iQ-Check® *Cronobacter* spp.

Catalog #: 3578137

User Guide

Test for the real-time PCR detection of *Cronobacter* spp. in infant formulas and environmental samples

BIO-RAD

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I. INTRODUCTION

Cronobacter spp., formerly named *Enterobacter sakazakii*, is a ubiquitous pathogen that has been isolated from foods, environment and clinical sources (1). It is sadly associated with rare but fatal (case-fatality rate may reach 80%) infant infections linked to the consumption of reconstituted Powdered Infant Formulas. Neonates, low birth weight and immunocompromised newborns are particularly at risks because of the high mortality rate, but the largest number of cases occurs in older population (2).

The resistance of the organism to desiccation, the low cell numbers that have been reported to cause disease and the high amount of baby milk products sales have lead many authorities to a strong monitoring. For example, the EU regulation (3, 4) requires the absence of the bacteria in 10 g of sample. To comply with regulations and to fulfill the HACCP approaches, industrials have even stricter targets and need to select highly sensitive and specific methods. If the classical microbiological methods offer standardized results (5), they are however long and tedious, and do not ensure the full answer to those needs.

iQ-Check *Cronobacter* spp. is a simple and rapid qualitative test allowing the detection of specific *Cronobacter* spp. DNA sequences in infant formulas and environmental samples. Up to 94 samples can be processed to obtain a reliable result in less than 24 hours including the single enrichment. This allows a more flexible workflow and a higher throughput to implement effective measures to improve the safety and hygiene.

II. THE iQ-Check *Cronobacter* spp. TECHNOLOGY

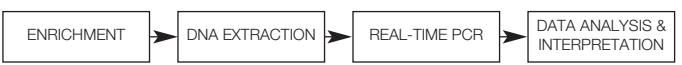
The iQ-Check *Cronobacter* spp. is a test based on gene amplification and detection by real-time PCR. Ready-to-use PCR reagents contain DNA primers and a DNA probe specific for *Cronobacter* spp., as well as DNA polymerase and nucleotides. Detection and data analysis are optimized for use with a Bio-Rad real-time PCR instrument, such as the Chromo4™ System, the MiniOpticon™ or the CFX96™.

PCR is a powerful technique used to generate many copies of target DNA. During the PCR reaction, several cycles of heating and cooling allow DNA denaturation, by heat, followed by primers binding to the target region. The DNA polymerase then uses these primers and deoxynucleotide triphosphates (dNTPs) to extend the DNA, creating copies of the target DNA. These copies are called amplicons.

In real-time PCR, specific probes are used to detect the DNA during the amplification, by hybridizing to the amplicons. These probes are linked to a fluorophore which fluoresces only when hybridized to the target sequence; FAM is the fluorophore linked to the probe hybridizing to the *Cronobacter* spp. specific DNA sequence. In the absence of target DNA, no fluorescence will be detected. As the amount of amplicons increases with each round of amplification, fluorescence intensity also increases. During each PCR cycle, at the annealing step, the optical module or detector measures this fluorescence, whereas the associated software plots the fluorescence intensity versus number of cycles. This method allows a simple determination of the presence or absence of *Cronobacter* spp. in a sample.

A synthetic DNA "internal control" is included in the reaction mix. This control is amplified with a specific probe at the same time as the *Cronobacter* spp. target DNA sequence, and detected by a second fluorophore. It allows for the validation of any negative result.

This test allows the detection of *Cronobacter* spp. in infant formula and environmental samples previously enriched by culture in buffered peptone water (supplemented or not). It includes the following four main steps:



III. KIT COMPONENTS

The iQ-Check *Cronobacter* spp. kit contains sufficient reagents for 96 tests.

Reference ID	Reagent	Quantity Provided
A	Lysis solution	1 bottle (20 mL)
B	Fluorescent probes	1 tube (0.55 mL)
C	Amplification mix	1 tube (4.4 mL)
D	Negative PCR control	1 tube (0.5 mL)
E	Positive PCR control	1 tube (0.25 mL)

IV. SHELF LIFE AND STORAGE

Once received, the kit must be stored between +2°C and +8°C. Reagents stored between +2°C and +8°C can be used until the expiration date indicated on the reagent tube.

V. MATERIAL REQUIRED BUT NOT SUPPLIED

Equipment

- Stomacher®, masticator or equivalent for homogenizing test samples.
- Incubator for sample microbiological enrichment.
- Specific for extraction in 1.5 mL tube
  - Bench top centrifuge (max. 10,000-12,000 g).
  - Dry heat block (100°C ± 5°C).
- Specific for extraction with deepwell plate,
  - Dry heat block (100°C ± 5°C),
  - or agitator-incubator* for deepwell plates, such as a "Thermomixer" (Eppendorf).
- Vortex apparatus.
- Magnetic stir plate.
- 20 µL, 200 µL and 1000 µL micropipettes.
- Combitip pipettes or equivalent repeat pipettors.
- Bio-Rad real-time PCR system, e.g. Chromo4, MiniOpticon or CFX96 systems.
- Bio-Rad iQ-Check™ Prep system for automated DNA Extraction and PCR plate set-up.

* Contact Bio-Rad for detailed information on instruments recommended by our technical department.

Note: We recommend using a universal power source (UPS) with the thermal cycler and iQ-Check Prep System.

Supplies

- Enrichment medium: buffered peptone water, (E.g. Bio-Rad cat. #: 3564684 for 500 g; 3554179 for 225 mL x 6 bottles; 3555795 for 3 L x 4 bags; 3555790 for 5 L x 2 bags).
- Specific for infant formulas sample enrichment: selective element: vancomycin.
- Specific for extraction in 1.5 mL tube:
  - 1.5 mL conical screwcap sterile tubes (E.g. Bio-Rad cat.#: 2240110).
- Stomacher bag with incorporated filter.
- Optional for extraction:
  - 1 mL deepwell plate, Bio-Rad cat. #: 3590132.
  - Plastic sealing film, Bio-Rad cat. #: 3590139.
  - Pre-pierced sealing film, such as "X-Pierce™ Sealing Films" (Bio-Rad cat.#: 3593977, x100 - for North America only, cat. #: 3600040, x100).
- RAPID'Sakazakii agar (Bio-Rad cat #: 3564976, 500 g)
- PCR plates, tubes, sealing tape and caps, see real-time PCR system user guide for iQ-Check kits.
- 1 mL and 10 mL pipettes.
- Sterile filter tips, adaptable to 20 µL, 200 µL and 1000 µL micropipettes.
- Tips for Combitip pipettes or equivalent repeat pipettors, sterile, individual package.
- 2 mL and 5 mL sterile test tubes.
- Powder-free gloves.
- Distilled sterile water.
- Bleach 5%.
- Cleaning agent such as DNA AWAY® or RNase AWAY®.

VI. PRECAUTIONS AND RECOMMENDATIONS

- This test must be performed by adequately trained personnel.
- Samples and enrichment cultures must be handled as potentially infectious material and eliminated according to local rules and regulations.
- All potentially infectious material should be autoclaved before disposal.
- The quality of results depends on strict compliance with the following Good Laboratory Practice (for example the EN ISO 7218 standard), especially concerning PCR:
  - The laboratory equipment (pipettes, tubes, etc.) must not circulate from one work station to another.

- The high risk of cross-contamination by using micropipettes during sample preparation must be considered. We recommend to have dedicated micropipettes respectively for DNA positive area and DNA negative area.
- It is essential to use a positive control and a negative control for each series of amplification reactions.
- Do not use reagents after their expiration date.
- Vortex reagents from the kit before using them to ensure homogeneity.
- Periodically, verify the accuracy and precision of pipettes, as well as correct functioning of the instruments.
- Change gloves often, especially if you suspect they are contaminated.
- Clean work spaces periodically with at least 5% bleach and other decontaminating agent such as DNA AWAY.
- Use powder-free gloves and avoid fingerprints and writing on caps of tubes. Both cases will interfere with data acquisition.

• It is strongly advised to follow the general requirements described in the standard EN ISO 22174:2005 "Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food pathogens – General requirements and definitions".

VII. PROTOCOL

It is strongly recommended to read the entire protocol before starting the test.

A. Sample Enrichment

It is necessary to respect strictly the temperature indicated. The use of a ventilated incubator is recommended. The Buffered Peptone Water Broth must be at Room Temperature before use.

1. Environmental samples, single enrichment

• Homogenize n g of sample in 9 x n mL (for example, 30 g in 270 mL) in the buffered peptone water, in a stomacher bag with incorporated filter.
Note: within the scope of the NF VALIDATION, test portions weighing more than 30 g have not been tested.

• Incubate without shaking for **18 hours ± 2 hours at 37°C ± 1°C**.
Note: the enriched samples can be stored between 2°C and +8°C for 48 hours following the end of the incubation at 37°C (Standard DNA extraction protocol must be then applied).

• Follow the Easy or Standard protocol for the DNA extraction.

2. Infant formulas samples, double enrichment

- Homogenize n g of sample in 9 x n mL (for example, 30 g in 270 mL) of buffered peptone water supplemented with vancomycin (10 µg/mL).
  Note: within the scope of the NF VALIDATION, test portions weighing more than 30 g have not been tested.
- Incubate without shaking for **20 hours** ± 2 hours at 37°C **± 1°C**.
- After this first enrichment, transfer in buffered peptone water (1/10 ratio ; for example, 1 mL of sample in 9 mL of buffered peptone water) for the secondary enrichment
- Incubate without shaking for **4 hours** ± 1 hour at 37°C **± 1°C**.
  Note: the enriched samples (secondary enrichment) can be stored between 2°C and +8°C for 48 hours following the end of the incubation at 37°C.
- Follow the Easy or Standard protocol for the DNA extraction.

B. DNA Extraction

Two protocol formats are proposed for DNA extraction:

- the "easy protocol" is using 100 µL of enriched sample in 100 µL of lysis reagent;
- the "standard protocol" is using 1.0 mL of enriched sample, followed by a centrifugation before the lysis in 200 µL of lysis reagent.

General recommendations:

Before starting the test, turn on the heat block to preheat it and set it to 95°C - 100°C.

- In general, avoid shaking the enrichment bag and collecting large fragments of food debris. For food samples with a fatty supernatant, collect the sample just below this layer.
- Open tubes and wells carefully to avoid any possible cross contaminations.
- Cool the deepwell plate before pipetting directly through pre-pierced sealing film.
- Pipette the lysis reagent while it is stirring at medium speed with the magnetic bar contained in the bottle, in order to keep it in suspension.

Easy protocol

1. Aliquote **100 µL of homogenized lysis reagent A** to tubes or in wells of a deepwell plate.
2. Add **100 µL** of decanted enriched sample .
   Mix by pipetting up and down and close the tubes with caps or seal the deepwell plate with pre-pierced sealing film.
3. Incubate in the appropriate heat block at 95°C - 100°C for 10 to 15 minutes or in the plate incubator-agitator for 15 to 20 minutes at 1,300 rpm.
4. Vortex tubes at high speed.
5. If using a deepwell plate, allow to cool down to room temperature.
6. Centrifuge at 10,000-12,000 g at least 2 minutes for tubes. Centrifugation is not needed for deepwell plate.

If you choose to temporarily stop the procedure, this is the recommended stopping point.

The supernatant can be stored for up to 1 year at -20°C. Before reusing it, always allow it to thaw, homogenize, and then centrifuge tubes at 10,000-12,000 g for 5 minutes.

Standard protocol

1. Collect 1 mL of decanted enriched sample into a tube.
2. Centrifuge at 10,000-12,000 g. for 5 minutes.
3. Discard all the supernatant.
4. Add 200 µL of the lysis reagent (reagent A) to the pellet.
5. Resuspend pellet by pipetting the reagent up and down in the tube.
6. Vortex tubes at high speed.
7. Place the tube in the heat block at 95°C - 100°C for 10 to 15 minutes.
8. Vortex at high speed.
9. Centrifuge at 10,000-12,000 g for 5 minutes

If you choose to temporarily stop the procedure, this is the recommended stopping point.

The supernatant can be stored for up to 1 year at -20°C. Before reusing it, always allow it to thaw, homogenize, and then centrifuge tubes at 10,000-12,000 g for 5 minutes.

C. Real-time PCR

1. Instrument and software setup

For instrument and software setup, follow instructions in the real-time PCR system user guide for iQ-Check kits.

2. PCR mix preparation

2.1. Prepare a PCR mix containing the amplification solution (**reagent C**) and the fluorescent probes (**reagent B**) according to the PCR mix calculation guide found in Appendix A. To find the correct volumes to use, add the total number of samples and controls to be analyzed, and find the corresponding volumes in the table. At least one positive and one negative control must be included in each PCR run.

After preparation, the PCR mix (reagent B + C) must be used immediately or is stable for 1 hour at 4°C.

2.2. Pipette **45 µL** of this PCR mix in each well according to your plate setup.

2.3. Add 5 µl of sample or reagent D (negative control) or reagent E (positive control). Do not vortex the sample before pipetting. Seal hermetically the wells of the plate or strips. It is important to avoid bubbles at the bottom of the wells by pipetting carefully. As an optional step to eliminate any bubbles, centrifuge the sealed PCR plate or the PCR strips (quick spin).

2.4. Place the plate or strips in the thermal cycler. Be sure to place the plate correctly: A1 well at the upper left corner. Close the reaction module.

3. PCR Start

To start the PCR run, follow instructions in the real-time PCR system user guide for iQ-Check kits.

D. Data Analysis and Results Interpretation

Data can be analyzed directly at the end of the PCR run or at a later time by opening the stored data file. Follow instructions in the corresponding real-time PCR system user guide for iQ-Check kits, for opening data files and setting the data analysis parameters.

Once the data analysis parameters have been set, results are interpreted by analyzing the Cq values of each sample (the cycle at which the amplification curve crosses the threshold).

A complete automated analysis is available with the Opticon™ Monitor Software, for the Chromo 4 system (see Chromo 4 User Guide for iQ-Check kits, #: 93269). The CFX Manager™ IDE allows a complete automated analysis for the CFX 96 and the Mini Opticon (User Guide #: 93893b).

1. Controls

Before interpreting sample results, it is necessary to verify the positive and negative controls.

For the experiment to be valid, the controls must have the following results, as summarized in the table below, otherwise the PCR reaction needs to be repeated.

	***Cronobacter* spp. detection (FAM)**	**Internal Control detection**
Negative control	Cq = N/A*	28 ≤ Cq ≤ 40
Positive control	26 ≤ Cq ≤ 36	Not significant

* The software indicates a Cq value of N/A (not applicable) when the fluorescence of a sample does not rise significantly above the background noise, and hence does not cross the threshold.

2. Samples

A **positive** *Cronobacter* sample must have a Cq value ≥ 10 for the FAM fluorophore.

If the Cq value is below 10, verify that as raw data the curve is a regular amplification curve (with a flat base line, followed by a rapid increase of fluorescence and then a flattening out). If the curve seems correct, it may be considered a positive *Cronobacter* sample.

If there is no Cq value (Ct=N/A) for FAM, or the curve is not a typical amplification curve, the internal control for that sample must then be analyzed:

- This sample is considered as a **negative** *Cronobacter* sample if there is no Cq value in FAM, and the internal control has a Cq ≥ 28.
- Should the internal control also not have a Ct value (Cq = N/A), then it is not possible to interpret the result. Such a result probably indicates an inhibition of the PCR reaction. The sample needs to be diluted (1/10, in distilled sterile water), and the PCR repeated.

- Should the Cq value for the internal control be < 28 it is not possible to interpret the result. Verify that the threshold was correctly placed, or that the curve as raw data is a regular amplification curve. If the curve does not have a characteristic shape, it will be necessary to repeat the PCR test.

Interpretation of sample results is summarized in the following table:

***Cronobacter* spp. detection (FAM)**	**Internal control detection**	**Interpretation**
Cq ≥ 10	Not significant	Positive
Cq = N/A	Cq ≥ 28	Negative
Cq = N/A	Cq = N/A	Inhibition**

** When both *Cronobacter* spp. and internal control detection give a Cq value = N/A, the sample must be tested again but diluted (1/10).

VIII. CONFIRMATION OF POSITIVE RESULTS

In the context of the NF VALIDATION Certified method, all positive iQ-Check *Cronobacter* spp. results need to be confirmed by one of the following ways:

1. Using standard tests described in the standardized ISO method (ISO TS 22964:2006) with colonies (including purification step).
2. Using the RAPID'*Sakazakii* chromogenic medium.
   - **For environmental samples**: use the RAPID'*Sakazakii* method. Samples are sub-cultured in mLST (1/10 ratio, for example 1 mL in 9 mL mLST) prior streaking onto the RAPID'*Sakazakii* medium.
   - **For dairy samples**: direct streaking of the secondary enrichment onto the RAPID'*Sakazakii* medium.

In the event of results that are not in agreement, between iQ-Check *Cronobacter* spp. and one of the confirmation options listed above, the laboratories should follow the necessary steps to ensure the validity of their results.

IX. CONFIRMATION OF SINGLE COLONIES USING iQ-Check

iQ-Check *Cronobacter* spp. may also be used for confirming single isolated Cronobacter colonies on agar plates.

1. Pick an isolated colony, from an agar plate, selective or non-selective, with a tooth-pick or sterile loop, or other adapted consumable (e.g. pipette tip).

2. Resuspend the colony in 100 µL tryptone salt or distilled sterile water in a microfuge tube. Homogenize using a vortex.
3. Use 5 µL of the supernatant with 45 µL of PCR mix (see section VII.C.Real-time PCR) and follow the rest of the iQ-Check *Cronobacter* spp. protocol for the data and result interpretation.

X. TEST PERFORMANCES AND VALIDATIONS

iQ-Check *Cronobacter* spp. is specific for the *Cronobacter* genus. With this kit it is possible to detect 1-10 CFU/25 g sample, according to the recommended enrichment.

BRD 07/23 – 01/13

ALTERNATIVE ANALYSIS METHODS FOR AGRIBUSINESS

http://nf-validation.afnor.org

NF VALIDATION

iQ-Check *Cronobacter* is certified NF VALIDATION as an alternative method to the reference method NF EN ISO/TS 22964, for the detection of *Cronobacter* spp. in all products for human and animal consumption, as well as environmental samples. The validation followed the protocol of the NF EN ISO 16140: 2003 standard, and includes the use of the iCycler iQ, Chromo4, MiniOpticon, CFX96 or of CFX96 Deep Well systems. The associated software are the Opticon Monitor (V3.4 and later), the iQ5 Optical system software (V1.0 and later) and the CFX Manager IDE (V1.2 and later). Certificate number: **BRD 07/23 – 01/13**. Valid until: refer to the certificate available on the AFNOR CERTIFICATION website.

XI. REFERENCES

1 - *Cronobacter* spp. ("*Enterobacter sakazakii*" sensu lato) : implication dans la contamination des préparations en poudre pour nourrissons et enfants en bas âge, Isabelle Proudy, Rev. can. microbiol. 55 : 473–500 (2009) doi:10.1139/W08-131.

2 - FAO/WHO [Food and Agriculture Organization of the United Nations/World Health Organization]. 2008. *Enterobacter sakazakii* (*Cronobacter* spp.) in powdered follow-up formulae. Microbiological Risk Assessment Series No. 15. Rome. 90pp.

3 - Commission Regulation EC 2073 /2005 of 15 November 2005 on microbiological criteria for foodstuffs.

4 - Commission Regulation EC 1141/2007 of 5 December 2007 amending regulation EC 2073 /2005 on microbiological criteria for foodstuffs.

5 - ISO TS 22964:2006 standard, Milk and Milk products – Detection of *Enterobacter sakazakii*.

APPENDIX - PCR Mix Calculation Guide

To find the correct volumes to use when preparing the PCR mix, add the total number of samples and controls to be analyzed, and find the corresponding volumes of reagent B and reagent C in the table.

Total number of samples & controls	Probes Reagent B (µL)	Amplification mix Reagent C (µL)
1	5	40
2	11	86
3	16	130
4	22	173
5	27	216
6	32	259
7	38	302
8	43	346
9	49	389
10	54	432
11	59	475
12	65	518
13	70	562
14	76	605
15	81	648
16	86	691
17	92	734
18	97	778
19	103	821
20	108	864
21	113	907
22	119	950
23	124	994
24	130	1000
25	135	1100
26	140	1100
27	146	1200
28	151	1200
29	157	1300
30	162	1300
31	167	1300
32	173	1400
33	178	1400
34	184	1500
35	189	1500
36	194	1600
37	200	1600
38	205	1600
39	211	1700
40	216	1700
41	221	1800
42	227	1800
43	232	1900
44	238	1900
45	243	1900
46	248	2000
47	254	2000
48	259	2100

Total number of samples & controls	Probes Reagent B (µL)	Amplification mix Reagent C (µL)
49	265	2100
50	270	2200
51	275	2200
52	281	2200
53	286	2300
54	292	2300
55	297	2400
56	302	2400
57	308	2500
58	313	2500
59	319	2500
60	324	2600
61	329	2600
62	335	2700
63	340	2700
64	346	2800
65	351	2800
66	356	2900
67	362	2900
68	367	2900
69	373	3000
70	378	3000
71	383	3100
72	389	3100
73	394	3200
74	400	3200
75	405	3200
76	410	3300
77	416	3300
78	421	3400
79	427	3400
80	432	3500
81	437	3500
82	443	3500
83	448	3600
84	454	3600
85	459	3700
86	464	3700
87	470	3800
88	475	3800
89	481	3800
90	486	3900
91	491	3900
92	497	4000
93	502	4000
94	508	4100
95	513	4100
96	518	4100

In the United States, for technical assistance, please call (800) 4BIORAD. Select option 2 for technical support and option 2 again for the Food Science Division. To place an order, please call (800) 4BIORAD and press option 1 for customer service. Orders can also be faxed to (800) 879-2289.

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