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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

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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).

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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.

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— the enrichment broth, modified lauryl sulfate tryptose broth (mLST), has been replaced by *Cronobacter* selective broth (CSB);

— the isolation agar, *Enterobacter sakazakii* isolation agar (ESIA) has been replaced by chromogenic *Cronobacter* isolation (CCI) agar;

— several confirmation tests have been replaced by other tests according to Table 1 of this document.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.

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[10] 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 *Cronobacter* isolation

CSB *Cronobacter* 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 ± 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 °C ± 3 °C

7.12 Water baths, capable of operating between 47 °C and 50 °C and at 37 °C ± 1 °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[3] for food and animal feed, and ISO 18593[4] 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.[5][,[6]

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 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 α-D-glucopyranoside substrate	D-Arabitol
L-Lysine decarboxylase	D-Sorbitol
L-Ornithine decarboxylase	D-Sucrose
Methyl Red (optional)	α-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) α-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 α-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.

10.5.3.4 L-Lysine decarboxylase

Using a loop or wire (7.3), inoculate the L-lysine decarboxylation medium (B.5.3) with each of the selected colonies (10.5.2) just below the surface of the medium. Incubate the tubes at 37 °C (7.2) for 24 h ± 2 h.

A violet colour after incubation indicates a positive reaction. A yellow colour indicates a negative reaction.

10.5.3.5 L-Ornithine decarboxylase

Using a loop or wire (7.3), inoculate the L-ornithine decarboxylation medium (B.5.4) with each of the selected colonies (10.5.2) just below the surface of the medium. Incubate the tubes at 37 °C (7.2) for 24 h ± 2 h.

A violet colour after incubation indicates a positive reaction. A yellow colour indicates a negative reaction.

10.5.3.6 Fermentation of various carbohydrates

Using a loop or wire (7.3), inoculate the carbohydrate fermentation medium (B.5.5) with each of the selected colonies (10.5.2) just below the surface of the medium. Incubate the tubes at 37 °C (7.2) for 48 h ± 2 h.

A yellow colour after incubation indicates a positive reaction. A red colour indicates a negative reaction.

10.5.3.7 Methyl red (MR) (optional)

Using a loop or wire (7.3), inoculate a tube of MR-VP broth (B.5.6) with each of the selected colonies (10.5.2) just below the surface of the medium. Incubate the tubes at 37 °C (7.2) for 48 h ± 2 h. Add five drops of methyl red solution (B.5.6.2) to each tube. A distinct red colour indicates a positive reaction. A yellow colour indicates a negative reaction.

10.5.3.8 Voges-Proskauer (VP) (optional)

Using a loop or wire (7.3), inoculate a tube of MR-VP broth (B.5.6) with each of the selected colonies (10.5.2) just below the surface of the medium. Incubate the tubes at 37 °C (7.2) for 24 h ± 2 h. Add 0,2 ml 40 % KOH (B.5.6.3.1) and 0,6 ml 1–naphthol solution (B.5.6.3.2). Let stand 1 h ± 0,5 h. An eosin pink colour around the surface of the medium indicates a positive reaction.

10.6 Interpretation of biochemical results

Cronobacter spp. are oxidase negative and are able to hydrolyse 4-Nitrophenyl α-D-glucopyranoside substrate and to produce acid from sucrose. They are unable to decarboyxlate L-lysine and to produce acid from D-sorbitol. Only very few *Cronobacter* strains give a positive methyl red reaction, and a negative Voges Proskauer test is also very uncommon. *Cronobacter* spp. can be distinguished from closely related species by the characteristics listed in Annex C.

NOTE *Cronobacter* spp. refers to the descriptions found in References [9] and [11].

11 Expression of results

In accordance with the results of the biochemical tests and their interpretation, indicate if *Cronobacter* spp. is detected or not detected in a test portion of *x* g or *x* ml of product (see ISO 7218).

12 Performance characteristics of the method

12.1 Interlaboratory study

The accuracy (precision) of the method was determined in an interlaboratory study to determine the specificity, sensitivity and, when possible, the LOD_{50} of the method. The data are summarized in Annex D. The values derived from the interlaboratory study may not be applicable to food types other than those given in Annex D.

12.2 Sensitivity

The sensitivity is defined as the number of samples found positive divided by the number of samples tested at a given level of contamination. The results are thus dependent on the level of contamination of the sample.

12.3 Specificity

The specificity is defined as the number of samples found negative divided by the number of blank samples tested.

12.4 LOD_{50}

The LOD_{50} is the concentration (cfu/sample) for which the probability of detection is 50 %.

13 Test report

The test report shall contain at least the following information:

a) the sampling method used, if known;

b) the method used, with reference to this document, i.e. ISO 22964;

c) test results obtained;

d) all operating details not specified in this document or regarded as optional, together with details of any incidents which may have influenced the test results;

e) all information necessary for the complete identification of the sample.

Annex A
(normative)

Diagram of the test procedure

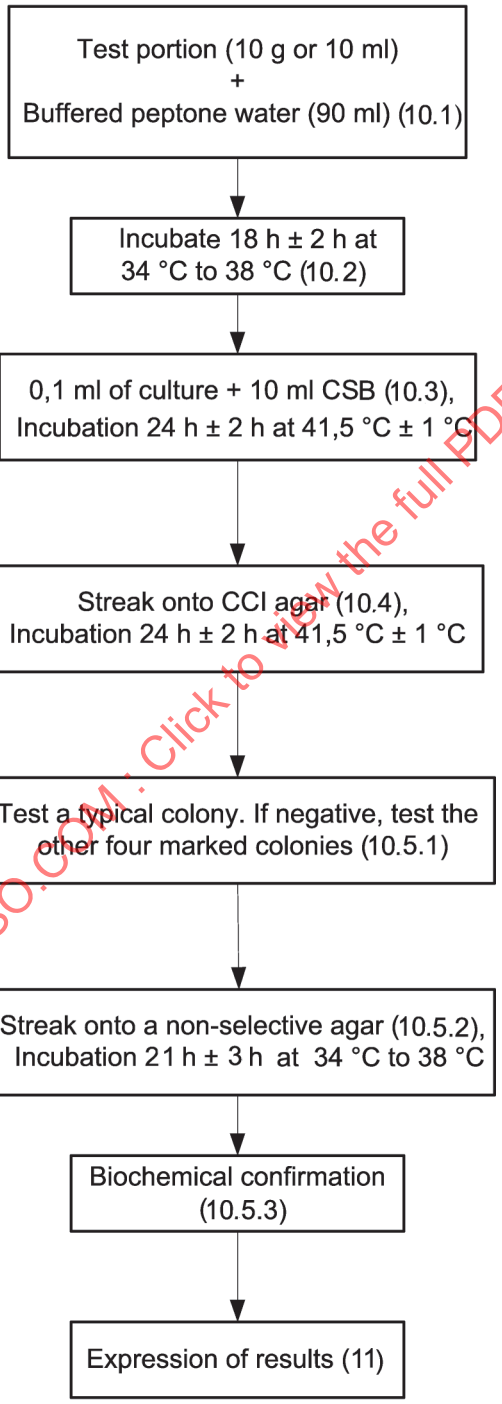

Figure A.1 — Flow diagram of the procedure for detection of *Cronobacter* spp. in food, animal feed and environmental samples from the food production area

Annex B
(normative)

Composition, preparation and performance testing of culture media and reagents

B.1 Buffered peptone water (BPW)

B.1.1 Composition

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

[a] For example enzymatic digest of casein.

B.1.2 Preparation

Dissolve the ingredients in 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 medium into flasks (7.5) of suitable capacity to obtain the portions necessary for the test.

Sterilize for 15 min in the autoclave (7.1) set at 121 °C. Store the medium in closed flasks at 5 °C (7.11) for up to 6 months.

B.2 *Cronobacter* selective broth (CSB)

B.2.1 Base medium

B.2.1.1 Composition

Enzymatic digest of animal tissues	10,0 g
Meat extract	3,0 g
Sodium chloride (NaCl)	5,0 g
Bromocresol purple	0,04 g
Sucrose ($C_{12}H_{22}O_{11}$)	10,0 g
Water	1 000 ml

B.2.1.2 Preparation

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

Adjust the pH, if necessary, so that after sterilization it is 7,4 ± 0,2 at 25 °C. Dispense 10 ml of the base medium into tubes (7.7). Sterilize the tubes at 121 °C for 15 min in the autoclave (7.1). Store the medium at 5 °C (7.11) for up to 6 months.

B.2.2 Vancomycin solution

B.2.2.1 Composition

Vancomycin hydrochloride (CAS Number 1404-93-9)	10 mg
Water	10 ml

B.2.2.2 Preparation

Dissolve the vancomycin in the distilled water. Mix and sterilize by filtration through filter pore size 0,2 µm.

The vancomycin solution may be kept at 5 °C (7.11) for 15 days.

B.2.3 Complete medium

Add aseptically 0,1 ml of vancomycin solution (B.2.2) to 10 ml of base medium (B.2.1) so as to obtain a final vancomycin concentration of 10 mg per litre of CSB.

The complete CSB may be kept at 5 °C (7.11) for 1 day.

B.3 Chromogenic *Cronobacter* isolation (CCI) agar

B.3.1 Composition

Tryptic digest of casein	7,0 g
Yeast extract	3,0 g
Sodium chloride (NaCl)	5,0 g
5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside	0,15 g
Sodium desoxycholate ($C_{24}H_{39}NaO_4$)	0,25 g
Ammonium iron(III) citrate ($C_6H_8O_7$ Fe NH_3)	1 g
Sodium thiosulfate ($Na_2S_2O_3$)	1 g
Agar	9,0 g to 18,0 g[a]
Water	1 000 ml

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

B.3.2 Preparation

Grind the 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside with the sodium chloride in a pestle and mortar (7.10) until the 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside is an evenly distributed fine powder. Dissolve the other components in the water by boiling, remove from the heat and add the salt: 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside mixture. Adjust the pH (7.4), if necessary, so that after sterilization it is 7,3 ± 0,2 at 25 °C.

Sterilize in the autoclave (7.1) at 121 °C for 15 min. Cool to between 47 °C and 50 °C. Pour about 18 ml to 20 ml of CCI agar into sterile empty Petri dishes (7.8) and allow to solidify on a cool even surface.

The medium may be kept at 5 °C (7.11) for up to 14 days.

B.4 Tryptone soya agar (TSA) (example of non-selective medium)

B.4.1 Composition

Enzymatic digest of casein	15,0 g
Enzymatic digest of soya	5,0 g
Sodium chloride (NaCl)	5,0 g
Agar	9,0 g to 18,0 g[a]
Water	1 000 ml
[a] Depending on the gel strength of the agar.	

B.4.2 Preparation

Dissolve each of the components in the water by boiling. Adjust the pH, if necessary, so that after sterilization it is 7,3 ± 0,2 at 25 °C. Sterilize in the autoclave (7.1) at 121 °C for 15 min. Cool to between 47 °C and 50 °C. Pour about 18 ml to 20 ml of TSA into sterile empty Petri dishes (7.8) and allow to solidify on a cool even surface.

Store medium in accordance with ISO 11133.

B.5 Media and reagents for biochemical characterization

B.5.1 Reagent for detection of oxidase

B.5.1.1 Composition

N,*N*,*N*′,*N*′-Tetramethyl-*p*-phenylenediamine dihydrochloride ($C_{10}H_{16}N_2 \cdot 2HCl$)	1,0 g
Water	100 ml

B.5.1.2 Preparation

Dissolve the component in water immediately before use.

B.5.2 α-Glucosidase enzymatic assay solution

B.5.2.1 Phosphate buffer, 0,3 M (pH 7,0)

B.5.2.1.1 Composition

Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$)	1,75 g
Sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$)	4,64 g
Water	90 ml

B.5.2.1.2 Preparation

Dissolve the components in sterile water and store at 5 °C (7.11) for not more than 15 days.

B.5.2.2 4-Nitrophenyl α-D-glucopyranoside substrate

B.5.2.2.1 Composition

4-Nitrophenyl α-D-glucopyranoside ($C_{12}H_{15}NO_8$)	0,4 g
Water	10 ml

B.5.2.2.2 Preparation

Dissolve the component in the water at 50 °C immediately before use.

B.5.2.3 Complete medium

Add 10 ml of *4*-Nitrophenyl α-D-glucopyranoside substrate (B.5.2.2) to 90 ml of Phosphate buffer (B.5.2.1) so as to obtain a final *4*-Nitrophenyl α-D-glucopyranoside concentration of 0,4 g per 100 ml of α-glucosidase enzymatic assay solution.

The complete α-glucosidase enzymatic assay solution may be kept at 5 °C (7.11) for one day.

B.5.2.4 Physiological salt solution 0,85 % NaCl

B.5.2.4.1 Composition

Sodium chloride (NaCl)	0,85 g
Water	100 ml

B.5.2.4.2 Preparation

Dissolve the components in the water and store at 5 °C (7.11) for not more than 15 days.

B.5.3 L-Lysine decarboxylation medium

B.5.3.1 Composition

L-lysine monohydrochloride ($C_6H_{14}N_2O_2$·HCl)	5 g
Yeast extract	3 g
Glucose ($C_6H_{12}O_6$)	1 g
Bromocresol purple	0,015 g
Water	1 000 ml

B.5.3.2 Preparation

Dissolve each of the components in the water, by heating if necessary. Adjust the pH, if necessary, so that after sterilization it is 6,8 ± 0,2 at 25 °C. Dispense 5 ml of L-lysine decarboxylation medium into tubes (7.7).

Sterilize the tubes at 121 °C in the autoclave (7.1) for 15 min. Store the poured tubes at 5 °C (7.11) for up to 3 months.

B.5.4 L- Ornithine decarboxylation medium

B.5.4.1 Composition

L-Ornithine monohydrochloride ($C_5H_{12}N_2O_2$·HCl)	10 g
Yeast extract	3 g
Glucose ($C_6H_{12}O_6$)	1 g
Bromocresol purple	0,015 g
Water	1 000 ml

B.5.4.2 Preparation

Dissolve each of the components in the water, by heating if necessary. Adjust the pH, if necessary, so that after sterilization it is 6,8 ± 0,2 at 25 °C.

Dispense 5 ml of L-ornithine decarboxylation medium into tubes (7.7). Sterilize the tubes at 121 °C in the autoclave (7.1) for 15 min. Store tubes at 5 °C (7.11) for up to three months.

B.5.5 **Media for fermentation of carbohydrates** (peptone water with phenol red, D-arabitol, α-Methyl-D-glucoside, D-sorbitol and D-sucrose)

B.5.5.1 Basic medium

B.5.5.1.1 Composition

Enzymatic digest of casein	10 g
Sodium chloride (NaCl)	5 g
Phenol red	0,02 g
Water	1 000 ml

B.5.5.1.2 Preparation

Dissolve each of the components in the water, by heating if needed. Adjust the pH, if necessary, so that after sterilization it is 6,8 ± 0,2 at 25 °C.

Dispense the basic medium into flasks (7.5) of suitable capacity. Sterilize in the autoclave (7.1) at 121 °C for 15 min. Store the medium in at 5°C (7.11) for up to six months.

B.5.5.2 Carbohydrate solution D-arabitol ($C_5H_{12}O_5$), α-Methyl-D-glucoside ($C_7H_{14}O_6$), D-sorbitol ($C_6H_{14}O_6$) and D-sucrose ($C_{12}H_{22}O_{11}$) 80 mg/ml

B.5.5.2.1 Composition

Carbohydrate	8 g
Water	100 ml

B.5.5.2.2 Preparation

Dissolve separately each of the four carbohydrate components in the water so as to obtain four carbohydrate solutions. Sterilize all by filtration through 0,2 µm filter. Store the medium at 5 °C (7.11) for up to six months.

B.5.5.3 Complete carbohydrate fermentation media

B.5.5.3.1 Composition

Basic medium (B.5.5.1)	875 ml
Carbohydrate solution (B.5.5.2)	125 ml

B.5.5.3.2 Preparation

For each carbohydrate, add the prepared carbohydrate solution (B.5.5.2) aseptically to basic medium (B.5.5.1) and mix. Dispense 10 ml of complete medium of each carbohydrate aseptically into sterile tubes (7.7).

The complete carbohydrate media should be prepared the day of use.

B.5.6 Methyl red (MR) and Voges-Proskauer (VP)-reactive compounds

B.5.6.1 MR/VP base medium

B.5.6.1.1 Composition

Enzymatic digest of animal tissues	7 g
D-glucose	5 g
Potassium phosphate dibasic (K_2HPO_4)	5 g
Water	100 ml

B.5.6.1.2 Preparation

Dissolve each of the components in the water, by heating if needed. Adjust the pH, if necessary, so that after sterilization it is 6,9 ± 0,2 at 25 °C.

Dispense 10 ml MR/VP medium into tubes (7.7). Sterilize the tubes in the autoclave (7.1) at 121 °C for 15 min. Store the medium in at 5 °C (7.11) for up to six months.

B.5.6.2 Methyl red (MR) reagent

B.5.6.2.1 Composition

Methyl red ($C_{15}H_{15}N_3O_2$)	0,03 g
Ethanol	85,5 ml
Water	4,5 ml

B.5.6.2.2 Preparation

Dissolve the methyl red in the ethanol:water solution. No sterilization is necessary. Store at room temperature (20 °C to 25 °C).

B.5.6.3 Voges-Proskauer (VP) reagent

B.5.6.3.1 40 % KOH

B.5.6.3.1.1 Composition

Potassium hydroxide (KOH)	40 g
Water	100 ml

B.5.6.3.1.2 Preparation

Dissolve the potassium hydroxide in the water. No sterilization is necessary. Store at room temperature (20 °C to 25 °C).

B.5.6.3.2 5 % 1-naphthol

B.5.6.3.2.1 Composition

1-naphthol ($C_{10}H_7OH$)	5 g
Ethanol	100 ml

B.5.6.3.2.2 Preparation

Dissolve the 1-naphthol in the ethanol. No sterilization is necessary. Store at room temperature (20 °C to 25 °C).

B.6 Performance testing of culture media and reagents

Test the performance of the culture media and reagents in accordance with the methods and criteria described in ISO 11133, see Table B.1.

Table B.1 — Performance testing for the quality assurance of the culture media

Medium	Type[a]	Function	Incubation	Control strains	WDCM numbers[c]	Method of control	Criteria	Characteristic reactions
BPW[g]	L	Productivity	(18 ± 2) h / 34 °C to 38 °C	*Cronobacter sakazakii* *Cronobacter muytjensii*	00214 [b] 00213	Qualitative	Turbidity (1–2) [e]	—
CSB	L	Productivity	(24 ± 2) h / (41,5 ± 1) °C	*Cronobacter sakazakii* + *Staphylococcus aureus* [d]	00214 [b] 00032 or 00034	Qualitative	Colour change of CSB > 10 colonies on CCI	Yellow colour of CSB; Small to medium-sized (1 mm to 3 mm) blue to blue-green colonies on CCI
				Cronobacter muytjensii + *Staphylococcus aureus* [d]	00213 00032 or 00034			
		Selectivity	(24 ± 2) h / (41,5 ± 1) °C	*Staphylococcus aureus* [d]	00032 or 00034	Qualitative	Total or partial inhibition on TSA ≤ 100 colonies	Purple colour of CSB
CCI	S	Productivity	(24 ± 2) h / (41,5 ± 1) °C	*Cronobacter sakazakii* *Cronobacter muytjensii*	00214 [b] 00213	Qualitative	Good growth (2) [f]	Small to medium-sized (1 mm to 3 mm) blue to blue-green colonies
		Selectivity		*Staphylococcus aureus* [d]	00032 or 00034	Qualitative	No growth (0) [f]	—
		Specificity		*Enterobacter cloacae*	00083	Qualitative	Growth (1–2) [f]	Colonies do not have a green or blue-green colour

[a] L = liquid medium, S = solid medium

[b] Strains to be used as a minimum.

[c] Refer to the reference strain catalogue available at www.wfcc.info for information on culture collection strain numbers and contact details; WDCM: World Data Centre for Microorganisms.

[d] Strain free of choice; one of the strains has to be used as a minimum.

[e] Turbidity is categorized as: 0 — no turbidity; 1 — slight turbidity; 2 — good turbidity (see also ISO 11133).

[f] Growth is categorized as: 0 — no growth; 1 — weak growth (partial inhibition); 2 — good growth (see also ISO 11133).

[g] For multipurpose media, refer to ISO 11133.

Annex C
(informative)

Distinction of *Cronobacter* spp. from other genera

Table C.1 — Distinction of *Cronobacter* spp. from other genera

	Cronobacter [a]					*Enterobacter* and related species[b]							
	C. dublinensis	*C. malonaticus*	*C. condimenti*/ *C. muytjensii*	*C. sakazakii*	*C. turicensis*/ *C. universalis*	*E. aerogenes*	*E. cancerogenus*	*E. cloacae* [c]	*E. gergoviae* [e]	*F. helveticus* [d]	*E. hormaechei*	*F. pulveris* [d]	*S. turicensis* [d]
Oxidase	0	0	0	0	0	0	0	0	0	0	0	0	0
Hydrolysis of 4-NP α-D-glucoside	100	100	100	100	100	0	0	1	0	100	0	100	100
Lysine decarboxylase	0	0	0	0	0	98	0	0	90	0	0	0	0
Ornithine decarboxylase	100	95	100	94	90	98	100	96	100	0	91	0	0
Methyl Red	0	0	0	1	0	5	0	5	5	91	57	83	100
Voges-Proskauer	100	100	100	99	100	98	100	100	100	9	100	17	0
Acid from:													
D-Arabitol	17	0	0	0	10	100	0	15	97	91	0	83	100
α-Methyl-D-glucoside	100	100	0	100	100	95	0	85	2	0	83	0	0
D-Sorbitol	0	0	0	0	0	100	0	95	0	0	0	0	0
D-Sucrose	100	100	100	100	100	100	0	97	98	0	100	100	0

Numbers indicate percentage of positive strains.

[a] From References [7], [8], [9] and [11].

[b] From References [12], [13] and [14].

[c] Similar percentages are obtained for other *E. cloacae*-complex species e.g. *E. asburiae, E. amnigenus, E. intermedium, E. nimipressuralis* (with the exception *E. nimipressuralis* does not produce acid from D-sucrose).

[d] *Franconibacter helveticus, Franconibacter pulveris* and *Siccibacter turicensis* can occur as presumptive positive colonies on CCI agar.

[e] Similar percentages are obtained for *E. pyrinus*.