.2 Enrichment in a selective medium (CSB)

The selective enrichment medium is inoculated with the culture obtained in 5.1 and incubated at 41,5 °C ± 1 °C for 24 h ± 2 h.

### 5.3 Plating out and identification on chromogenic agar (CCI agar)

The chromogenic agar is streaked for isolation with the enrichment culture obtained in 5.2 and incubated at 41,5 °C ± 1 °C for 24 h ± 2 h.

### 5.4 Confirmation

Typical colonies are selected from the chromogenic agar, purified on a non-selective agar such as TSA and biochemically characterized.

## 6 Culture media and reagents

For current laboratory practice, see ISO 7218 and ISO 11133.

Composition of culture media and reagents and their preparation are described in [Annex B](#).

For performance testing of culture media see ISO 11133 and/or [Annex B](#).

## 7 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications.

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

### 7.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave).

As specified in ISO 7218.

### 7.2 Incubators, capable of operating in the range 34 °C to 38 °C, 37 °C ± 1 °C and 41,5 °C ± 1 °C.

**7.3 Sterile loops**, of approximate diameter 3 mm (10 µl volume), and of 1 µl volume, and inoculation needle or wire.

**7.4 pH meter**, having an accuracy of calibration of  $\pm 0,1$  pH unit at 25 °C.

**7.5 Flasks and bottles**, with closures, of suitable capacities for use in the preparation of enrichment broths and agars and their storage.

**7.6 Sterile graduated pipettes or automatic pipettes**, of nominal capacities 10 ml, 1 ml and 0,1 ml.

**7.7 Tubes** (plugged or with caps) **or culture bottles**, of appropriate capacity, with non-toxic metallic caps with liners or plastic disposable caps (see ISO 7218).

**7.8 Petri dishes**, of diameter approximately 90 mm.

**7.9 Spectrophotometer**, capable of measuring absorption of light with a wavelength of 405 nm.

**7.10 Pestle and mortar**.

**7.11 Refrigerators**, capable of operating at  $5\text{ °C} \pm 3\text{ °C}$

**7.12 Water baths**, capable of operating between 47 °C and 50 °C and at  $37\text{ °C} \pm 1\text{ °C}$ .

**7.13 Drying cabinet (or oven ventilated by convection)**, capable of being maintained between 25 °C and 50 °C.

## 8 Sampling

Sampling is not part of the method specified in this document. See the specific International Standard dealing with the product concerned. If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the interested parties come to an agreement on this subject.

A recommended sampling method is given in ISO/TS 17728<sup>[3]</sup> for food and animal feed, and ISO 18593<sup>[4]</sup> for sampling of surfaces.

It is important that the laboratory receives a sample which is truly representative and which has not been damaged or changed during transport or storage (see ISO 7218).

## 9 Preparation of test sample

Prepare the test sample from the laboratory sample in accordance with the specific International Standard dealing with the product concerned: see ISO 6887 (all parts). If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

## 10 Procedure (as shown in [Annex A](#))

### 10.1 Test portion

In general, to prepare the primary dilution, add 10 g or 10 ml of the test sample ([Clause 9](#)) to 90 ml of pre-enrichment medium ([B.1](#)) (BPW), to yield a tenfold dilution. Pre-warm the BPW to room temperature

before use. For specific products, follow the procedures specified in ISO 6887 (all parts). For dry milk, follow ISO 6887-5.

This document has been validated for test portions of 10 g. A smaller size of the test portion may be used without the need of additional validation/verification providing that the same ratio between pre-enrichment broth and test portion is maintained. A larger test portion than that initially validated may be used, if a validation/verification study has shown that there are no negative effects on the detection of *Cronobacter* spp.

NOTE 1 Validation can be conducted in accordance with the appropriate documents of ISO 16140 (all parts). Verification for pooling samples can be conducted in accordance with the protocol described in ISO 6887-1:2017, Annex D (verification protocol for pooling samples for qualitative tests).

NOTE 2 Large sample sizes can compromise the recovery of stressed *Cronobacter* spp. when interfering microflora are present, such as probiotics.<sup>[5][6]</sup>

For preparing quantities larger than 10 g, BPW should be pre-warmed between 34 °C and 38 °C (7.2) before inoculated with the test portion.

## 10.2 Pre-enrichment

Incubate the inoculated pre-enrichment medium prepared in accordance with 10.1 between 34 °C and 38 °C (7.2) for 18 h ± 2 h.

## 10.3 Enrichment

After incubation of the inoculated pre-enrichment medium, mix well and transfer 0,1 ml of the obtained culture 10.2 into 10 ml of CSB (B.2) and mix well. Incubate at 41,5 °C (7.2) for 24 h ± 2 h.

## 10.4 Isolation of presumptive *Cronobacter* spp.

Allow the CCI (B.3) plates to equilibrate at room temperature if they are stored at a lower temperature. If necessary, dry the surface of the plates (7.13) following the procedure given in ISO 11133.

From enrichment culture, mix well and inoculate, by means of a 10 µl loop (7.3), the surface of the CCI<sup>17</sup> agar (B.3) to obtain well-separated colonies. Incubate the plate at 41,5 °C (7.2) for 24 h ± 2 h.

After incubation, examine the chromogenic plate for the presence of typical colonies of presumptive *Cronobacter*.

Typical *Cronobacter* colonies on CCI are small to medium-sized (1 mm to 3 mm) and blue to blue-green in colour. Non-*Cronobacter* colonies are often white or white with a green centre, grey or black. Some naturally pigmented colonies of non-*Cronobacter* can appear yellow or red.

## 10.5 Confirmation

### 10.5.1 General

For confirmation, sub-culture from the selective medium CCI (see 10.4) five marked typical or suspect colonies. In the case that colonies are not well separated, it might be necessary to streak a typical colony first on the selective agar (B.3) again.

If on the dish there are fewer than five typical or suspect colonies, take all the marked colonies for confirmation.

Use pure cultures for biochemical confirmation.

### 10.5.2 Purification of colonies

Streak the selected colonies onto the surface of a non-selective agar such as TSA (B.4) so as to gain well-isolated colonies.

Incubate the plates inverted between 34 °C and 38 °C (7.2) for 21 h ± 3 h.

If the cultures on the non-selective agar are mixed, sub-culture the suspect colony onto a further plate of the non-selective agar and incubate between 34 °C and 38 °C (7.2) for 21 h ± 3 h to obtain a pure culture.

It is possible to first test the most characteristic colony from the selective agar plate. If positive, it is not necessary to test other colonies. If negative, progress through the other selected colonies until either all are negative or a positive is found.

Strains can be kept on the non-selective agar at 5 °C (7.11), but cannot be stored for more than seven days. Fresh subcultures of the colonies should be obtained before performing confirmation tests.

### 10.5.3 Biochemical confirmation

#### 10.5.3.1 General

Carry out the confirmation tests listed in Table 1.

**Table 1 — Confirmation tests for *Cronobacter* spp.**

Oxidase	Acid from:
Hydrolysis of 4-Nitrophenyl $\alpha$ -D-glucopyranoside substrate	D-Arabitol
L-Lysine decarboxylase	D-Sorbitol
L-Ornithine decarboxylase	D-Sucrose
Methyl Red (optional)	$\alpha$ -Methyl-D-glucoside (optional)
Voges-Proskauer (optional)	

NOTE 1 If shown to be reliable, miniaturized galleries for the biochemical identification of *Cronobacter* spp., can be used (see ISO 7218).

NOTE 2 Other alternative procedures can be used to confirm the isolate as *Cronobacter* spp., provided that the suitability of the alternative procedure has been verified (see also ISO 7218).

#### 10.5.3.2 Oxidase

Using a platinum-iridium or plastic loop (7.3), take a portion of a well-isolated colony from each individual plate (10.5.2) and streak it on to a filter paper moistened with the oxidase reagent (B.5.1); the appearance of a mauve, violet or deep blue colour within 10 s indicates a positive reaction. If a commercially available oxidase test kit is used, follow the manufacturer's instructions.

#### 10.5.3.3 Hydrolysis of 4 Nitrophenyl (PNP) $\alpha$ -D-glucopyranoside substrate

Using a loop or wire (7.3), suspend an individual colony grown on the non-selective agar such as TSA (10.5.2) in 2 ml of physiological salt solution, 0,85 % NaCl (B.5.2.4). Add 2 ml of the  $\alpha$ -Glucosidase enzymatic assay solution (B.5.2). Incubate in a water bath at 37 °C (7.12) for 4 h and measure the formation of yellow colouration in a spectrophotometer (7.9) at 405 nm. A minimal absorption of 0,3 at 405 nm after 4 h, equivalent to 16 mM PNP, can be considered positive.