U.S. FOOD & DRUG ADMINISTRATION

Bacteriological Analytical Manual

Chapter 29: *Cronobacter*

September 2026 Edition

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Revision History

September 2026:

1. Reduced the number of enrichment culture aliquots subjected to centrifugation from three to one.
2. Added a secondary enrichment step and a corresponding DNA extraction procedure as an equally acceptable approach for powdered infant formula analysis.
3. Added procedures for enrichment, qPCR screening, and cultural confirmation of environmental samples.
4. Added a preferred double-quenched *Cronobacter* probe for qPCR.
5. Added an equally acceptable approach for preparing the internal amplification control for qPCR.
6. Increased the annealing temperature for qPCR.
7. Updated the required and optional chromogenic agars.
8. Updated the inclusivity and exclusivity tables.

November 2023:

1. Removed the use of SmartCycler for qPCR.
2. Removed the requirement to perform qPCR without IAC and to perform qPCR on two aliquots per sample.
3. Updated the 7500 Fast software version.
4. Added ROX to the qPCR assay and revised the 7500 Fast analysis settings on threshold and baseline.
5. Revised the stock concentrations and volumes of each qPCR component without changing their final concentrations.
6. Added an extra wash step to the pellet to improve the quality of the resultant DNA extract.
7. Added temperature and time tolerance ranges for incubation of enrichment cultures and chromogenic agars.
8. Added optional use of CCI agar and ESIA agar incubated at their respective temperatures as well as R&F agar incubated at 41.5°C.

9. Added more figures and detailed descriptions of colony phenotypes and morphologies on different agars.
10. Removed the option to use RAPID ID 32E for cultural confirmation.
11. Revised the chapter with updated nomenclature of *Cronobacter* and non-*Cronobacter* species.
12. Updated other parts of the chapter to improve clarity and provide detailed instructions with additional text and figures.

April 2012:

Sections D.1.a, D.1.b, D.2.3; Correction: The fluorescence is recorded at the end of each annealing step, not at the end of each extension step.

March 2012:

New Chapter (This chapter has replaced the method for Isolation and Enumeration of *Enterobacter sakazakii* from Dehydrated Powdered Infant Formula.

Introduction

Cronobacter is a Gram-negative rod-shaped bacterium within the family *Enterobacteriaceae* [1]. The organism was referred to as "yellow-pigmented *Enterobacter cloacae*" until it was renamed *Enterobacter sakazakii* in 1980 [2]. Urmenyi and Franklin reported the first two known cases of meningitis caused by *E. sakazakii* in 1961 [3]. Subsequently, cases of meningitis, septicemia, and necrotizing enterocolitis due to *E. sakazakii* have been reported worldwide [4]. Although most documented cases involve infants, infections in adults have also been reported. Overall, case-fatality rates vary considerably with some reaching as high as 80% [5]. While the reservoir for *E. sakazakii* (now *Cronobacter*) remained unknown, a growing number of reports implicated powdered infant formula (PIF) as a vehicle for infection [6].

Between 2008 and 2014, evidence obtained using amplified fragment length polymorphism, phenotypic arrays, automated ribotyping, 16S rRNA gene sequencing, DNA-DNA hybridization, multilocus sequence typing, and genome-scale phylogeny resulted in a nomenclature change [1, 7, 8]. *E. sakazakii* was reclassified into a new genus, *Cronobacter*, comprising seven species: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter muytjensii*, *Cronobacter dublinensis*, *Cronobacter universalis*, and *Cronobacter condimenti*. *Cronobacter dublinensis* contains three subspecies (Table 1) [1, 7, 8].

Table 1. Biochemical tests for the identification of *Cronobacter* and differentiation of *Cronobacter* species and subspecies [1, 7, 8] .

Biochemical tests[a]	*C. sakazakii*	*C. malonaticus*	*C. turicensis*	*C. universalis*	*C. muytjensii*	*C. condimenti*	*C. dublinensis* subsp. dublinensis	*C. dublinensis* subsp. lactaridi	*C. dublinensis* subsp. lausannensis
Motility	+[b]	V	+	V	+	−	+	+	+
Indole production	−	−	−	−	+	+	+	+	V
Dulcitol	−	−	+	+	+	−	−	−	−
Lactulose	+	+	+	+	+	−	+	+	−
Malonate	−	+	V	+	+	+	+	−	−
Maltitol	+	+	+	+	−	−	+	+	−
Palatinose	+	+	+	V	V	−	+	+	+
Putrescine	+	V	+	−	+	−	+	+	V
Melezitose	−	−	+	+	−	−	+	−	−
Turanose	+	+	+	−	V	−	+	V	−
myo-Inositol	V	V	+	+	+	−	+	+	−
cis-Aconitate	+	+	+	V	+	−	+	+	+
trans-Aconitate	−	+	−	−	+	−	+	+	+
4-Aminobutyrate	+	+	+	V	V	−	+	+	+
1-0-Methyl a-D-glucopyranoside	+	+	+	+	−	+	+	+	+
D-Sorbitol	−	−	−	−	−	−	−	−	−
D-Sucrose	+	+	+	+	+	+	+	+	+
Oxidase	−	−	−	−	−	−	−	−	−
Hydrolysis of 4-NP α-D-glucoside	+	+	+	+	+	+	+	+	+
Lysine decarboxylase	−	−	−	−	−	−	−	−	−
Ornithine decarboxylase	+	+	+	+	+	+	+	+	+
Methyl Red	−	−	−	−	−	−	−	−	−
Voges-Proskauer test	+	+	+	+	+	+	+	+	+
D-Arabitol	−	−	−	−	−	−	−	−	−

[a]The biochemical tests listed in the bottom ten rows are from ISO 22964:2017 and detailed procedures are described in the standard. They can be used for the differentiation of *Cronobacter* from other genera.
[b]+, > 90% Positive; V, 20-80% positive; −, < 10% positive.

Method

The method described here comprises both a real-time PCR component [9] for rapid screening and a cultural component for the detection and isolation of *Cronobacter* spp.[10]. The qPCR targets *Cronobacter* partial macromolecular synthesis operon, spanning the 3' end of the ribosomal protein S21 *(rpsU*) gene and the 5' end of the DNA primase (*dnaG*) gene [9]. Chromogenic agars are used to isolate cultures for confirmation. A pre-enrichment step is used to grow the bacteria to a level (≥ 10^3 CFU/mL) detectable by qPCR and chromogenic agars. The cultural component of this method constitutes a complete recovery and isolation procedure and can therefore be employed as a standalone method in the absence of qPCR technology. The qPCR assay described in Section E is a screening method; as such, positive results should always be confirmed using the cultural method. The qPCR assay should also be used to confirm pure cultures as *Cronobacter* spp. Flowcharts of the entire procedures for analyzing PIF and environmental samples are presented in Figures 1a and 1b.

The inclusivity of this method was determined by analyzing 50 different *Cronobacter* strains, isolated from foods, clinical samples, environmental surfaces, and nationally/internationally recognized culture depositories, representing the seven *Cronobacter* species. The source of each strain is listed in the Inclusivity Table (A). Each strain was grown in brain heart infusion (BHI) broth, diluted in buffered peptone water (BPW), and tested according to this method. The exclusivity of this method was determined by testing 40 strains representing 34 non-*Cronobacter* species. The source of each strain is listed in the Exclusivity Table (B). Each strain was grown in BHI broth and tested according to this method.

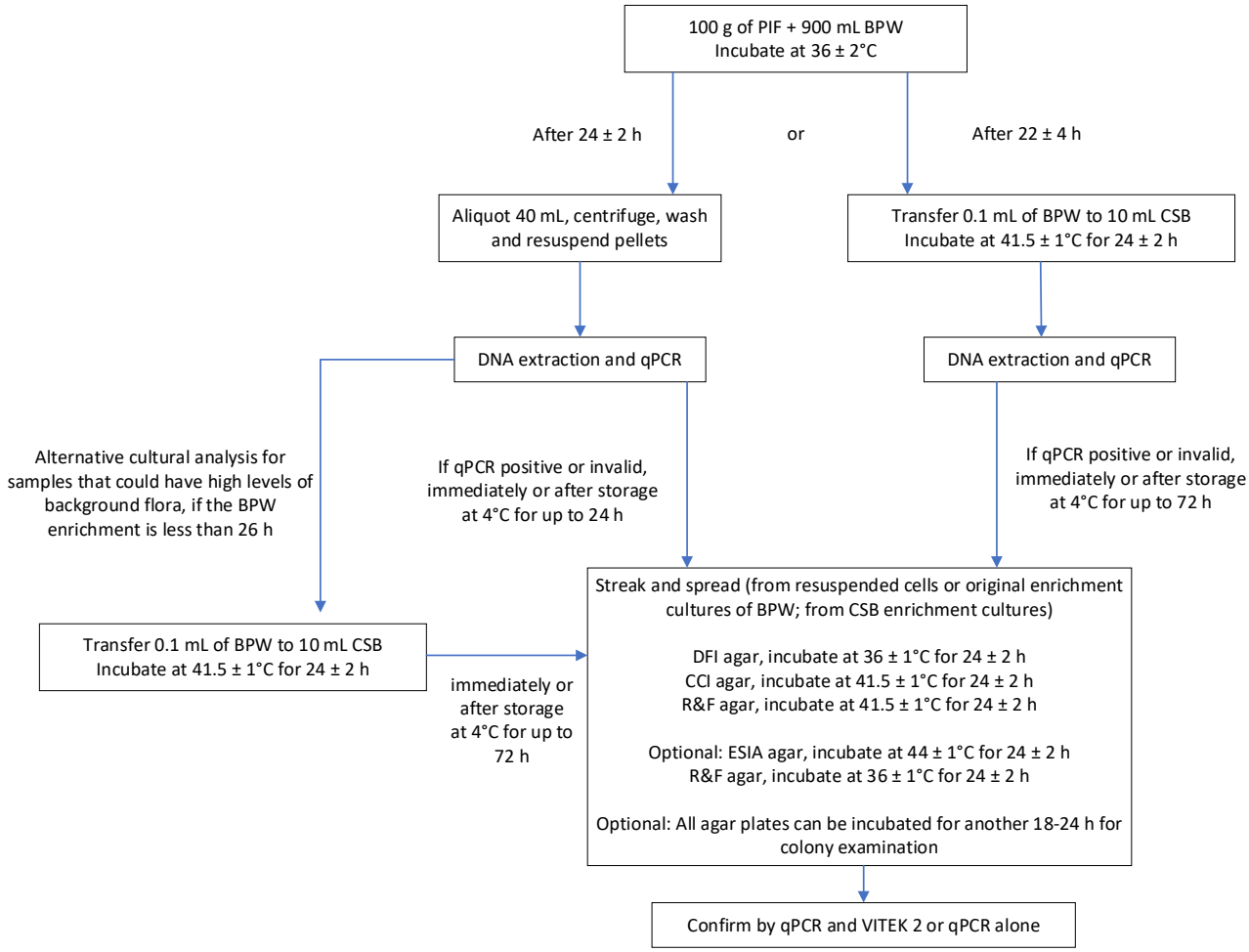


Figure 1a. Flowchart for PIF analysis. The cultural protocol can be used as a standalone protocol without requiring qPCR. Secondary enrichment in CSB is beneficial for isolating *Cronobacter* from samples with high levels of background flora. Performing qPCR after the two-step BPW-CSB enrichment yields qPCR results one day later than qPCR performed directly after BPW enrichment and centrifugation.

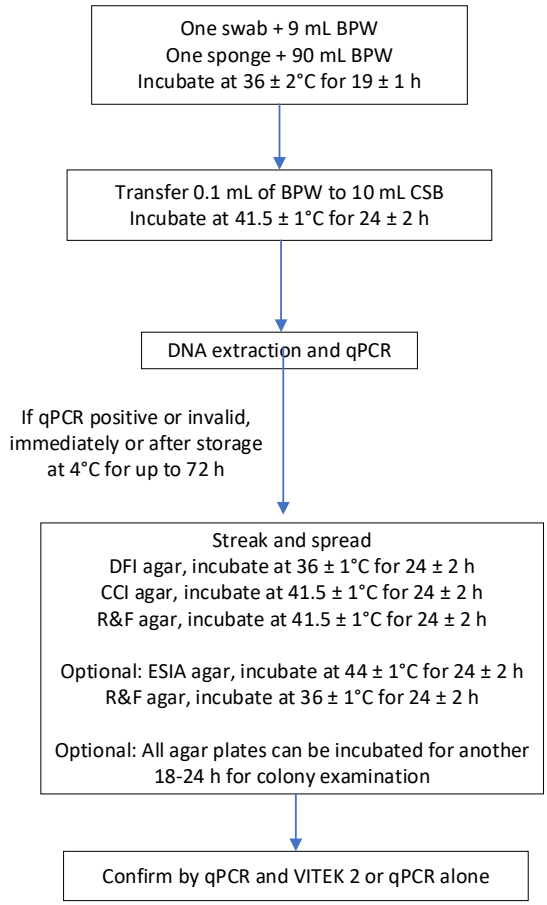


Figure 1b. Flowchart for environmental sample analysis. The cultural method can be used as a standalone protocol without requiring qPCR.

A. Equipment and materials

1. Balance with capacity of 2 kg and sensitivity of 0.1 g
2. Incubators, 36 ± 2°C, 36 ± 1°C, 41.5 ± 1°C, and, optionally, 44 ± 1°C
3. Sterile Erlenmeyer flasks with polyethylene screw caps equipped with Teflon liners, 2 L
4. Sterile sample bags, 2 L or 4 L
5. Micropipettes and tips to dispense 1 µL, 100 µL, 150 µL, 200 µL and 1,000 µL volumes
6. Pipets, 1, 5, and 10 mL, graduated in 0.1 mL units
7. Sterile inoculating loops, 3 mm loop size
8. Glass or plastic spreading rods (e.g., hockey stick) 3-4 mm diameter with 45-55 mm spreading area
9. Sterile utensils for sample handling (see BAM Chapter 1)

10. Centrifuge with a swinging bucket rotor, capable of 3,000 × *g*
11. Microcentrifuge, capable of 15,000 × *g*
12. Polypropylene centrifuge tubes, 50 mL tubes with conical bottoms; 1.5 mL microcentrifuge tubes
13. Vortex mixer
14. Water bath, capable of 100°C
15. Heating block, capable of 100°C
16. Thermometer to check the temperature of heating blocks/water baths.
17. Thermal cyclers: Applied Biosystems™ 7500 Fast real-time PCR system (ThermoFisher Scientific, Inc., Waltham, MA)
18. Instrument-specific 96-well microwell plates, strips, caps and tube/strip installing tools.
19. Optical adhesive covers
20. Petri dishes, plastic, sterile, 15 × 100 mm
21. VITEK® 2 Compact (bioMérieux, Hazelwood, MO)
22. Qubit™ 4 Fluorometer (ThermoFisher Scientific) with High Sensitivity Kit (Cat. No. Q33230, ThermoFisher Scientific) or other DNA quantification instrument
23. Green Fluorescent Protein (GFP) strain, *Cronobacter sakazakii* G-78 (Microbiologics, Saint Cloud, MN)
24. Sterile swabs. Pre-moistened swabs are used for dry and semi-dry surfaces and dry swabs are used for wet surfaces. The following or equivalent:
    a. Neogen™ Swab-sampler in 10 ml D/E neutralizing broth (catalog# RS96010DE, www.neogen.com) (Polyester)
    b. Puritan® dry cotton swab (catalog# 25-806 1PC, 25-806 2PC, www.puritanmedproducts.com) (Cotton)
    c. World Bioproducts PUR-Blue™ swab sampler with (catalog# BLU-10DE) or without (catalog# BLU-DRY, www.worldbioproducts.com) 10 ml D/E neutralizing broth (Polyurethane)
    d. Healthlink® dry swab transporter (catalog# 4159BX, www.hardydiagnostics.com) (Polyester)
25. Sterile sponges. Pre-moistened sponges are used for dry and semi-dry surfaces and dry sponges are used for wet surfaces. The following or equivalent:
    a. World Bioproducts EZ Reach™ sponge sampler with (catalog# EZ-10DE-PUR) or without (catalog# EZ-DRY-PUR,

www.worldbioproducts.com) 10 ml D/E neutralizing broth (Polyurethane)

b. Nasco Whirl-Pak® dry sponge probe (catalog# B01475WA, www.enasco.com) (Cellulose)

c. Neogen™ Sponge-sticks with (catalog# SSL10DE, www.neogen.com) or without (catalog# SSL100) 10 ml D/E neutralizing broth (Cellulose)

B. Media and reagents

1. Phosphate-buffered saline (BAM R59)
2. Buffered peptone water (BPW) (BAM M192)
3. *Cronobacter* Selective Broth (CSB) (BAM M205). CSB base medium can be stored at 4ºC for up to 6 months. Vancomycin solution can be stored at 4ºC for up to 15 days. Once vancomycin is added to CSB, the complete medium should be used within 1 day.
4. Brilliance *Enterobacter sakazakii* agar (DFI formulation) (BAM M201). After the plates have been poured and dried for up to 48 h, they can be stored at 2-8ºC in the dark for up to 2 weeks. Commercial media identical in formulation to BAM M201 can be used after verification following the guidelines of the microbiology management and QA/QC management of each laboratory.
5. *Enterobacter sakazakii* chromogenic plating agar (R&F agar) (BAM M202, Cat. No. M-0700, R & F Laboratories, Downers Grove, IL). Plates should be poured, dried, and stored similarly to DFI agar. The final pH value of the prepared agar medium should be between 6.8 and 7.1. Prepared plates of R&F agar can be stored for up to 60 days.
6. Chromogenic *Cronobacter* Isolation (CCI) agar (BAM M203). Plates should be poured, dried, and stored similarly to DFI agar.
7. *Enterobacter sakazakii* Isolation Agar (ESIA) (BAM M204). Prepare the medium according to the manufacturer's instructions. Plates should be poured, dried, and stored similarly to DFI agar.
8. PrepMan™ Ultra Sample Preparation Reagent (Cat. No. 4318930, ThermoFisher Scientific)
9. iQ™ Supermix qPCR master mix (Cat. No. 170-8860, Bio-Rad, Hercules, CA). This 2× mix contains 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM of each dNTP, 50 U/mL iTaq™ DNA polymerase and 6 mM $MgCl_2$.
10. VITEK® 2 GN ID Card (Cat. No. 21341, bioMérieux, St Louis, MO)
11. Platinum™ Taq DNA Polymerase (Cat. No. 10966-018, ThermoFisher Scientific).

12. ROX passive reference dye (Cat. No. 12223012, ThermoFisher Scientific). Reagents from another manufacturer can be used after verification following the guidelines of the microbiology management and QA/QC management of each laboratory.
13. Proteinase K (Cat. No. 4333793, ThermoFisher Scientific). Reagents from another manufacturer can be used after verification following the guidelines of the microbiology management and QA/QC management of each laboratory.
14. Primers and probes (Table 2). qPCR primers are commercially synthesized with basic desalt purification and reconstituted in PCR-grade water to 100 µM for long-term storage. They are diluted to 2.5-40 µM working stock concentrations. qPCR probes are commercially synthesized with HPLC purification and reconstituted to 2.5-5 µM in single-use aliquots in 1× PCR-grade low-EDTA Tris-EDTA (TE; 10 mM Tris, 0.1 mM EDTA, pH 8.0) buffer to avoid repeated freeze-thaw cycles. Primers and probes should be stored frozen (-20 to -70°C). Discard any remaining thawed probe and do not refreeze. The *Cronobacter* probe is a ZEN/Iowa Black™ FQ double-quenched probe, available for purchase from Integrated DNA Technologies (IDT) (Coralville, IA). A TAMRA probe can be used as an alternative when the ZEN/Iowa Black double-quenched probe is unavailable.
15. Internal amplification control DNA [11] One of the following methods can be used to prepare the internal amplification control (IAC) DNA.
    a. Construction of a plasmid. IAC DNA can be constructed by generating a 200 bp sequence that is synthesized and inserted into a pZErO-2 or equivalent vector and transformed into *Escherichia coli* pDMD801 or another competent strain. A sequence representation of the internal amplification control is shown in Figure 2a (GenBank accession no. FJ357008). The plasmid is extracted from the transformed *Escherichia coli* cells using a Qiagen Plasmid Mini Kit (Cat. No. 12125, Qiagen, Valencia, CA) or equivalent, following the manufacturer's instructions, and quantified using a Qubit™ 4 Fluorometer. To avoid repeated freeze-thaw cycles, plasmids can be diluted into single-use aliquots in PCR-grade 1× low-EDTA TE buffer (10 mM Tris and 0.1 mM EDTA, pH 8.0).
    b. Synthesis of IDT gBlock™ fragments (linear double-stranded DNA fragments). Due to the requirements of gBlock™ synthesis, the 200 bp IAC sequence is modified to remove some internal repeats (Figure 2b). The primer and probe binding regions remain unchanged. The total length, overall GC content, and *Tm* of the IAC remain unchanged. The copy number of gBlock™ IAC is the same as that of the plasmid IAC in PCR.
    c. Commercially synthesized IAC DNA fragment. The IAC fragment

can also be commercially synthesized using other methods and diluted in 1× PCR-grade low-EDTA TE buffer or molecular biology-grade water to a concentration that reliably yields a Ct of no less than 24 when *Cronobacter* DNA extracted from 40 mL overnight BPW cultures is present and no more than 32 with water as a negative control.

Table 2. Primers and probes for the qPCR assay.

Oligos	**Name**	**Sequences (5' to 3')**
Cronobacter forward primer	CronoF	GGGATATTGTCCCCTGAAACAG
Cronobacter reverse primer	CronoR	CGAGAATAAGCCGCGCATT
Cronobacter probe	CronoP	6FAM-AGAGTAGTAGTTGTAGAGGCCGTGCTTCCGAAAG-ZEN/Iowa Black™ FQ (backup) 6FAM-AGAGTAGTAGTTGTAGAGGCCGTGCTTCCGAAAG-TAMRA
Internal control forward primer	IACF	CTAACCTTCGTGATGAGCAATCG
Internal control reverse primer	IACR	GATCAGCTACGTGAGGTCCTAC
Internal control probe	IACP	Cy5-AGCTAGTCGATGCACTCCAGTCCTCCT-Iowa Black RQ-Sp.



Figure 2a. Illustration of the internal amplification control (IAC) DNA. The 200 bp IAC is in black. T7 promoter is represented in a box, and M13, IAC forward and reverse primers and probe are represented by underlines (complete IAC sequence,
TACAGCACCCTAGCTTGGTAGAATCGATCAGCTACGTGAGGTCCTACGACGAT
CGCCAAGCATGCCCTAGCTAAGATGCATCGATTGCTCATCACGTACGTTAGGTCGAC
TAGGAGGACTGGAGTGCATCGACTAGCTAAGATGGTTCGATTGCTCATCACGAAGG
TTAGGTCGACTACGAACGAGTCGTACGTTAGGTT).

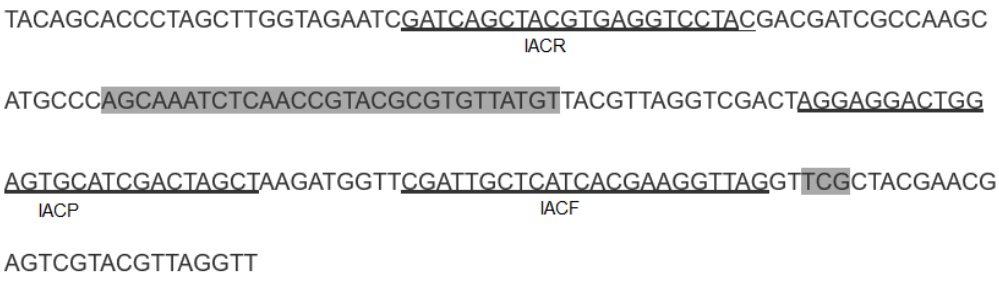


Figure 2b. Illustration of the 200 bp IAC DNA modified specifically for gBlock™ fragment synthesis. The IAC primers and probes are represented by underlines. The grey-highlighted regions indicate where the modifications to the IAC sequence are made in the gBlock™ fragment. (Complete modified IAC sequence,
TACAGCACCCTAGCTTGGTAGAATCGATCAGCTACGTGAGGTCCTACGACGA
TCGCCAAGCATGCCCAGCAAATCTCAACCGTACGCGTGTTATGTTACGTTAG
GTCGACTAGGAGGACTGGAGTGCATCGACTAGCTAAGATGGTTCGATTGCT
CATCACGAAGGTTAGGTTCGCTACGAACGAGTCGTACGTTAGGTT)

C. Preparation of infant formula samples

1. Always wear double gloves. Change outer gloves, wipe the balance and work area clean after processing each sample.
2. Sterilize the container margins (i.e., the areas surrounding the opening) prior to withdrawing a sample.
3. Aseptically weigh 100 ± 0.2 g of powdered infant formula using sterile scoops and transfer to a 2 L sterile Erlenmeyer flask or a 2-4 L sterile sample bag.
4. Add 900 mL of sterile buffered peptone water (BPW) (1:10 dilution) pre-warmed to 36 ± 2°C. Gently massage and/or swirl by hand until no visible clumps remain.
5. Either of the following two enrichment procedures can be used: (1) Incubate for 22-26 h at 36 ± 2°C, then thoroughly mix the BPW enrichment culture and proceed to DNA extraction and qPCR. Retain the BPW enrichment culture at 4°C for potential subsequent analysis or troubleshooting. (2) Incubate for 18-26 h at 36 ± 2°C, then thoroughly mix and transfer 0.1 mL of the BPW enrichment culture to 10 mL of sterile *Cronobacter* selective broth (CSB) and incubate at 41.5 ± 1°C for 24 ± 2 h. Thoroughly mix the CSB enrichment culture and proceed to DNA extraction and qPCR. Retain the CSB enrichment culture at 4°C for potential subsequent analysis or troubleshooting.

D. Preparation of environmental samples

1. When sampling dry surfaces, swabs (cotton, polyester, or polyurethane) or sponges (cellulose or polyurethane) should be pre-moistened in 10 mL of Dey/Engley (D/E) neutralizing broth. Swabs are typically stored in tube containers and sponges are typically stored in bag containers. Before sampling, press swabs against the inner tube walls or squeeze the sponges within the bags to remove excess broth. Sample environmental surfaces using firm, even pressure, swabbing vertically (approximately 10 strokes), then flip the sampler and use the other side to swab horizontally (approximately 10 strokes) and diagonally (approximately 10 strokes). When returning the samplers to their containers, ensure they are fully submerged in the D/E neutralizing broth. When sampling wet surfaces, dry swabs or sponges should be used and then transferred to D/E neutralizing broth immediately after sampling. Cotton swabs or those with large tips appear to absorb liquid more effectively than other swab types. For samples that cannot be collected using standard-sized swabs or sponges as described in section A, refer to applicable sampling compliance guidance documents for additional instructions.
2. Add the swab or sponge, along with any remaining D/E neutralizing broth, to 90 mL of BPW. Q-tip swabs submerged in 1 mL of D/E neutralizing broth can be transferred to 9 mL of BPW. Thoroughly massage or use a stomacher to expel the collection broth into the enrichment broth.

3. Incubate for 19 ± 1 h at 36 ± 2°C, then thoroughly mix the enrichment culture.
4. Transfer 0.1 mL of the enrichment culture to 10 mL of *Cronobacter* selective broth (CSB), incubate at 41.5 ± 1°C for 24 ± 2 h, and thoroughly mix the resulting enrichment culture.

E. qPCR screening of *Cronobacter*

1. DNA extraction

    a. From the BPW enrichment culture, remove 40 mL from each sample and transfer it into a 50 mL centrifuge tube. Centrifuge the aliquot at 3,000 × *g* for 10 min in a swinging bucket centrifuge (fixed-angle centrifuges are not recommended due to difficulties separating the fats from the pellet). Aspirate the supernatant from each centrifuge tube. If necessary, use sterile swabs or equivalent tools to remove the fat precipitate on the side wall within the centrifuge tube. Resuspend the resultant pellet (approximately 200 µL pellet volume) in 1,400 µL of phosphate buffered saline (PBS) by vortexing at maximum speed for at least 20 sec. Certain PIF samples may form very large pellets; in such cases, it may be necessary to increase the volume of PBS and the volume of the subsequent transfer accordingly; refer to applicable sampling compliance guidance documents for specific instructions. Certain PIF samples may contain ingredients (e.g., rice starch) that prevent the formation of small, solid pellets after centrifugation; refer to applicable sampling compliance guidance documents for specific instructions. Transfer 800 µL of resuspended cells (in PBS) to a 1.5 mL microcentrifuge tube and centrifuge at 3,000 × *g* for 5 min. Store the remaining resuspended cells on ice or at 4°C until needed. Depending on the presence or absence of bacterial cells and the efficiency of fat removal in the previous step, up to four layers may be visible after centrifugation. The top layer consists of fat residues, the second layer is the supernatant, the third layer is the bacterial cell pellet (brown/yellow in color), and the bottom layer consists of milk particles. If necessary, discard the supernatant and any fat residues from each centrifuge tube and use sterile cotton tip swabs or equivalent tools to remove any precipitate from the inner walls of the centrifuge tube. Add 400 µL of PrepMan™ Ultra Sample Preparation Reagent to each tube and vortex at maximum speed until fully suspended. Heat the sample for 10 min at 100°C in a boiling water bath or heating block, then cool to room temperature for 2 min. Centrifuge the sample for 2 min at a minimum speed of 15,000 × *g*. Transfer 50 µL of the supernatant to a new tube for qPCR analysis, or store at -20ºC for up to 3 days prior to qPCR analysis.

    b. From the CSB enrichment culture, transfer 1.8 mL to a 2 mL

microcentrifuge tube and centrifuge at 15,000 × g for 2 min. Discard the supernatant and resuspend the pellet in 600 µL of PBS, followed by centrifugation at 15,000 × g for 2 min. Discard the supernatant and resuspend the cell pellet in 150 µL of molecular biology-grade Tris-EDTA buffer (TE; 10 mM Tris, 1 mM EDTA, pH 8.0) before adding 10 µL of proteinase K (20 mg/mL). Perform lysis by heating the sample at 56°C for 15 min followed by 100°C for 10 min and then cooling to room temperature for 2 min. Centrifuge the lysate at 15,000 × g for 2 min and transfer 50 µL of the supernatant to a new tube for qPCR analysis or store at -20°C prior to qPCR.

2. qPCR Controls

   Include a positive control (prepared by 1:10 dilution of DNA extracted from a pure culture of *Cronobacter* spp., e.g., ATCC 29544, or the GFP strain *Cronobacter sakazakii* G-78) and a no-template (water) control in each qPCR run. The qPCR assay is designed to detect very low levels of *Cronobacter* cells. If large amounts of cells are present following enrichment, the qPCR may yield high FAM signals (e.g., Ct < 12). In such cases, the DNA extract may be diluted (typically 1:10 or 1:100) and qPCR rerun.

3. qPCR with ABI 7500 Fast Thermal Cycler (software version 2.3)

   Prepare qPCR reactions from the reaction components and final concentrations listed in Table 3. Create a "new experiment" on the 7500 Fast instrument. Assign each experiment a unique name. Select the following parameters:

   a. In "Experimental Properties", select quantitation-standard curve as the experiment type, TaqMan reagents, and standard ramp speed.

   b. In "Plate Setup", select ROX as the reference dye, FAM as the reporter dye for the *Cronobacter* probe and Cy5 as the reporter dye for the IAC probe, none as the quencher for the *Cronobacter* probe if the ZEN/Iowa Black™ FQ double-quenched probe is used (preferred), or TAMRA as the quencher for the *Cronobacter* probe if the TAMRA probe is used when the ZEN/Iowa Black™ FQ probe is unavailable. Select none as the quencher for the IAC probe. Assign appropriate wells on the thermal cycler block.

   c. In "Run Method", set the reaction volume per well to 25 µL. Select the following qPCR cycling conditions: 95°C for 3 min followed by 40 cycles of 95°C for 15 sec, 56°C for 40 sec and 72°C for 15 sec. Fluorescence is recorded at the end of each annealing step.

Table 3. qPCR reaction components for ABI 7500 Fast with IAC.

Component	Volume/reaction	Final Concentration
iQ Supermix	12.5 µL	50 mM KCl, 20 mM Tris-HCl, 0.2 mM each dNTP, 0.625 U iTaq DNA polymerase and 3 mM $MgCl_2$
ROX	0.05 µL (25 µM solution)	50 nM
CronoF	0.25 µL (40 µM stock solution)	400 nM
CronoR	0.25 µL (40 µM stock solution)	400 nM
CronoP	1.5 µL (5 µM stock solution)	300 nM
IACF	0.25 µL (15 µM stock solution)	150 nM
IACR	0.25 µL (15 µM stock solution)	150 nM
IACP	1.5 µL (2.5 µM stock solution)	150 nM
IAC DNA	1 µL (3000 plasmid copies or gBlock™ DNA copies per µL)	3000 copies
Taq Polymerase	0.25 µL (10 U/µL)	2.5 U
$MgCl_2$	1.5 µL (50 mM solution)	3 mM
DNA extract or control	2 µL	2 µL
PCR-grade water	Appropriate amount to reach 25 µL	Appropriate amount to reach 25 µL

4. Data analysis. Set the threshold and baseline values according to Table 4. The threshold values are based on the plot of ΔRn vs. Cycle. ROX is required for screening qPCR and recommended for confirmatory qPCR. If ROX is unavailable, confirmatory qPCR may be performed without it; in this case, a threshold of 250,000 and the baseline settings described in Table 4 should be used. Representative screenshots of the graph and table views are shown in Figure 3. If the qPCR result is positive, the sample is considered qPCR-positive and should proceed to cultural confirmation (Section F). If the qPCR result is negative, the sample is considered negative, and analysis is halted.

   A DNA extract is considered qPCR-positive if a Ct value is obtained for FAM and the amplification curve is sigmoidal. The multicomponent plot in the 7500 Fast software displays individual signals for FAM, Cy5, ROX, and TAMRA, which can be used to evaluate non-typical plots. Review of IAC reactions is not required when the FAM reaction is positive. If no Ct value is obtained for FAM, or if a Ct value is obtained but the curve does not exhibit a typical sigmoidal shape, the IAC reaction for that DNA extract must be evaluated:

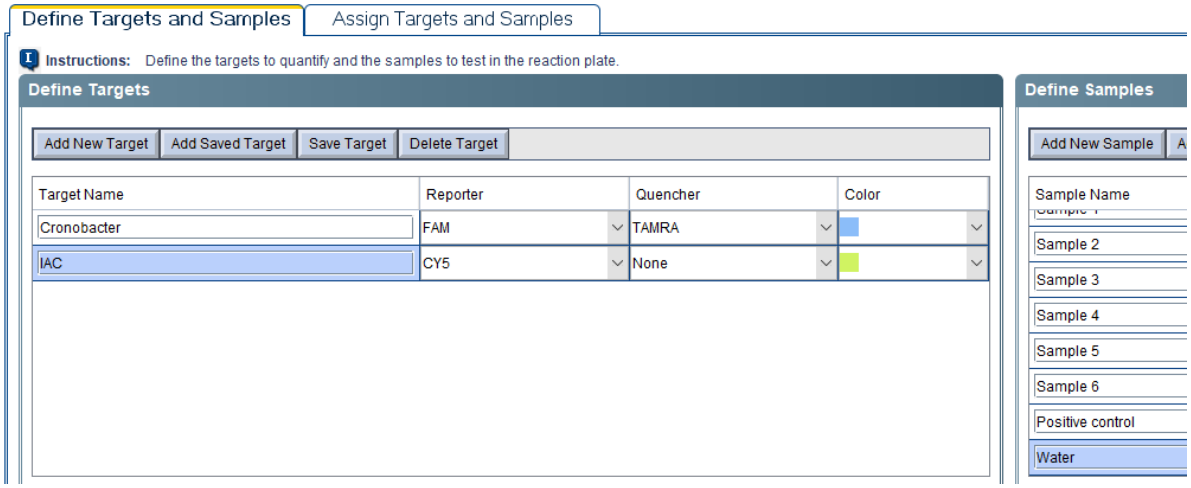
   a. The DNA extract is considered negative if the Cy5 amplification curve is sigmoidal and the difference in Cy5 Ct values between the DNA extract and the water negative control in the same run is less than 4.

   b. If no Ct value is obtained for Cy5 or if the difference in Ct values between the DNA extract and the water negative control in the

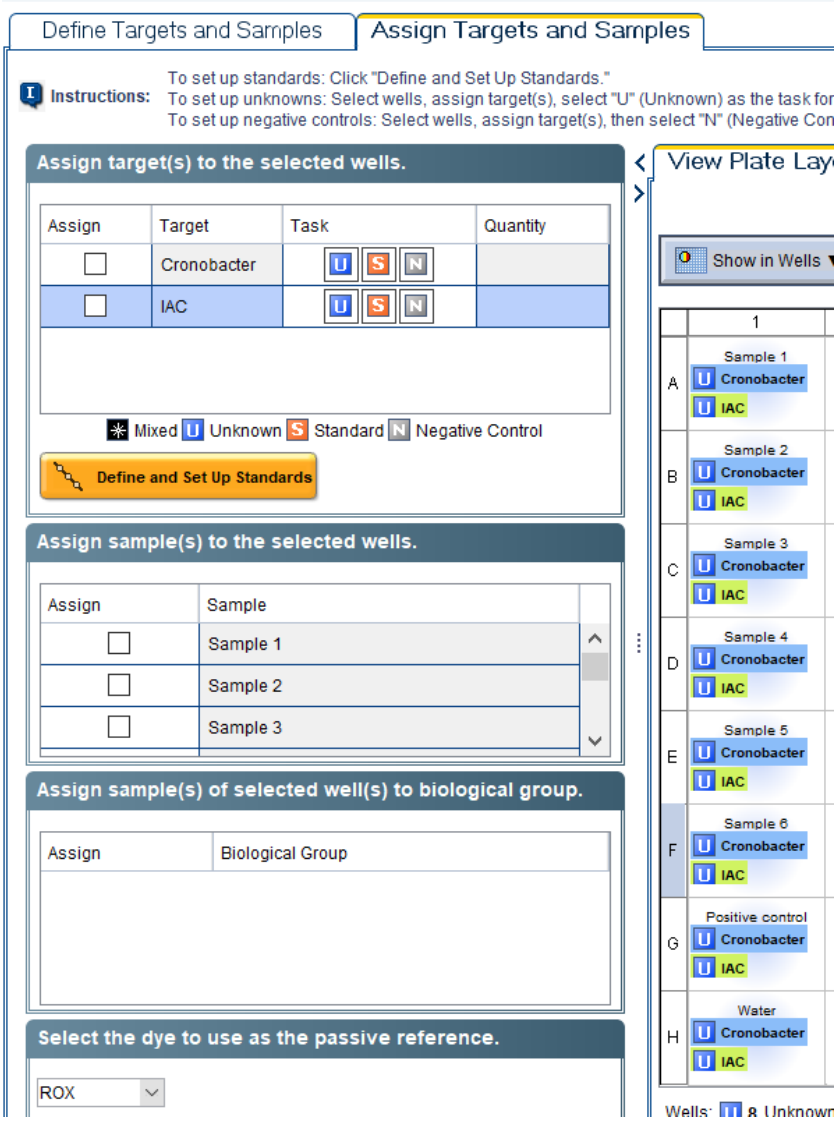
same run is 4 or greater, an inhibitory substance may be present in the sample, and the qPCR result is considered invalid. In such cases, the DNA extract should be diluted (1:10 in PCR-grade water) or centrifuged for further purification and the qPCR repeated; alternatively, proceed directly to cultural analysis (Section F).

Table 4. Threshold and baseline settings for *Cronobacter* and IAC reactions.

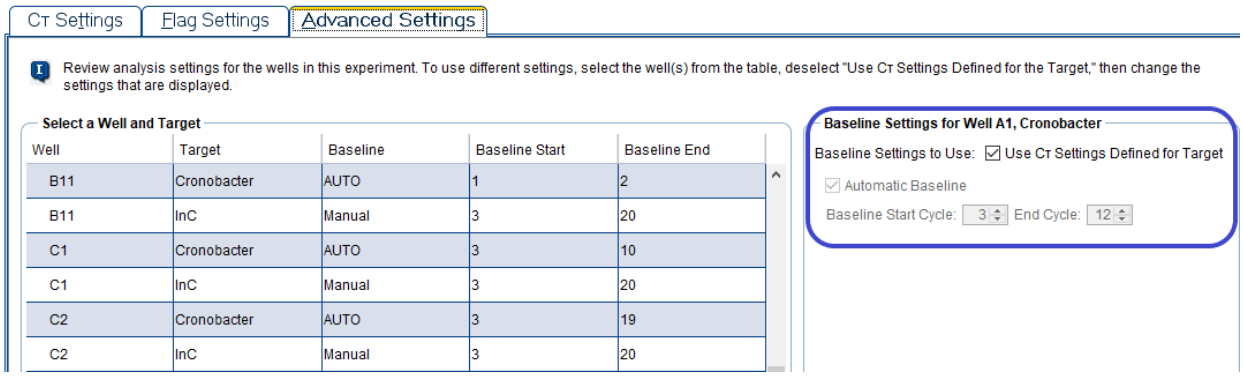
qPCR targets	Threshold	Baseline
FAM-*Cronobacter* for screening	0.25 for ZEN/Iowa Black™ FQ quencher; 0.15 for TAMRA quencher	Initially set to auto baseline for all samples. For any sample, if the baseline start < 3, manually change the baseline start to 3; if the baseline end < 15, manually change the baseline end to 15.
FAM-*Cronobacter* for confirmation	0.25 for either quencher	The same as above
Cy5-IAC for both screening and confirmation	0.05	3 to 20



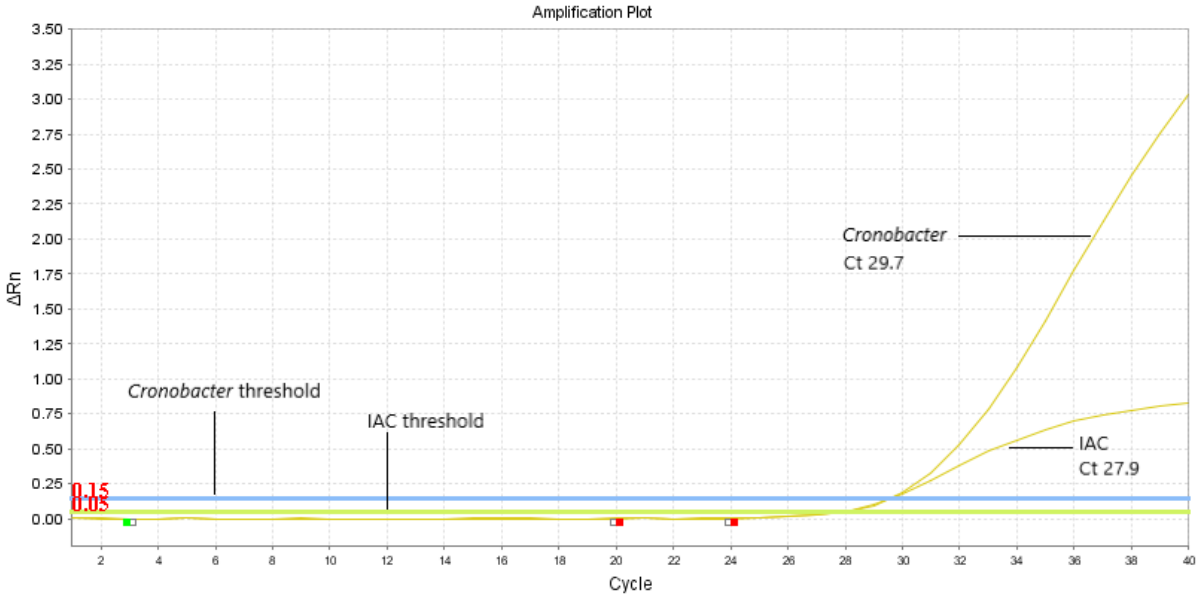
(a)


Define Targets and Samples
Assign Targets and Samples
Instructions:
To set up standards: Click "Define and Set Up Standards."
To set up unknowns: Select wells, assign target(s), select "U" (Unknown) as the task for
To set up negative controls: Select wells, assign target(s), then select "N" (Negative Con
Assign target(s) to the selected wells.
Assign
Target
Task
Quantity
Cronobacter
IAC
Mixed
Unknown
Standard
Negative Control
Define and Set Up Standards
Assign sample(s) to the selected wells.
Assign
Sample
Sample 1
Sample 2
Sample 3
Assign sample(s) of selected well(s) to biological group.
Assign
Biological Group
Select the dye to use as the passive reference.
ROX
View Plate Lay
Show in Wells
1
A
Sample 1
Cronobacter
IAC
B
Sample 2
Cronobacter
IAC
C
Sample 3
Cronobacter
IAC
D
Sample 4
Cronobacter
IAC
E
Sample 5
Cronobacter
IAC
F
Sample 6
Cronobacter
IAC
G
Positive control
Cronobacter
IAC
H
Water
Cronobacter
IAC

(b)


CT Settings
Flag Settings
Advanced Settings
Review analysis settings for the wells in this experiment. To use different settings, select the well(s) from the table, deselect "Use CT Settings Defined for the Target," then change the settings that are displayed.
Select a Well and Target
Well
Target
Baseline
Baseline Start
Baseline End
B11 Cronobacter AUTO 1 2
B11 InC Manual 3 20
C1 Cronobacter AUTO 3 10
C1 InC Manual 3 20
C2 Cronobacter AUTO 3 19
C2 InC Manual 3 20
Baseline Settings for Well A1, Cronobacter
Baseline Settings to Use:
Use CT Settings Defined for Target
Automatic Baseline
Baseline Start Cycle: 3
End Cycle: 12

(c)



(d)

Figure 3. Representative screenshots of the 7500 Fast results. (a) Defining samples and targets, (b) Assigning targets to each sample. (c) Adjusting baseline of individual well. (d) Amplification curves, thresholds when the *Cronobacter* probe is the TAMRA probe, and Ct values of *Cronobacter* and IAC reactions.

F. Isolation of *Cronobacter*

For qPCR-positive samples, samples exhibiting inhibition during qPCR screening, or samples not analyzed by qPCR, streak a loopful of the remaining resuspended cells from the centrifuged BPW enrichment culture (obtained in E.1) or a loopful of the original BPW enrichment culture (obtained in C.5) onto the surface of one DFI (M201) agar plate [12], one R&F agar plate (M202) [13], and one CCI agar plate (M203), [14] using sterile inoculation loops (Figure 1). If CSB is used as the secondary enrichment, streak a loopful of the CSB enrichment culture (obtained in D.4) onto the same agar types. In addition, spread 100 µL of the resuspended cells from the centrifuged BPW enrichment culture, the original BPW enrichment culture, or the CSB enrichment culture evenly onto the surface of one DFI agar plate, one R&F agar plate, and one CCI agar plate using sterile spreading rods (Figure 1). Incubate DFI agar plates at 36 ± 1°C for 24 ± 2 h. Incubate R&F and CCI agar plates at 41.5 ± 1°C for 24 ± 2 h. Examine all plates for typical or suspect *Cronobacter* colonies (Figure 4) for further confirmation. If cultures overgrow on the plates, streak a 3 mm loopful (~10 µL) of lawn material to at least three quadrants of a new plate to obtain single colonies. Plates can be kept for up to 3 days at 4°C during the investigation of confluent growth.

Using a sterile loop or needle, pick at least one suspect *Cronobacter* colony, if present, from each chromogenic agar plate for further confirmation. If no isolated

colonies are available, areas of suspect confluent growth should be re-streaked for purification. If isolated colonies from streak plates are selected for further purification or direct confirmation, spread plates of the same agar type exhibiting confluent growth may not require sub-culturing. At least one isolated suspect colony from each chromogenic agar type should be picked for confirmation as described in Sections H and I. Additional colonies per agar type may be selected if the goal is to isolate multiple strains from the same sample.

Note: The BPW enrichment cultures can be stored at 4°C for up to 24 h before streaking. The CSB enrichment cultures can be stored at 4°C for up to 72 h before streaking.

Optionally, after streaking and spread plating onto the required agars described above, enrichment cultures can be streaked onto the surface of one ESIA agar plate (M204) [15] and/or one R&F agar plate (Figure 1). Incubate the optional R&F agar plate at 36 ± 1°C for 24 ± 2 h. Incubate the optional ESIA agar plate at 44 ± 1°C for 24 ± 2 h.

Optionally, the plates can be incubated for an additional 18-24 h to increase colony size and improve color development for some strains.

Optionally, for BPW enrichment cultures that will undergo further cultural analysis, particularly when high levels of background flora are expected, transfer 0.1 mL of the enrichment culture to 10 mL of sterile *Cronobacter* selective broth (CSB) provided the enrichment duration has not exceeded 26 h. BPW enrichment cultures should be maintained at 36 ± 2°C prior to transfer to CSB. Incubate the CSB cultures at 41.5 ± 1°C for 24 ± 2 h. Following incubation, streak and spread plate the cultures onto the chromogenic agar plates as described above.

Use standard long-wave UV light (360–400 nm), also known as UV-A or black light, to examine the GFP control strain G-78. G-78 appears pale to yellow green on DFI and CCI agars, light green on ESIA agar, and blue-black on R&F agar.

G. Identification of *Cronobacter* colonies

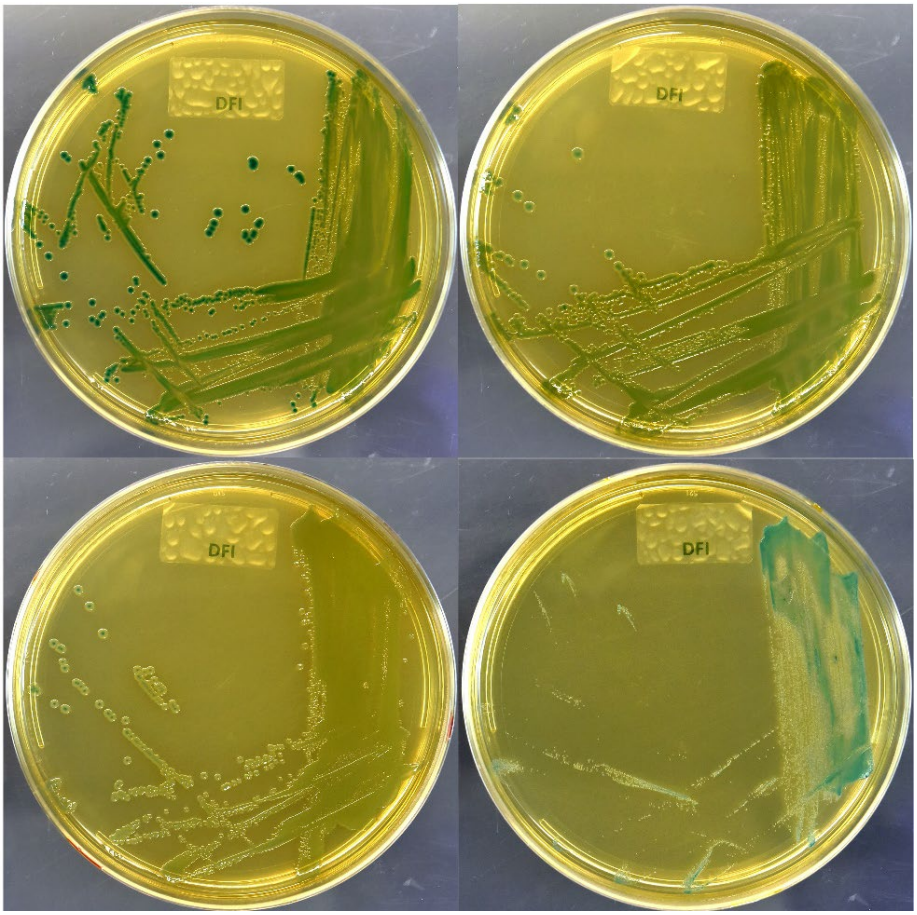
Presumptive *Cronobacter* colonies on DFI and CCI agar appear dark green, pale green, or yellow green. Some colonies have a green center with a white or yellow border. Rare strains appear predominantly yellow with a very faint green center (Figure 4a, 4b, 4f, and 4g).

Presumptive *Cronobacter* colonies on R&F agar appear blue to black or blue to grey against the red background. Rare strains may appear light grey when grown at 36°C (Figure 4c and 4d). The red background can appear purplish red depending on the strain or lighting conditions. *Cronobacter* does not alter the color of R&F agar; however, the presence of background microflora can change

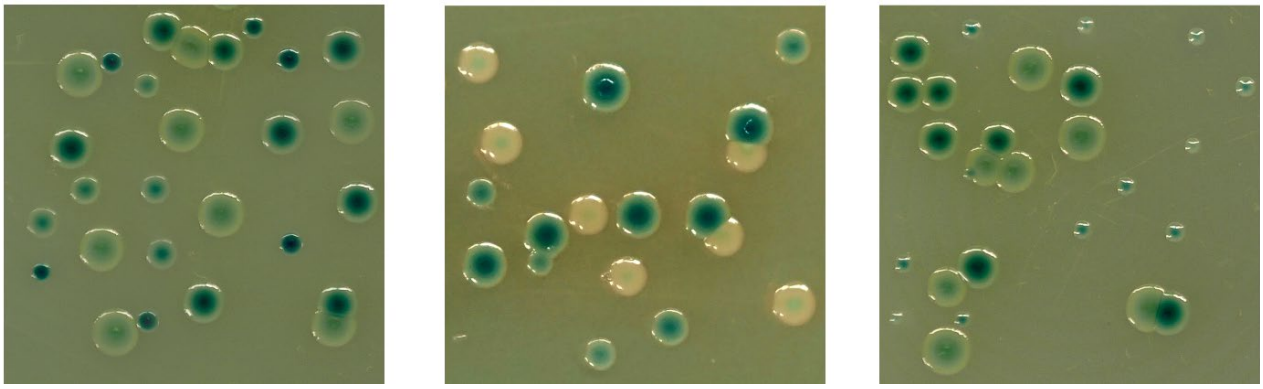
the color of R&F agar from red to yellow, which may cause *Cronobacter* colonies to appear green to black (Figure 4e).

Presumptive *Cronobacter* colonies on ESIA agar appear dark blue to green or blue to green. Some strains exhibit very light green pigmentation and thus appear predominantly blue, especially as isolated colonies (Figure 4h, 4i, and 4j).

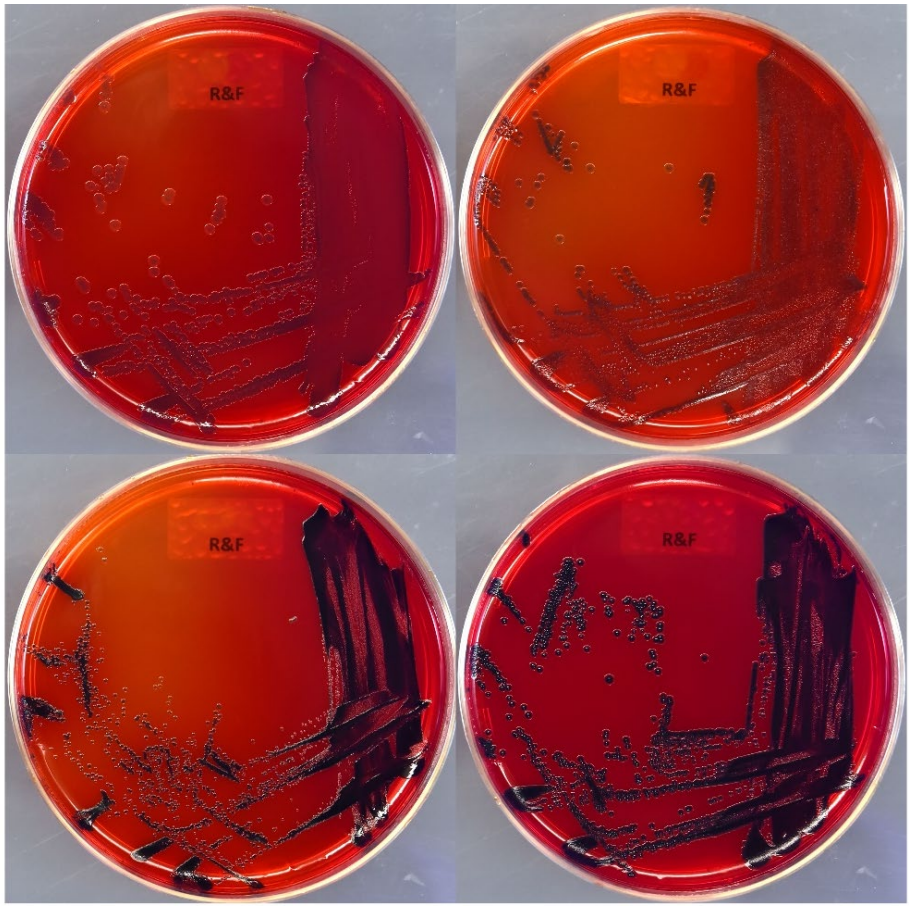
Some strains produce very small colonies on any of the agars after 24 h of incubation. Careful attention should be paid to suspect colonies exhibiting weak green coloration. Typical colonies are 2-3 mm in diameter; however, colonies of some rare strains may be as small as 1 mm in diameter.



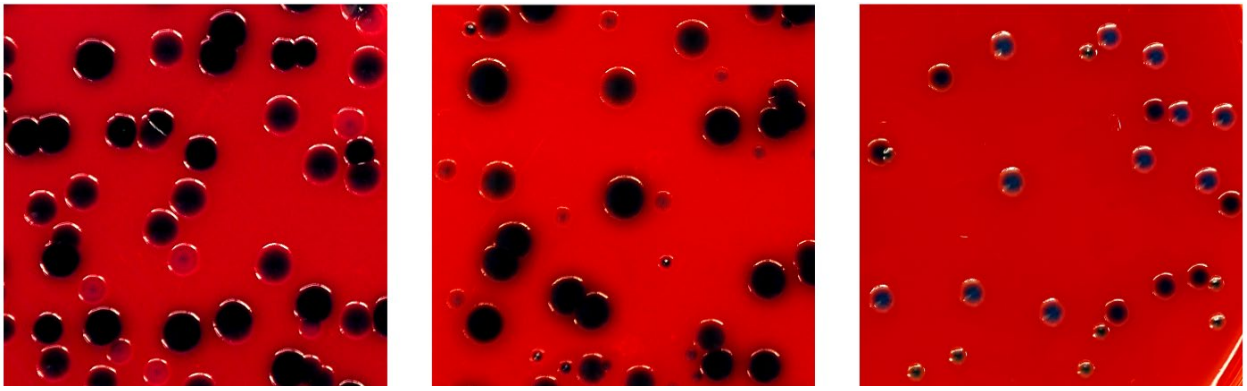
(a) *Cronobacter* colonies on DFI agar photographed against a dark background. The coloration can be green (upper left), pale green (upper right), or yellow green (bottom left). Rare strains may produce very small colonies (bottom right**).**



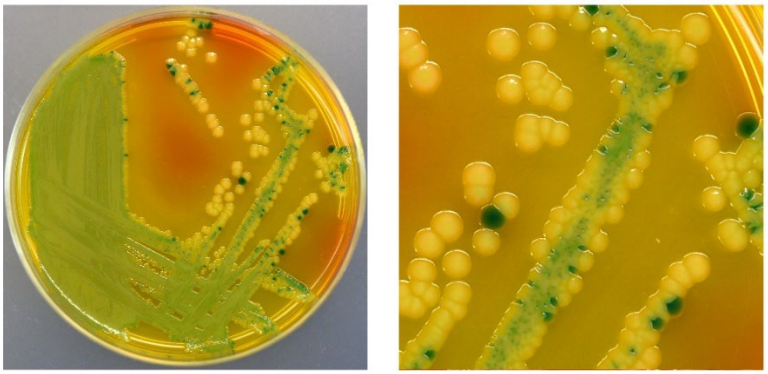
(b) Mixed *Cronobacter* colonies on DFI agar photographed against a dark background. The green coloration tends to be concentrated at the colony center. Rare strains may appear predominantly yellow with a very faint green center as shown in the center image. Note that the zoomed images make colonies appear larger than actual size.



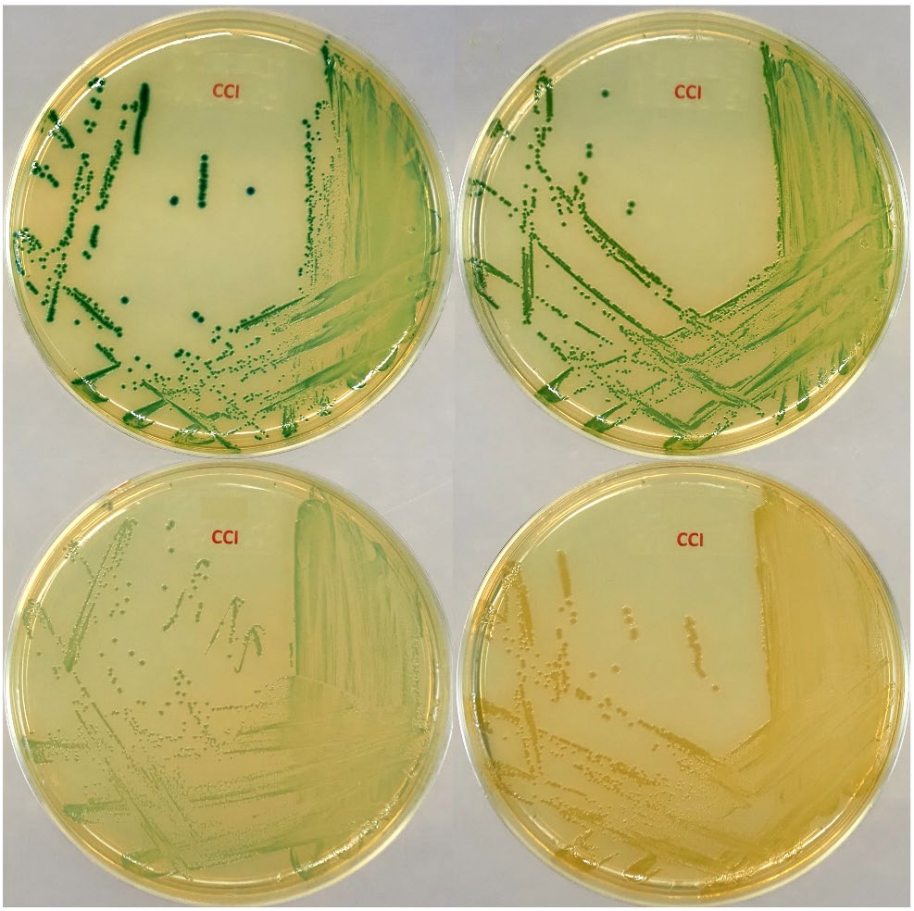
(c) *Cronobacter* colonies on R&F agar. Colony coloration can be blue grey (upper right) or blue black (bottom right). Rare strains may appear light grey when grown at 36°C (upper left) or produce small colonies (bottom left).



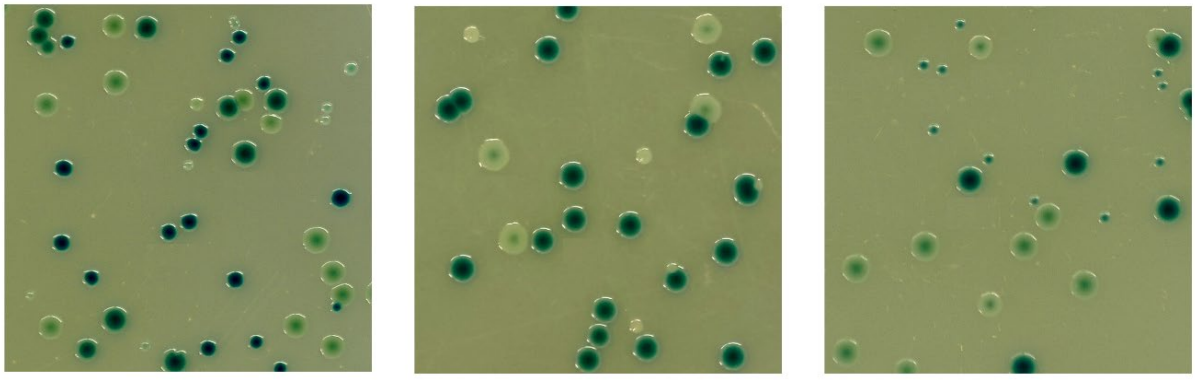
(d) Mixed *Cronobacter* strains on R&F agar.



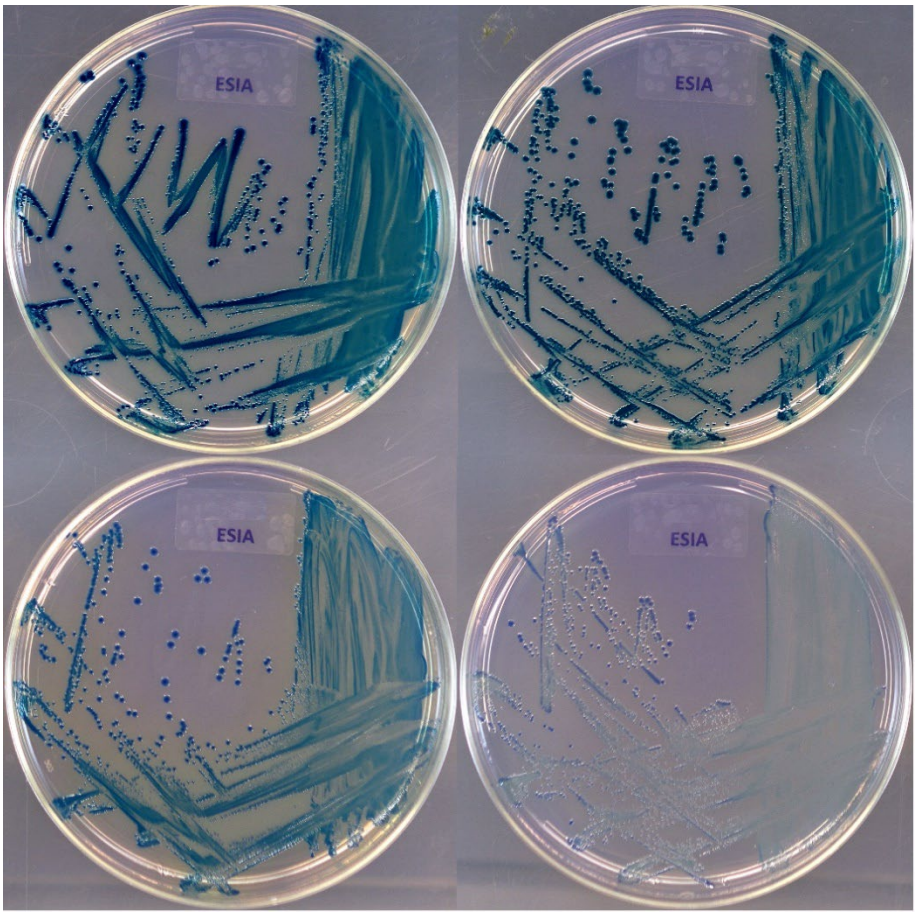
(e) *Cronobacter* colonies mixed with background flora on R&F agar. The background flora change the agar background color from red to yellow, causing *Cronobacter* colonies to appear green to black against the yellow background.



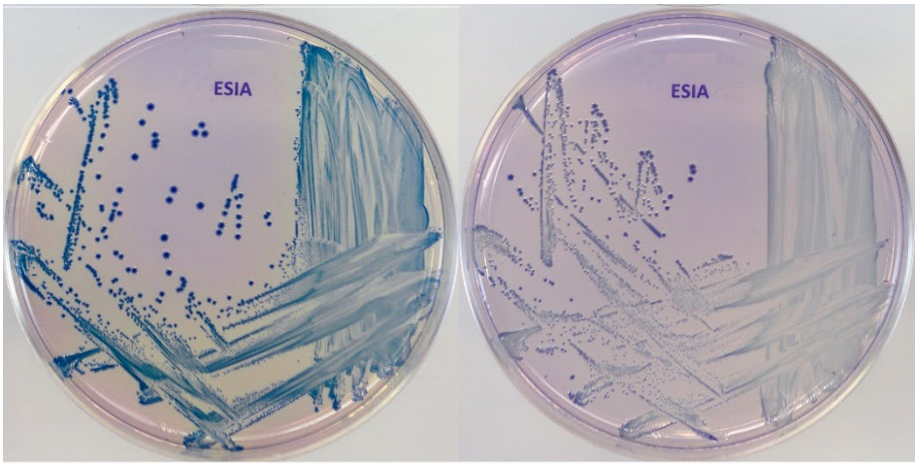
(f) *Cronobacter* colonies on CCI agar photographed against a light background. Colony coloration can be dark green (upper left), green (upper right), pale green (bottom left), or yellow green (bottom right).



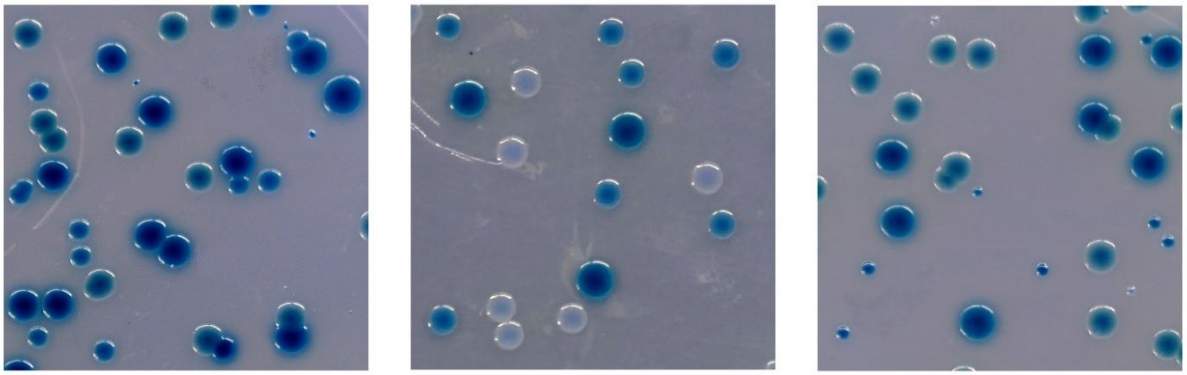
(g) Mixed *Cronobacter* colonies on CCI agar photographed against a dark background**.** Rare strains may produce very small colonies, as shown in all three images**.**



(h) *Cronobacter* colonies on ESIA agar photographed against a dark background. Colony coloration can be dark blue green (upper) or blue green, with isolated colonies more blue and heavy growth areas more green (bottom left). Some strains exhibit very light green pigmentation and thus appear predominantly blue (bottom right).



(i) Same two plates from the bottom of (h) photographed against a light background. The colonies appear greener than when photographed against a dark background.



(j) Mixed *Cronobacter* colonies on ESIA agar photographed against a dark background. Rare strains may exhibit light blue coloration, as shown in the center image.

Figure 4. Colors and morphologies of *Cronobacter* colonies.

H. qPCR confirmation

From each chromogenic agar type, prepare DNA from at least one isolated suspect colony if one is available. DNA can also be prepared from the nutrient-rich, general-purpose agar plates used for further purification. Plates can be held at 4°C for no more than 24 h prior to DNA extraction. Transfer one colony to 300 µL of PCR-grade dH_2O in a 1.5 mL microcentrifuge tube, resuspend by vortexing or pipetting, and heat for 5 min in a boiling water bath or a heating block set to 100°C. Chill the tubes on ice and centrifuge at 15,000 × *g* for 2 min. Use 2 µL of the resulting lysate as the DNA template for the qPCR assay described in Section E. If a colony is picked from a chromogenic agar plate directly for qPCR confirmation, subculture it onto TSA if VITEK 2 confirmation is subsequently needed.

I. Biochemical confirmation

Further purify suspect colonies picked from chromogenic agars onto TSA or other approved nutrient media, then confirm using the VITEK 2 biochemical identification system according to the manufacturer's instructions.

When qPCR is used as the initial method to confirm suspect isolates, secondary confirmation of the same colony by VITEK 2 is highly recommended. When VITEK 2 is used as the initial method to confirm suspect isolates and yields a positive identification of the *Cronobacter sakazakii* group, secondary confirmation of the same colony by qPCR is required. When VITEK 2 is used as the initial method to confirm suspect isolates and yields negative identification for *Cronobacter*, secondary confirmation of the same colony by qPCR is not required.

The biochemical tests listed in Table 1 can be used for the identification of *Cronobacter*. Other commercial biochemical test kits can be used if properly validated. *Franconibacter* and *Siccibacter* share some biochemical characteristics with *Cronobacter* and thus should be included when validating alternative biochemical test kits.

J. Optional: enumeration of *Cronobacter*

Use the three-tube, three-level Most Probable Number (MPN) procedure (BAM Manual, Appendix 2; Most Probable Number Determination from Serial Dilutions: https://www.fda.gov/food/laboratory-methods-food/bam-appendix-2-most-probable-number-serial-dilutions). Aseptically weigh out in triplicate, 100 g, 10 g, and 1 g of the powdered infant formula and add to 2 L, 250 mL, and 125 mL Erlenmeyer flasks or equivalently sized sample bags, respectively, and proceed with sample preparation, culture isolation, and confirmation. Calculate the MPN of *Cronobacter* cells/g of sample based on the number of positive "tubes" at each dilution level.

K. Subtyping of *Cronobacter*

Confirmed *Cronobacter* isolates should be further characterized by whole genome sequencing using standardized GenomeTrakr methods and the resulting data submitted to GenomeTrakr.

References

1. Iversen C, Mullane N, McCardell B, Tall BD, Lehner A, Fanning S, Stephan R, Joosten H: ***Cronobacter*** **gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter genomospecies* 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactaridi* subsp. nov.** International Journal of Systematic and Evolutionary Microbiology 2008, **58**(Pt 6):1442-1447.

2. Farmer JJI, Asbury MA, Hickman FW, Brenner DJ, The Enterobacteriaceae Study Group: **A New Species of "*Enterobacteriaceae*" Isolated from Clinical Specimens**. International Journal of Systematic and Evolutionary Microbiology 1980, **30**(3):569-584.

3. Urmenyi AM, Franklin AW: **Neonatal death from pigmented coliform infection**. Lancet 1961, **1**(7172):313-315.

4. Nazarowec-White M, Farber JM: ***Enterobacter sakazakii*****: a review**. International Journal of Food Microbiology 1997, **34**(2):103-113.

5. Lai KK: ***Enterobacter sakazakii*** **infections among neonates, infants, children, and adults. Case reports and a review of the literature**. Medicine (Baltimore) 2001, **80**(2):113-122.

6. Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment (JEMRA), Nutrition and Food Safety (NFS), (SSA) SSAoFN: ***Enterobacter sakazakii*** **and other microorganisms in powdered infant formula: meeting report**; 2004.

7. Stephan R, Grim CJ, Gopinath GR, Mammel MK, Sathyamoorthy V, Trach LH, Chase HR, Fanning S, Tall BD: **Re-examination of the taxonomic status of *Enterobacter helveticus*, *Enterobacter pulveris* and *Enterobacter turicensis* as members of the genus *Cronobacter* and their reclassification in the genera *Franconibacter* gen. nov. and *Siccibacter* gen. nov. as *Franconibacter helveticus* comb. nov., *Franconibacter pulveris* comb. nov. and *Siccibacter turicensis* comb. nov., respectively**. International Journal of Systematic and Evolutionary Microbiology 2014, **64**(Pt 10):3402-3410.

8. Joseph S, Cetinkaya E, Drahovska H, Levican A, Figueras MJ, Forsythe SJ: Cronobacter condimenti sp. nov., isolated from spiced meat, and Cronobacter universalis sp. nov., a species designation for Cronobacter sp. genomospecies 1, recovered from a leg infection, water and food ingredients. International Journal of Systematic and Evolutionary Microbiology 2012, **62**(Pt 6):1277-1283.

9. Seo KH, Brackett RE: **Rapid, specific detection of Enterobacter sakazakii in infant formula using a real-time PCR assay**. Journal of Food Protection 2005, **68**(1):59-63.

10. Chen Y, Song KY, Brown EW, Lampel KA: **Development of an improved protocol for the isolation and detection of *Enterobacter sakazakii* (*Cronobacter*) from powdered infant formula**. Journal of Food Protection 2010, **73**(6):1016-1022.

11. Deer DM, Lampel KA, Gonzalez-Escalona N: **A versatile internal control for use as DNA in real-time PCR and as RNA in real-time reverse transcription PCR assays**. Letters in Applied Microbiology 2010, **50**(4):366-372.

12. Druggan P, Iversen C: **Culture media for the isolation of *Cronobacter* spp.** Int J Food Microbiol 2009, **136**(2):169-178.

13. Restaino L, Frampton EW, Lionberg WC, Becker RJ: **A chromogenic plating medium for the isolation and identification of *Enterobacter sakazakii* from foods, food ingredients, and environmental sources**. Journal of Food Protection 2006, **69**(2):315-322.

14. de Benito A, Gnanou Besse N, Desforges I, Gerten B, Ruiz B, Tomas D: Validation of standard method EN ISO 22964:2017 - **Microbiology of the food chain - Horizontal method for the detection of *Cronobacter* spp**. International Journal of Food Microbiology 2019, **288**:47-52.

15. Iversen C, Forsythe SJ: **Comparison of media for the isolation of *Enterobacter sakazakii***. Applied and Environmental Microbiology 2007, **73**(1):48-52.

Appendix

Table A. Inclusivity testing results for *Cronobacter*.

Isolate ID	Species	Source	DFI Agar	R&F Agar (36ºC)	R&F Agar (41.5ºC)	CCI Agar	ESIA Agar	*Cronobacter* selective broth	qPCR
LMG 26250/E1021	*Cronobacter condimenti*	spiced sausage	Typical	Typical	Typical	Typical	Typical	Growth	Positive
2-14	*Cronobacter dublinensis*	nuts	Typical	Typical	Typical	Typical	Typical	Growth	Positive
CDC 5960-70	*Cronobacter dublinensis*	clinical	Typical	Typical	Typical	Typical	Typical	Growth	Positive
CR1304	*Cronobacter dublinensis*	thyme	Typical	Typical	Typical	Typical	Typical	Growth	Positive
CR1523	*Cronobacter dublinensis*	unknown	Typical	Typical	Typical	Typical	Typical	Growth	Positive
E1028A	*Cronobacter dublinensis*	environment	Typical	Typical	Typical	Typical	Typical	Growth	Positive
E1028B	*Cronobacter dublinensis*	environment	Typical	Typical	Typical	Typical	Typical	Growth	Positive
LMG 23825T/E464	*Cronobacter dublinensis*	environment	Typical	Typical	Typical	Typical	Typical	Growth	Positive
O#19	*Cronobacter dublinensis*	unknown	Typical	Typical	Typical	Typical	Typical	Growth	Positive
2149	*Cronobacter malonaticus*	clinical	Typical	Typical	Typical	Typical	Typical	Growth	Positive
2193-01/827	*Cronobacter malonaticus*	clinical	Typical	Typical	Typical	Typical	Typical	Growth	Positive
E608 A	*Cronobacter malonaticus*	clinical	Typical	Typical	Typical	Typical	Typical	Growth	Positive
E615	*Cronobacter malonaticus*	clinical	Typical	Typical	Typical	Typical	Typical	Growth	Positive
E754	*Cronobacter malonaticus*	clinical	Typical	Typical	Typical	Typical	Typical	Growth	Positive
GK1349	*Cronobacter malonaticus*	environment	Typical	Typical	Typical	Typical	Typical	Growth	Positive
LMG 23826T/E825	*Cronobacter malonaticus*	clinical	Typical	Typical	Typical	Typical	Typical	Growth	Positive
Md25g	*Cronobacter malonaticus*	insect	Typical	Typical	Typical	Typical	Typical	Growth	Positive

ATCC 51329T/LMG51329	*Cronobacter muytjensii*	lab control	Typical	Typical	Typical	Typical	Typical	Growth	Positive
CDC 3523-75	*Cronobacter muytjensii*	clinical	Typical	Typical	Typical	Typical	Typical	Growth	Positive
E456	*Cronobacter muytjensii*	unknown	Typical	Typical	Typical	Typical	Typical	Growth	Positive
E488	*Cronobacter muytjensii*	unknown	Typical	Typical	Typical	Typical	Typical	Growth	Positive
E769	*Cronobacter muytjensii*	milk powder	Typical	Typical	Typical	Typical	Typical	Growth	Positive
GK1257	*Cronobacter muytjensii*	environment	Typical	Typical	Typical	Typical	Typical	Growth	Positive
GK1258	*Cronobacter muytjensii*	environment	Typical	Typical	Typical	Typical	Typical	Growth	Positive
305N	*Cronobacter sakazakii*	unknown	Typical	Typical	Typical	Typical	Typical	Growth	Positive
ATCC BAA894	*Cronobacter sakazakii*	clinical	Typical	Typical	Typical	Typical	Typical	Growth	Positive
CQ12	*Cronobacter sakazakii*	environment	Typical	Typical	Typical	Typical	Typical	Growth	Positive
CQ138	*Cronobacter sakazakii*	environment	Typical	Typical	Typical	Typical	Typical	Growth	Positive
CQ147	*Cronobacter sakazakii*	environment	Typical	Typical	Typical	Typical	Typical	Growth	Positive
crop1	*Cronobacter sakazakii*	unknown	Typical	Typical	Typical	Typical	Typical	Growth	Positive
crop8	*Cronobacter sakazakii*	unknown	Typical	Typical	Typical	Typical	Typical	Growth	Positive
KW18	*Cronobacter sakazakii*	mushroom	Typical	Typical	Typical	Typical	Typical	Growth	Positive
KW4	*Cronobacter sakazakii*	seaweed	Typical	Typical	Typical	Typical	Typical	Growth	Positive
Lc10s	*Cronobacter sakazakii*	insect	Typical	Typical	Typical	Typical	Typical	Growth	Positive
MARJ25	*Cronobacter sakazakii*	unknown	Typical	Typical	Typical	Typical	Typical	Growth	Positive
MB340	*Cronobacter sakazakii*	unknown	Typical	Typical	Typical	Typical	Typical	Growth	Positive

MB349	*Cronobacter sakazakii*	unknown	Typical	Typical	Typical	Typical	Typical	Growth	Positive
Md70g	*Cronobacter sakazakii*	insect	Typical	Typical	Typical	Typical	Typical	Growth	Positive
PB18	*Cronobacter sakazakii*	unknown	Typical	Typical	Typical	Typical	Typical	Growth	Positive
SP291-1 A1	*Cronobacter sakazakii*	unknown	Typical	Typical	Typical	Typical	Typical	Growth	Positive
2-15	*Cronobacter turicensis*	nuts	Typical	Typical	Typical	Typical	Typical	Growth	Positive
3-24	*Cronobacter turicensis*	nuts	Typical	Typical	Typical	Typical	Typical	Growth	Positive
CR342	*Cronobacter turicensis*	lettuce	Typical	Typical	Typical	Typical	Typical	Growth	Positive
CR447	*Cronobacter turicensis*	carrot	Typical	Typical	Typical	Typical	Typical	Growth	Positive
CR505	*Cronobacter turicensis*	carrot	Typical	Typical	Typical	Typical	Typical	Growth	Positive
E625	*Cronobacter turicensis*	infant food	Typical	Typical	Typical	Typical	Typical	Growth	Positive
Md1sN	*Cronobacter turicensis*	insect	Typical	Typical	Typical	Typical	Typical	Growth	Positive
z3032/E866/LMG23827	*Cronobacter turicensis*	clinical	Typical	Typical	Typical	Typical	Typical	Growth	Positive
E680	*Cronobacter universalis*	food	Typical	Typical	Typical	Typical	Typical	Growth	Positive
NCTC 9529/E797	*Cronobacter universalis*	water	Typical	Typical	Typical	Typical	Typical	Growth	Positive

Table B. Exclusivity testing results.

Isolate ID	Species	Source	DFI Agar	R&F Agar (36ºC)	R&F Agar (41.5ºC)	CCI Agar	ESIA Agar	*Cronobacter* selective broth	qPCR
BAC0013	*Bacillus cereus*	lab control	No growth	No growth	No growth	No growth	No growth	No growth	Negative
NES 28	*Bacillus licheniformis*	milk	No growth	No growth	No growth	No growth	No growth	No growth	Negative
NES 6	*Bacillus subtilis*	lab control	No growth	No growth	No growth	No growth	No growth	No growth	Negative
BB-12	*Bifidobacterium lactis*	clinical	No growth	No growth	No growth	No growth	No growth	No growth	Negative
ATCC 43864	*Citrobacter freundii*	lab control	Not typical	Not typical	Not typical	Not typical	Not typical	Growth	Negative
ATCC 25408	*Citrobacter koseri*	clinical	Not typical	Not typical	No growth	Not typical	Not typical	No growth	Negative
ATCC 35956	*Enterobacter asburiae*	clinical	Not typical	Not typical	No growth	Not typical	Not typical	Growth	Negative
ATCC 35314	*Enterobacter cancerogenus*	clinical	Not typical	Typical	No growth	Not typical	Not typical	No growth	Negative
ATCC 49141	*Enterobacter cloacae*	clinical	Not typical	Not typical	No growth	Not typical	Not typical	Growth	Negative
ATCC 23373	*Enterobacter cloacae*	lab control	Not typical	Not typical	Not typical	Not typical	Not typical	Growth	Negative
ATCC 13047	*Enterobacter cloacae*	clinical	Not typical	Not typical	Not typical	Not typical	Not typical	Growth	Negative
ATCC 35030	*Enterobacter cloacae*	lab control	Not typical	Not typical	Not typical	Not typical	Not typical	Growth	Negative
ATCC 49163	*Enterobacter hormaechei*	clinical	Not typical	Typical	Typical	Not typical	Not typical	Growth	Negative
NES 11	*Escherichia coli*	clinical	Not typical	Not typical	Not typical	Not typical	Not typical	No growth	Negative
NES 20	*Escherichia hermanii*	clinical	Not typical	Not typical	Not typical	Not typical	Not typical	No growth	Negative
LMG 23733; 1159/04	*Franconibacter helveticus*	fruit powder	Typical	Typical	Typical	Typical	Typical	No growth	Negative
LMG 23732; 513/05	*Franconibacter helveticus*	fruit powder	Typical	Typical	Typical	Typical	Typical	No growth	Negative
LMG 24058; 1160/04	*Franconibacter pulveris*	fruit powder	Typical	Not typical	Not typical	Typical	Typical	Growth	Negative

LMG 24057; 601/05	*Franconibacter pulveris*	fruit powder	Typical	Typical	Typical	Typical	Typical	No growth	Negative
ATCC 13048	*Klebsiella aerogenes*	clinical	Not typical	Not typical	Not typical	Not typical	Not typical	Growth	Negative
NES 3	*Klebsiella oxytoca*	clinical	Not typical	Not typical	Not typical	Not typical	Not typical	Growth	Negative
NES 14	*Klebsiella pneumoniae*	clinical	Not typical	Not typical	Not typical	Not typical	Not typical	Growth	Negative
ATCC 33110	*Kluyvera intermedia*	water	Not typical	Not typical	No growth	Not typical	Not typical	No growth	Negative
LGG	*Lactobacillus rhamnosus*	clinical	No growth	No growth	No growth	No growth	No growth	No growth	Negative
ATCC 11454	*Lactococcus lactis*	lab control	No growth	No growth	No growth	No growth	No growth	No growth	Negative
NES 9	*Leclercia adecarboxylata*	water	Not typical	Not typical	No growth	No growth	No growth	No growth	Negative
ATCC 33731	*Lelliottia amnigena*	lab control	Not typical	Typical	No growth	Not typical	Not typical	No growth	Negative
CFSAN024082	*Listeria monocytogenes*	produce	No growth	No growth	No growth	No growth	No growth	No growth	Negative
ATCC 49008	*Pantoea agglomerans*	lab control	Not typical	Not typical	No growth	No growth	Not typical	No growth	Negative
ATCC 33028	*Pluralibacter gergoviae*	clinical	Not typical	Not typical	Not typical	Not typical	Not typical	No growth	Negative
NES 15	*Providencia rettgeri*	lab control	Not typical	Not typical	Not typical	Not typical	Not typical	No growth	Negative
NES 51	*Salmonella enteritidis*	food	Not typical	Not typical	Not typical	Not typical	Not typical	No growth	Negative
NES 52	*Salmonella typhimurium*	food	Not typical	Not typical	Not typical	Not typical	Not typical	No growth	Negative
NES 18	*Serratia ficaria*	fruit	Not typical	Not typical	No growth	No growth	No growth	No growth	Negative
NES 4	*Serratia marcescens*	water	Not typical	Not typical	No growth	No growth	Not typical	No growth	Negative
SAFE 109	*Shigella flexneri*	clinical	Not typical	Not typical	Not typical	Not typical	No growth	No growth	Negative
SAFE 108	*Shigella sonnei*	clinical	Not typical	Typical	Typical	Typical	Not typical	No growth	Negative

LMG 23730; 508/05	*Siccibacter turicensis*	fruit powder	Typical	Not typical	Not typical	Typical	Typical	Growth	Negative
LMG 23731; 610/05	*Siccibacter turicensis*	fruit powder	Typical	Not typical	Not typical	Typical	Typical	Growth	Negative
25923	*Staphylococcus aureus*	lab control	No growth	No growth	No growth	No growth	No growth	No growth	Negative