TECHNICAL DATA SHEET

CSD METHOD

METHOD FOR THE DETECTION OF CRONOBACTERS

1 INTENDED USE

The CSD method allows the rapid detection of *Cronobacter* spp in human food products (especially milk powders and infant ingredients) and products from the production environment.
At the end of an enrichment phase with *Salmonella* Enrichment + CSD supplement, a subculture is carried out on CCI agar. The characteristic colonies will be confirmed.

The CSD method is NF VALIDATION certified, for the detection of *Cronobacter* spp, according to the validation protocol NF EN ISO 16140-2 of 2016, for the following categories:

- Infant milk powders, with and without probiotics; ingredients for test intakes from 0 to 50 g, with a 1/10th dilution.
- Infant milk powders, with and without probiotics; ingredients for test intakes from 50 to 375 g, with a ¼ dilution.
- Samples of the production environment

BKR 23/12 – 12/20
METHODES ALTERNATIVES D'ANALYSE POUR L'AGROALIMENTAIRE
Certifié par AFNOR Certification http://nf-validation.afnor.org/

Refer to the certificate available on the NF VALIDATION website for the end of validity date of the method. The reference method used for the validation is the standard NF EN ISO 22964 : 2017.

The CCI agar complies with the formulation described in the NF EN ISO 22964 standard of 2017.

The **CSD supplement** allows **the common enrichment of *Salmonella* and *Cronobacter*** in infant milk powders with and without probiotics, as well as ingredients and environmental products.

For the detection of *Salmonella* spp, refer to the IRIS *Salmonella* ® method, BKR 23/07-10/11, certified NF VALIDATION.

2 PRINCIPLES

The dilution step at ¼ or 1/10th of the product to be analysed is carried out in accordance with the recommendations of the NF EN ISO 6887 standards, with systematic addition of the CSD selective supplement.
After this addition, the enrichment broth turns green.

The **enrichment** broth is incubated at 41.5°C.
The detection step is carried out by transplanting the broth onto **CCI Agar.**

The possible confirmation stage can be carried out by the classic tests described in the standard or alternative methods certified by NF VALIDATION.

The non-detection of *Cronobacter* can be declared from 37 hours of analysis for weighing up to 50 g and from 39 hours for weighing up to 375 g.

3 TYPICAL COMPOSITION

The composition can be adjusted for optimum performance.

Salmonella Enrichment
For 1 litre of medium:

- Peptone ..... 10.00 g
- Sodium chloride ..... 5.00 g
- Phosphate buffer ..... 5.06 g

pH of the ready-to-use medium at 25 °C: 7.0 ± 0.2.

Note: The *Salmonella* Enrichment formula is in conformity with that of Buffered Peptone Water.

Salmonella Enrichment + Tween® 80
For 1 litre of medium:

- Peptone ..... 10.00 g
- Sodium chloride ..... 5.00 g
- Phosphate buffer ..... 5.06 g
- Tween® 80 ..... 10,00 g

pH of the ready-to-use medium at 25 °C: 7.0 ± 0.2.

Salmonella Enrichment double-strength buffered
For 1 litre of medium:

- Peptone ..... 10.00 g
- Sodium chloride ..... 5.00 g
- Phosphate buffer ..... 10.12 g

pH of the ready-to-use medium at 25 °C: 7.0 ± 0.2.

CCI Agar (chromogenic _Cronobacter_ Isolation Agar)
For 1 litre of medium:

- Tryptone ..... 7.0 g
- Yeast autolytic extract ..... 3.0 g
- Sodium chloride ..... 5.0 g
- Sodium deoxycholate ..... 0.25 g
- Ammoniacal iron citrate ..... 1.0 g
- Sodium thiosulphate ..... 1.0 g
- 5-bromo-4-chloro-3-indoxyl, α-D-glucopyranoside ..... 0.15 g
- Bacteriological agar agar ..... 14.2 g

pH of the ready-to-use medium at 25 °C: 7.3 ± 0.2.

4 PREPARATION

Preparation of dehydrated media _Salmonella_ Enrichment:

- Dissolve 20.0 g of dehydrated media (BK194) in 1 liter of distilled or demineralized water.
- Mix well, until complete dissolution.
- Divide according to the intended use into tubes or vials so that the mother suspension can be made up to 1/10th or ¼.
- Sterilize in an autoclave at 121°C for 15 minutes.
- Cool to room temperature.

✓ **Reconstitution:**
20.0 g/L

✓ **Sterilization:**
15 min at 121°C

Preparation of dehydrated *Salmonella* Enrichment double-strength buffered:

- Dissolve 25.1 g of dehydrated media (BK225) in 1 liter of distilled or demineralized water.
- Mix well, until complete dissolution.
- Divide according to the intended use into tubes or vials so that the mother suspension can be made up to 1/10th or ¼.
- Sterilize in an autoclave at 121°C for 15 minutes.
- Cool to room temperature.

✓ **Reconstitution:** **25.1 g/L**

✓ **Sterilization:** **15 min at 121°C**

Preparation of *Salmonella* Enrichment broth from 9x Concentrated broth:

- Mix sterilely 1 volume of Salmonella Enrichment 9x concentrated broth (BM233) with 8 volumes of sterile distilled or demineralized water.

Preparation of dehydrated CCI Agar:

- Dissolve 31.6 g of dehydrated media (BK200) in 1 liter of distilled or demineralized water.
- Slowly bring the medium to the boil under constant agitation.
- Dispense into bottles and autoclave for 15 minutes at 121°C.
- Cool to 44-47°C and pour into Petri dishes.
- Cool on a flat surface.

✓ **Reconstitution:** **31.6 g/L**

✓ **Sterilization:** **15 min at 121°C**

5 INSTRUCTIONS FOR USE

Always use good laboratory practices. Refer to standard NF EN ISO 7218.
NF VALIDATION certified protocol for test up to 50 g (infant milk powders, with and without probiotics; ingredients) and products from the production environment:

- Aseptically introduce (x) g of test material into 9 (x) mL of Salmonella Enrichment.
- Introduce the CSD supplement at a rate of 0.1 mL of liquid supplement BS095 per gram of sample (i.e. 2.5 mL per 25 g).
- Homogenize or stomacher if necessary.
- Incubate the broth at 41.5 ± 1.0 °C for **16 to 22 hours.**
- Isolate 10 µL from the enrichment obtained on the CCI Agar.
- Incubate at 41.5 ± 1 °C for 24 ± 3 hours.

✓ **Enrichment:** **At 1/10, 16-22 h at 41.5 °C**

✓ **Detection:** **Isolation 10 µL, 24 h at 41.5 °C**

Notes
It is also possible to follow the examples provided in standard NF EN ISO 18593 (See paragraph 9.2).
For surface samples after cleaning, which may contain disinfectant residues, it is recommended to use swabs, sponges or wipes already soaked in neutralizing solution, or to use a diluent containing 10% universal neutralizers and 90% *Salmonella* Enrichment, before adding the CSD supplement.

Protocol certified NF VALIDATION for test samples from 50 g to 375 g (infant milk powders, with and without probiotics; ingredients):

- Aseptically introduce (x) g of test material into 3 (x) mL of pre-warmed Salmonella Enrichment.
- Introduce the CSD supplement (BS095) at a rate of 0.1 mL per gram of sample (i.e. 37.5 mL per 375 g).
- Homogenize or stomacher if necessary.
- Incubate the broth at 41.5 ± 1.0 °C for **18 to 24 hours.**
- Isolate 10 µL from the enrichment obtained on the CCI Agar.
- Incubate at 41.5 ± 1 °C for 24 ± 3 hours.

✓ **Enrichment:** **At 1/4, 18-24 h at 41.5 °C**

✓ **Detection:** **Isolation 10 µL, 24 h at 41.5 °C**

Notes about NF VALIDATION certified protocols

- The method is certified only with the CCI agar provided by Solabia in different packagings.
- One CSD tablet Qsp 10 g (BS09908) can be added to Salmonella Enrichment for 10 g test samples.
- One CSD tablet Qsp 25 g (BS10008) can be added to Salmonella Enrichment for 25 g test samples.
- Several tablets can be added to the broth in accordance with the test sample (e.g. 3 BS100 tablets for a 75g test sample).
- Refer to the different parts of the NF EN ISO 6887 standard:
  - Use *Salmonella* Enrichment with Tween for suspensions and matrix enrichment with more than 20% fat.
  - Use *Salmonella* Double Buffered Enrichment or *Salmonella* Enrichment for acid and acidifying matrices, or milks with probiotics.
  - Add 0.1 g/L of α amylase for infant cereals.
- The enrichment broth, after incubation, can be stored for up to 3 days at 2-8°C before subculture on CCI Agar.
- The CCI agar, after incubation, can be stored for up to 48 hours at 2-8°C before reading and possible confirmation.

6 RESULTS

The appearance of the colonies on CCI Agar is as follows:

Microorganisms	Characteristic colonies
Cronobacter spp.	Colonies blue to blue-green, from 1 to 3 mm
Escherichia coli	White colonies, white with greenish centre
Salmonella spp, Proteus	Colonies with black centre
Gram-positive bacteria	Inhibited growth

See APPENDIX 1: PHOTO SUPPORT.

7 CONFIRMATION

All presumed positive results must be confirmed in one of the following ways:

Standard or validated methods ISO 16140-6

The formulation of the CCI agar is according to the formula described in ISO 22964, the following methods can be used:

- Implementation of the classic tests described in the CEN or ISO standardized methods (including the purification stage), starting from a blue to blue-green colony isolated on CCI Agar
- Implementation of methods certified according to ISO 16140-6 using characteristic colonies isolated on CCI.

Methods certified NF VALIDATION

Within the NF VALIDATION mark, all positive results must be confirmed in one of the following ways:

- Option 1: Implementation of the classical tests described in the CEN or ISO standard methods (including the purification step), starting from a blue to blue-green colony isolated on CCI Agar.
- Option 2: Implementation of a biochemical gallery from an isolated colony.
- Option 3: Use of any other NF VALIDATION certified method, of a different principle. The validated protocol of the second method must be respected as a whole, i.e. all the steps prior to the intermediate step from which confirmation is taken must be common to both methods. The two validated methods (one used in detection and the other in confirmation) must therefore have a common core.

NOTE:
In the event of conflicting results (positive by the alternative method, not confirmed by one of the options described above), the laboratory shall implement sufficient means to ensure the validity of the result rendered. It is possible, for example, to carry out biochemical tests or use nucleic probes as described in standard NF EN ISO 7218.

8 QUALITY CONTROL

Prepared medium: Amber agar, transparent

Cultural response after 24 hours incubation at 41.5°C (NF EN ISO 22964):

Microorganisms		Growth	Characteristics
Cronobacter sakazakii	WDCM 00214	Good, score 2	Blue-green colonies
Cronobacter muytjensii	WDCM 00213	Good, score 2	Blue-green colonies
Enterobacter cloacae	WDCM 00083	Good, score1- 2	White
Staphylococcus aureus	WDCM 00034	Inhibited, score 0	-

9 STORAGE / SHELF LIFE

***Salmonella* Enrichment, *Salmonella* Enrichment double-strength buffered:**
Dehydrated media: 2-30 °C.
Ready-to-use media in vials or flexible bags: 2-25 °C.

***Salmonella* Enrichment with Tween®:**
Ready-to-use media in vials or flexible bags: 2-25°C.

***Salmonella* Enrichment 9x Concentrated:**
Medium in flexible bags: 15-30 °C.

Supplements and tablets CSD: 2-8 °C.

CCI medium:
Dehydrated medium: 2-30 °C.
CCI medium pre-cast in Petri dishes: 2-8 °C.
Expiry dates are indicated on the labels.

***Salmonella* Enrichment with or without Tween®, *Salmonella* Enrichment double-strength buffered** prepared in vials or in tubes (*) : 180 days at 2-25°C.

CCI media prepared in Petri dishes: 1 month at 2-8°C.

(*) Benchmark value determined under standard preparation conditions, following manufacturer's instructions.

10 PACKAGING

***Salmonella* Enrichment:**
500 g bottle..... BK194HA
5 kg drum BK194GC
10 x 225 mL vials BM13608
3 x 3 L flexible bag..... BM13708
2 x 5 L flexible bag..... BM14408
40 x 5L flexible bag BM23708

***Salmonella* Enrichment + Tween® 80 (10 g/L):**
3 x 3 L flexible bag..... BM16308
2 x 5 L flexible bag..... BM19808
10 x 225 mL bottles BM22808

***Salmonella* Enrichment double-strength buffered:**
500 g bottle..... BK225HA
5 kg drum BK225GC
2 x 5 L flexible bag..... BM20008
10 x 225 mL vials BM20108

***Salmonella* Enrichment 9X Concentrated:**
4 x 2.5 L flexible bag..... BM23308

***Salmonella* Enrichment double-strength buffered with Tween®:**
2 x 5 L flexible bag..... BM22008

CCI Agar (Ø 90 mm):
500 g bottle..... BK200HA
20 plates (Ø 90 mm)..... BM15408
120 plates (Ø 90 mm)..... BM22608

CSD Supplement*:*
10 vials of 100 mL BS09508
120 tablets qsf 10 g..... BS09908
120 Tablets qsf 25 g..... BS10008

11 BIBLIOGRAPHY

NF EN ISO 16140-2. September 2016. Microbiology of the food chain - Method validation - Part 2: Protocol for the validation of alternative (commercial) methods to a reference method - Food microbiology

ISO 16140-6: 2019. Microbiology of the food chain - Method validation - Part 6: Protocol for the validation of alternative (commercial) methods for microbiological confirmation and typing.

NF EN ISO 6887. Microbiology of the food chain. Preparation of samples, mother suspension and decimal dilutions for microbiological examination. Parts 1 to 6.

NF EN ISO 7218. July 2024. Microbiology of the food chain — General requirements and guidance for microbiological examinations.

EN EN ISO 18593: July 2018. Microbiology of the food chain - Horizontal methods for sampling techniques from surfaces using contact plates and swabs

NF EN ISO 22964. June 2017. Microbiology of the food chain. Horizontal method for the search for *Cronobacter* spp.

12 ADDITIONAL INFORMATION

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CHROMOGENIC *CRONOBACTER* ISOLEMENT AGAR (CCI Agar)

Cronobacter detection

Growth obtained after 24 hours incubation at 41.5°C.