Bacteriological Analytical Manual
Chapter 29: *Cronobacter*

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Revision History:

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 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 extra wash buffer to the pellet to improve the quality of the resultant DNA extract.
7. Added temperature and time tolerance ranges for incubation of enrichment and 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 phenotype description of the colony 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 part of the chapter to improve clarity and to provide detailed instructions with additional texts 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.

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Introduction

Cronobacter is a Gram-negative rod within the family *Enterobacteriaceae* (9). The organism was called "yellow-pigmented *Enterobacter cloacae*" until it was renamed *Enterobacter sakazakii* in 1980 (6). Urmenyi and Franklin reported the first two known cases of meningitis caused by *E. sakazakii* in 1961 (16). Subsequently, cases of meningitis, septicemia, and necrotizing enterocolitis due to *E. sakazakii* have been reported worldwide (12). Although most documented cases involve infants, reports describe infections in adults as well. Overall, case-fatality rates vary considerably with some as high as 80 percent (11). While a reservoir for *E. sakazakii* is unknown, a growing number of reports suggest powdered infant formulas as a vehicle for infection (17).

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 has resulted in a nomenclature change (9, 10, 14). *E. sakazakii* was reclassified into a new genus, *Cronobacter*, comprising seven species including *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter muytjensii*, *Cronobacter dublinensis*, *Cronobacter universalis* and *Cronobacter condimenti*. *Cronobacter dublinensis* contains three subspecies (Table 1) (8, 9, 10, 14).

Table 1. Biochemical tests for the identification of *Cronobacter* and differentiation of species and subspecies of the genus *Cronobacter* (8, 9, 10, 14).

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]+, > 10% Positive; V, 20-80% positive; −, < 10% positive.

Method

The method described here contains both a real-time PCR method (13) for rapid screening and a cultural method for the detection/isolation of *Cronobacter* spp. (3). The qPCR targets *Cronobacter* partial macromolecular synthesis operon: the ribosomal protein S21 (*rpsU*) gene 3' end and the DNA primase (*dnaG*) gene 5' end (13). Chromogenic agars are used to isolate the culture for confirmation. A pre-enrichment step is used to grow the bacteria to an amount ($\geq 10^3$ CFU/ mL) detectable by qPCR and chromogenic agars. The cultural portion of this method is a complete detection/isolation method, so it can be used as a standalone method if qPCR technology is unavailable. The qPCR portion of the method is a screening method, whose positive results should always be confirmed with the cultural method. The qPCR method may be used to confirm pure cultures as *Cronobacter* spp. A flowchart of the entire procedure is described in Figure 1. This method was validated in pre-collaborative and collaborative studies (1, 2).

The inclusivity of this method was determined by analyzing 52 different *Cronobacter* strains representing the seven *Cronobacter* species that were isolated from foods, clinical samples, environmental surfaces, and nationally/internationally recognized culture depositories. The origin and source of each strain are listed in the Inclusivity Table (A). Each strain was enriched in brain heart infusion (BHI) broth and diluted in buffered peptone water (BPW) to approximately 10 times the limit of detection. The diluted cultures were then tested according to this method.

The exclusivity of this method was determined by testing 51 non-*Cronobacter* strains based on taxonomy in 2023. The source and origin of each strain are listed in the Exclusivity Table (B). Each strain was enriched in BHI broth. These incubated cultures were tested according to this method.

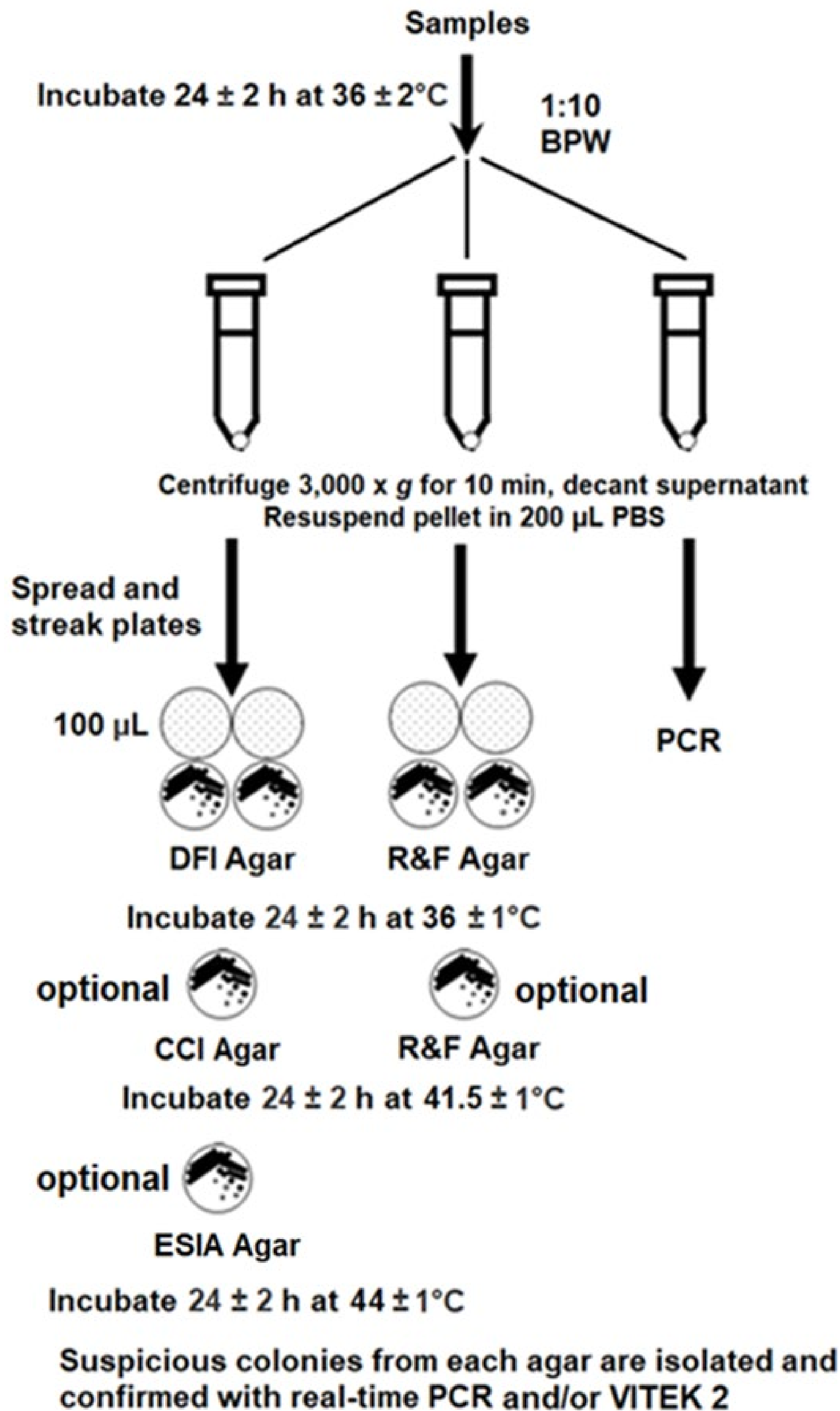


Figure 1. Flowchart of the complete procedure.

A. Equipment and materials

1. Balance with capacity of 2 kg and sensitivity of 0.1 g
2. Incubator, 36 ± 2°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. Micropipette and tips to dispense 1 µL, 100 µL, 150 µL, and 200 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. Centrifuge tubes, polypropylene, 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 block/water bath.
17. Thermal cyclers: Applied Biosystems™ 7500 Fast real-time PCR system (ThermoFisher Scientific, Inc., Waltham, MA),
18. 96-well microwell plate
19. Optical adhesive covers
20. Petri dishes, plastic, sterile, 15 × 150 mm
21. VITEK® 2 Compact (bioMerieux, Hazelwood, MO 63402)
22. Qubit 4 Fluorometer (ThermoFisher Scientific, Waltham, MA)
23. Green Fluorescent Protein (GFP) strain, *Cronobacter sakazakii* G-78 (Microbiologics, Saint Cloud, MN)

B. Media and reagents

1. Phosphate-buffered saline (BAM R59)
2. Buffered peptone water (BPW) (BAM M192)
3. Brilliance *Enterobacter sakazakii* agar (DFI formulation) (BAM M201, or Cat. No. CM1055, Oxoid, Lenexa, KS). After the plates have been poured and dried upside down in the dark at room temperature for up to 48 h, they can be placed in petri plate sleeves (cutting a 0.5" to 1" hole in the sleeves to allow condensation to escape) and stored upside down at 2-8 ºC in the dark for up to 2 weeks. Media made from another manufacturer that have the same formulation can be used after verification following guidelines of microbiology management and QA/QC management of each laboratory.
4. *Enterobacter sakazakii* chromogenic plating agar (R&F agar) (BAM M202, Cat. No. M-0700, R & F Laboratories, Downers Grove, IL). The plates should be poured, dried and stored similar to DFI agar. The final pH value of the prepared agar media should range between 6.8 and 7.1. The prepared plates can be stored for up to 60 days.
5. Chromogenic *Cronobacter* Isolation (CCI) agar (BAM M203, or Cat. No. CM1122, Oxoid). The plates should be poured, dried and stored similar to DFI agar.

6. *Enterobacter sakazakii* Isolation Agar (ESIA) (BAM M204, or Cat. No. CM1134, Oxoid, Lenexa, KS). Prepare media according to the instructions on the packaging label. The plates should be poured, dried and stored similar to DFI agar.
7. PrepMan™ Ultra sample preparation reagent (Cat. No. 4318930, Thermo Fisher Scientific, Waltham, MA)
8. iQ™ Supermix qPCR master mix (Cat. No. 170-8860, Bio-Rad, Hercules, CA 94547). 2 × mix contains 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP, 50 U/mL iTaq™ DNA polymerase and 6 mM $MgCl_2$.
9. VITEK® 2 GN ID Card (Cat. No. 21341, bioMérieux)
10. Platinum™ Taq DNA Polymerase (Cat. No. 10966-018, ThermoFisher Scientific, Waltham, MA)
11. ROX passive reference dye (Cat. No. 12223012, ThermoFisher Scientific, Waltham, MA). Reagent from another manufacturer can be used after verification following guidelines of microbiology management and QA/QC management of each laboratory.
12. Primers and probes (Table 2). qPCR primers are commercially synthesized with basic desalt purification and then reconstituted using PCR grade water to 100 µM for prolonged storage. They are diluted to 2.5 to 40 µM working stock concentrations. qPCR probes are commercially synthesized with HPLC purification and reconstituted to 2.5 to 5 µM in single use aliquots using 1× PCR grade low-EDTA Tris-EDTA (TE, 10 mM Tris, 0.1 mM EDTA, pH 8.0) buffer to avoid repeated freezing/thawing. Primers and probes need to be stored frozen (-20 to -70°C). Discard leftover thawed probes and avoid repeating freeze-thawing.
13. Internal amplification control DNA (4). Internal amplification control (IAC) DNA is constructed by generating a 200 bp sequence that is synthesized and inserted into a pZErO-2 vector or other vector and transformed into *Escherichia coli* pDMD801 or other competent strain. Partial sequence representation containing the internal control is shown below (GenBank accession no. FJ357008, Figure 2). The internal amplification control DNA (IAC in Figure 2) sequence is in grey, T7 promoter is represented in a box, and M13, internal control forward and reverse primers and probe targets are represented by arrows. The plasmid is extracted by Qiagen Plasmid Mini Kit (Cat. No. 12125, Qiagen, Valencia, CA 91355) or equivalent from the transformed *Escherichia coli* cells following manufacturer's instructions and quantified by Qubit fluorometer. To avoid repeated freezing/thawing, plasmids can be diluted to small aliquots and single use aliquots in PCR grade 1 × low-EDTA TE buffer containing 10 mM Tris and 0.1 mM EDTA at pH 8.0. The IAC DNA fragment can also be commercially synthesized and diluted in TE buffer to a stock solution which will reliably provide a Ct of no less than 24 when *Cronobacter* DNA extracted from 40 mL overnight BPW cultures are present and no more than 32 with water negative controls.

Table 2. Primers and probes for the qPCR assay.

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

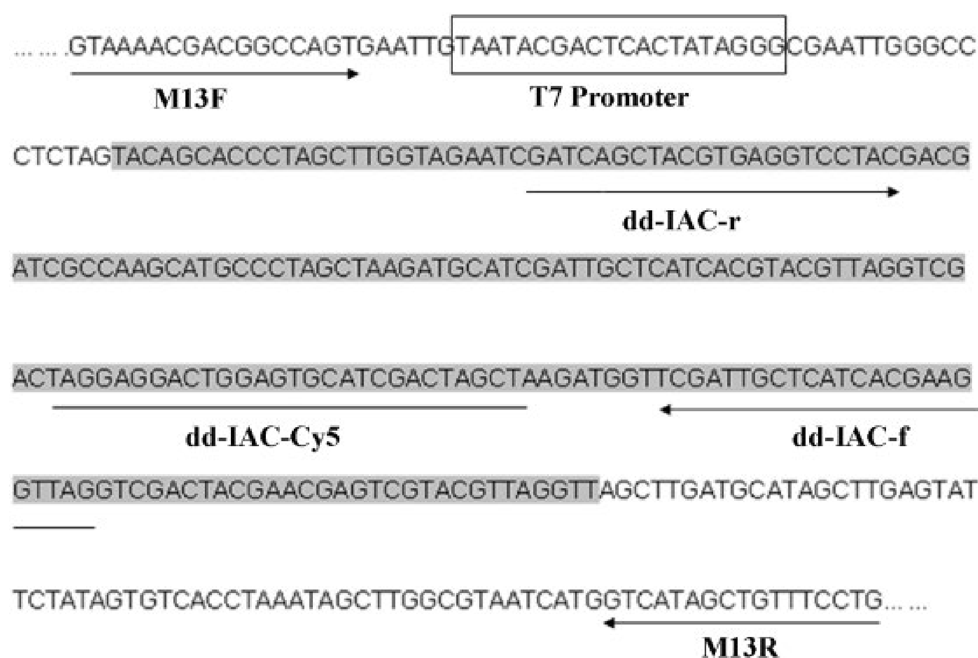


Figure 2. Illustration of the internal amplification control DNA. The 200 bp IAC is highlighted in grey (Complete sequence, TACAGCACCCTAGCTTGGTAGAATCGATCAGCTACGTGAGGTCCTACGACGATCGCCAAGCATGCCCTAGCTAAGATGCATCGATTGCTCATCACGTACGTTAGGTCGACTAGGAGGACTGGAGTGCATCGACTAGCTAAGATGGTTCGATTGCTCATCACGAAGGTTAGGTCGACTACGAACGAGTCGTACGTTAGGTT).

C. Preparation of infant formula samples for isolation of *Cronobacter*

1. Wear double gloves at all times. Change outer gloves, wipe clean the balance and working area after processing each sample.
2. Sterilize the container margins and the spoons used for sampling prior to withdrawing the samples.
3. Aseptically weigh out 100 ± 0.2 g of the powdered infant formula and add to 2 L sized Erlenmeyer flasks or 2 to 4 L sized sterile sample bags.
4. Add 900 mL (1:10 dilution) of sterile buffered peptone water (BPW) pre-warmed to 36 ± 2°C and gently shake by hand until the powder is uniformly suspended. Incubate for 24 ± 2 h at 36 ± 2°C.
5. Thoroughly mix the enrichment mix and remove three aliquots of 40 mL each from the incubated sample and place them into three 50 mL centrifuge tubes. Centrifuge the aliquots at 3,000 × *g* for ten minutes in a swinging bucket centrifuge (fixed angle centrifuges are not recommended because of problems separating the fats from the pellet).
6. Aspirate the supernatants of each centrifuge tube.
7. Use sterile cotton swabs or equivalent tools to remove the fat precipitate on the side wall of the centrifuge tube, if necessary.
8. Suspend the resultant pellet in 200 µL of phosphate buffered saline (PBS) by vortexing at maximum speed for at least 20 sec. One of the aliquots will be used for qPCR. Two of the aliquots will be used for culture confirmation if necessary.
   Note: One aliquot can be prepared first for qPCR screening. The other two aliquots can be prepared and centrifugated after obtaining qPCR positive results. The enrichment cultures can be stored at 4°C while waiting for the qPCR result.

D. qPCR screening of *Cronobacter*

1. DNA extraction. Transfer 200 µL suspended cells (in PBS) to a 1.5 mL microcentrifuge tube and add another 600 µL of PBS to mix. Centrifuge at 3,000 × *g* for 5 min. Depending on the presence and absence of bacterial cells and the efficiency of fat removal at the previous step, there could be 4 layers after centrifugation. The top layer is fat residues, the second layer is supernatant, the third layer is bacterial cell pellets which are brown/yellow, and the bottom pellets are milk particles. Discard the supernatants and any fat residues of each centrifuge tube. Use sterile cotton tips or equivalent tools to remove the precipitates on the side wall of the centrifuge tube if necessary. Add 400 µL of PrepMan™ Ultra sample preparation reagent to each tube and vortex at maximum speed to allow complete suspension. Heat the sample for 10 min at 100°C in a boiling water bath or heating block, then cool the sample to room temperature for 2 min. Centrifuge the sample for 2 min at a speed of at least 15,000 × *g*. Transfer 50 µL of the supernatant into a new tube for qPCR analysis.
2. Include a positive control (prepared by 1:10 dilution of DNA prepared from a pure culture of *Cronobacter* strain, e.g., ATCC 29544, or GFP strain, *Cronobacter sakazakii* G-78) and a no template (water) control in each qPCR run. The qPCR is designed to detect very low level of *Cronobacter* cells. If after enrichment, large amounts of cells are grown, the qPCR could yield high FAM signals (e.g., Ct < 12). One option is to dilute the DNA (1:10 or 1:100) and rerun the qPCR.

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 7500 Fast. Give each experiment a unique name. Select the following parameters:
   a. In "Experimental properties", select quantitation-standard curve as the type of experiments, TaqMan reagents and standard ramp speed.
   b. In "Plate Setup", select ROX as reference dye, FAM as reporter dye for *Cronobacter* probe and Cy5 as reporter dye for IAC probe, and TAMRA as quencher for *Cronobacter* probe and none as quencher for IAC probe. Assign appropriate sites on the cycler block.
   c. In "Run Method", set reaction volume per well to 25 µL. Select the qPCR conditions: 95°C for 3 min followed by 40 cycles of 95°C for 15 sec, 52°C for 40 sec and 72°C for 15 sec. The 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 (3×10^3 plasmid 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 for screening, 1 µL for confirmation	2 µL for screening, 1 µL for confirmation
PCR grade water	Appropriate amount to reach 25 µL	Appropriate amount to reach 25 µL

4. Data analysis. Set the threshold and baseline 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. Confirmatory PCR can be performed without ROX if it is not available; threshold of 250,000 and the baseline setting described in Table 4 should be used. Sample screenshots of the graph and table view are shown in Figure 3. If the qPCR result is positive, the sample is considered PCR-positive and proceed to the culture confirmation (Section E). If the qPCR result is negative, the sample is considered negative, and analysis is halted.

   DNA extracts that have Ct values for FAM and demonstrate sigmoidal amplification curves are considered PCR-positive. The multicomponent plot in 7500 FAST software displays individual signals of FAM, CY5, ROX and TAMRA which can be used to check non-typical plots. Review of IAC reactions is not required when the FAM reaction is positive. If there is no Ct value in FAM for a DNA extract, or if there is Ct value but the curve is not the typical sigmoidal shape, the IAC for that DNA extract must be analyzed:
   a. The DNA extract is considered negative if there is Ct value in Cy5 and the curve for Cy5 is sigmoidal.
   b. If there is no Ct value in Cy5, then there is possible inhibitory substance in the sample and qPCR result is invalid. The DNA extracts need to be diluted (1/10, in PCR grade water) or centrifuged for further purification, and the qPCR should be repeated; or directly proceed with culture analysis (Section E).

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

qPCR targets	**Threshold**	**Baseline**
FAM-*Cronobacter* for screening	0.15	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	The same as above
Cy5-IAC for both screening and confirmation	0.05	3 to 20

Define Targets and Samples | Assign Targets and Samples

Instructions: Define the targets to quantify and the samples to test in the reaction plate.

Define Targets

Add New Target | Add Saved Target | Save Target | Delete Target

Target Name	Reporter	Quencher	Color
Cronobacter	FAM	TAMRA	
IAC	CY5	None	

Define Samples

Add New Sample

Sample Name
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
Positive control
Water

(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	U S N	
	IAC	U S N	

Mixed U Unknown S Standard N 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

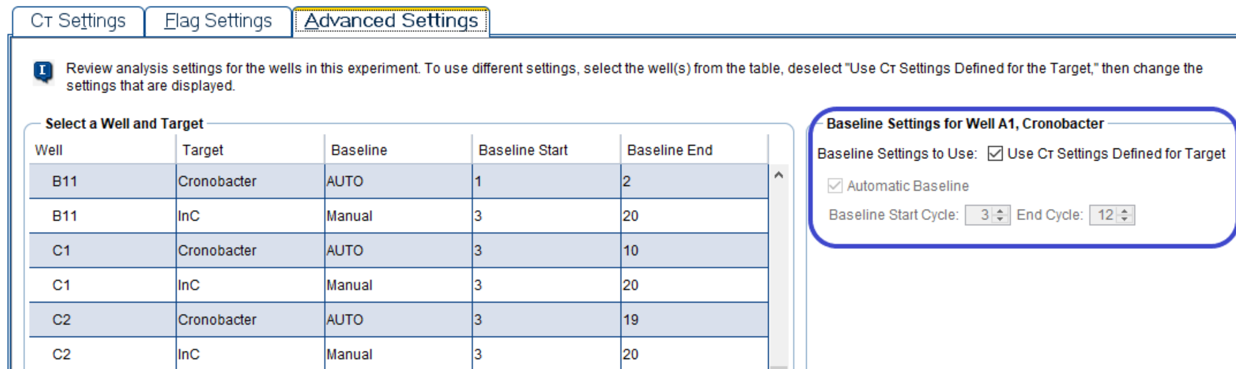
View Plate Lay

Show in Wells

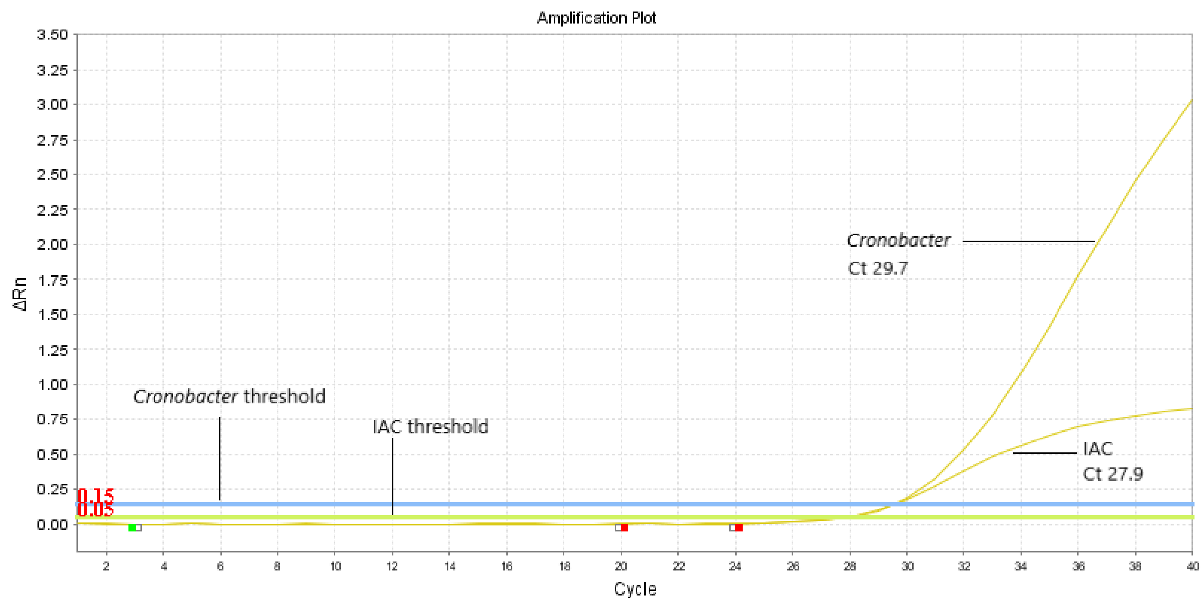
	1
A	Sample 1 U Cronobacter U IAC
B	Sample 2 U Cronobacter U IAC
C	Sample 3 U Cronobacter U IAC
D	Sample 4 U Cronobacter U IAC
E	Sample 5 U Cronobacter U IAC
F	Sample 6 U Cronobacter U IAC
G	Positive control U Cronobacter U IAC
H	Water U Cronobacter U IAC

Wells: U 8 Unknown

(b)



(c)



(d)

Figure 3. Sample 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 and Ct values of *Cronobacter* and IAC.

E. Isolation of *Cronobacter*

For qPCR-positive samples and samples that show inhibition in the qPCR screening, streak a loopful of suspended cells (obtained in C8) onto the surface of two DFI (M201) (5) and two R&F agars (M202) (15) with sterile inoculation loops as described in Figure 1. In addition, spread 100 µL aliquots of suspended cells evenly onto each of the two DFI chromogenic agars and two R&F *Cronobacter* chromogenic plating agars with sterile spreading rods. Incubate the agar plates at 36 ± 1°C for 24 ± 2 h. Observe plates for typical or suspect colonies of *Cronobacter* (Figure 4).

Optionally, with sterile inoculation loops, streak a loopful of suspended cells onto the surface of one CCI agar (M203) (8) or one ESIA agar (M204) (7) and/or one R&F agar

(Figure 1). Incubate the optional CCI or R&F agar plates at 41.5 ± 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 another 18 to 24 h to increase the size of the colonies and improve color development for some strains.

If the cultures overgrow on the plates, streak a 3 mm loopful (10 µL) of lawn materials to at least three quadrants of a new plate for isolation of single colonies. If isolated colonies from streak plates are picked for further purification or direct confirmation, spread plates of the same aliquot may have confluent growth and may not need to be sub-cultured. Plates can be kept for up to 3 days at 4°C during the investigation of confluent growth.

Use UV illumination to check 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.

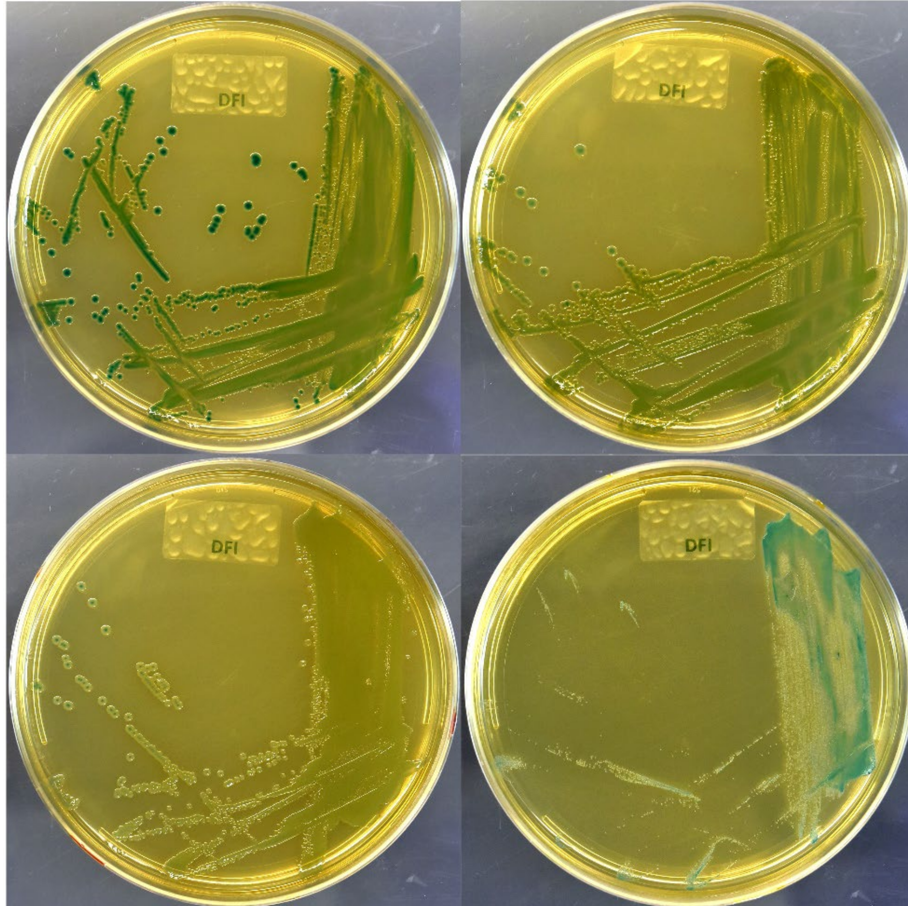
F. Identification of *Cronobacter*

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

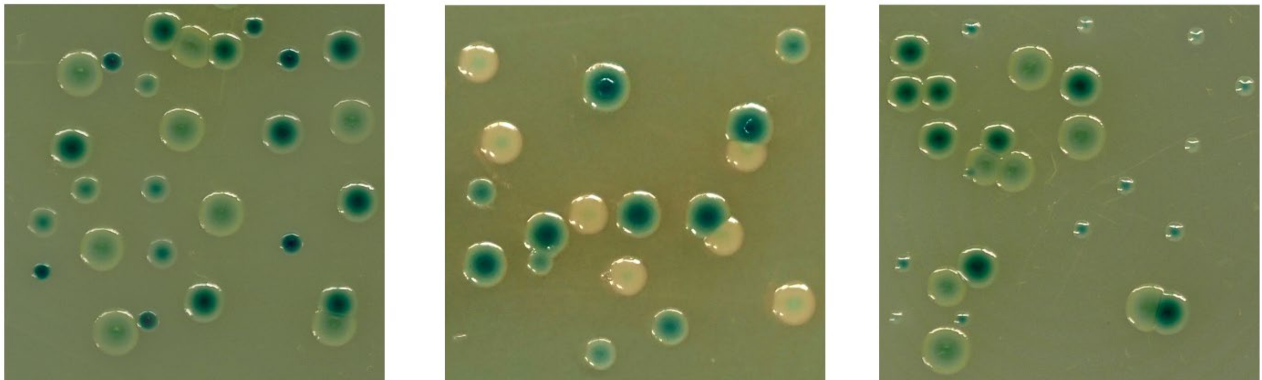
Presumptive *Cronobacter* colonies on R&F agar appear blue to black, or blue to grey with the red background. Rare strains can be light grey when grown at 36°C (Figure 4c and 4d). The red background can appear purplish red with different strains or under different light conditions. *Cronobacter* does not change the color of R&F agar, but the presence of background microflora can change the color of R&F agar from red to yellow, which may make *Cronobacter* colonies appear green to black (Figure 4e).

Presumptive *Cronobacter* colonies on ESIA agar appear dark blue to green and blue to green. Some strains have very light green color and thus appear mostly blue, especially for isolated colonies (Figure 4h, 4i and 4j).

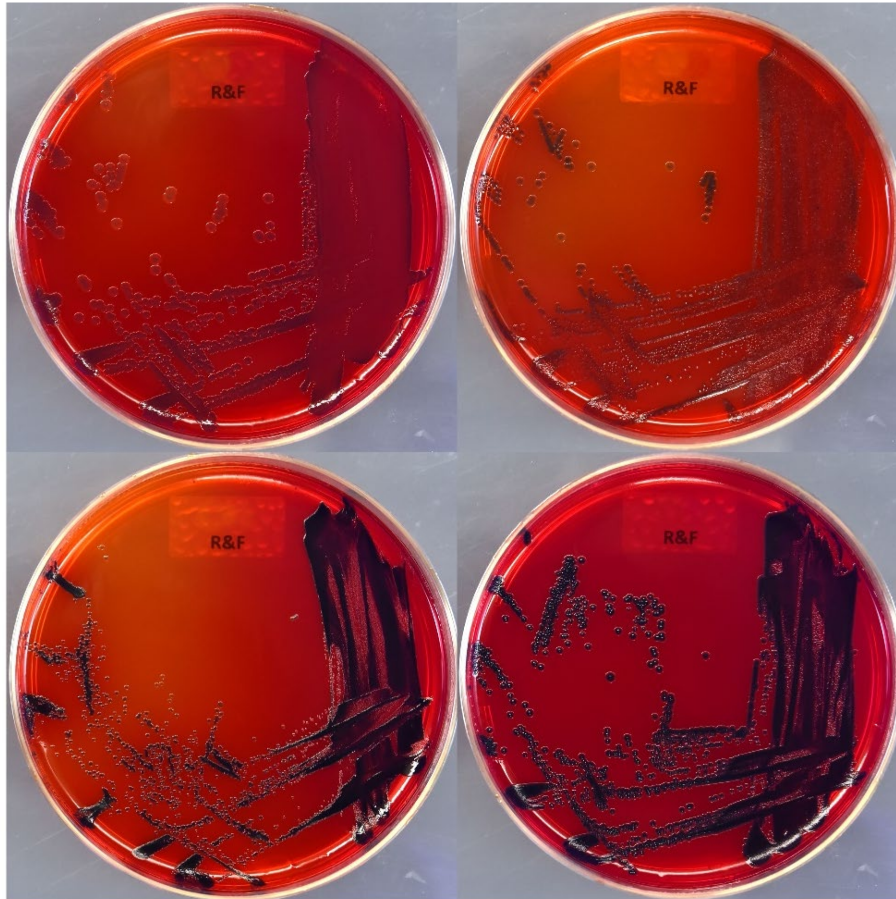
Some strains have very small colonies on any of the agars after 24 h incubation. Pay attention to suspect colonies with weak green color. Typical colonies are 2-3 mm in diameter, but some rare strains have colonies of 1 mm in diameter.



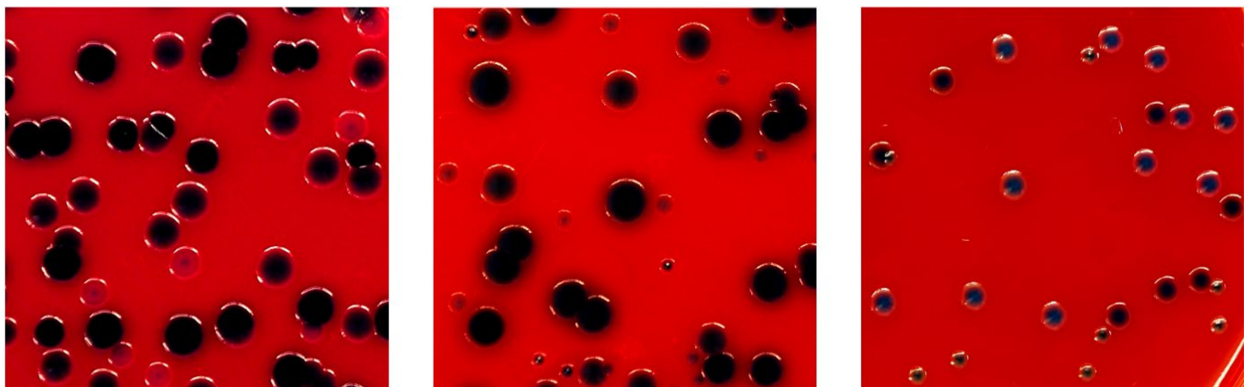
(a) *Cronobacter* colonies on DFI agar on a dark background. The colors can be green (upper left), pale green (upper right) and yellow green (bottom left). Rare strains can have very small colonies (bottom right**).**



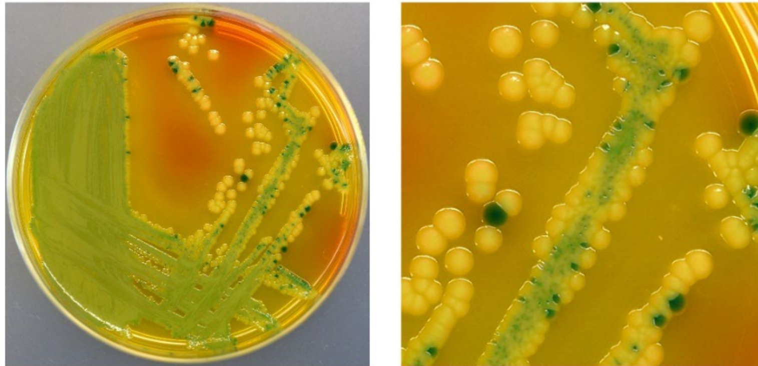
(b) Mixed *Cronobacter* colonies on DFI agar on a dark background. Rare strains can appear yellow with very weak green center. The zoomed pictures make colonies appear larger than they are.



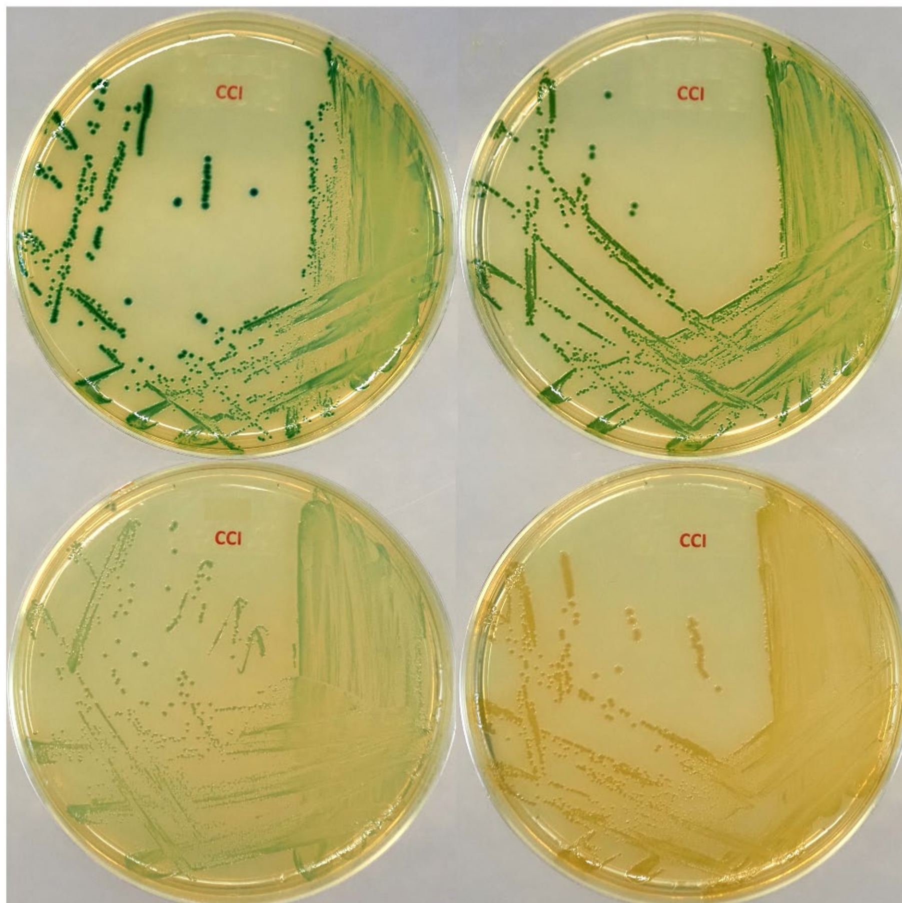
(c) *Cronobacter* colonies on R&F agar. The colors can be blue grey (upper right) and blue black (bottom right). Rare strains can be light grey when grown at 36°C (upper left) or have small colonies (bottom left).



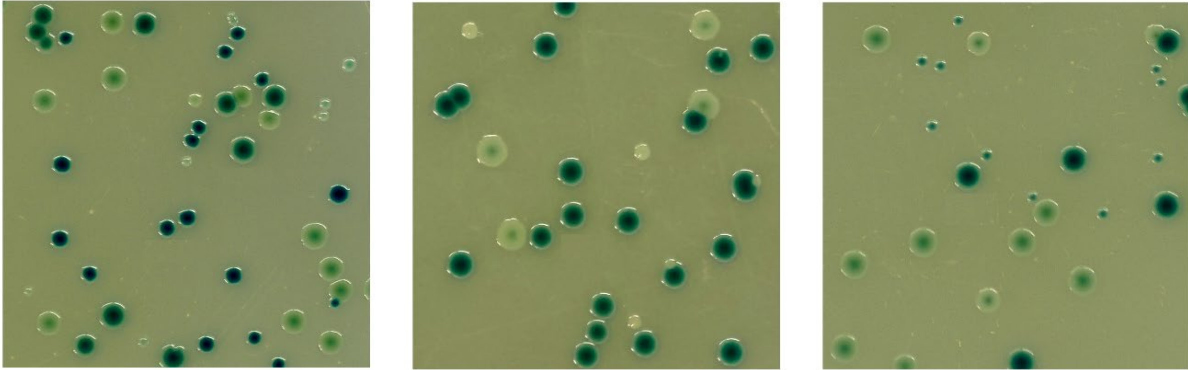
(d) Mixed *Cronobacter* colonies on R&F agar**.**



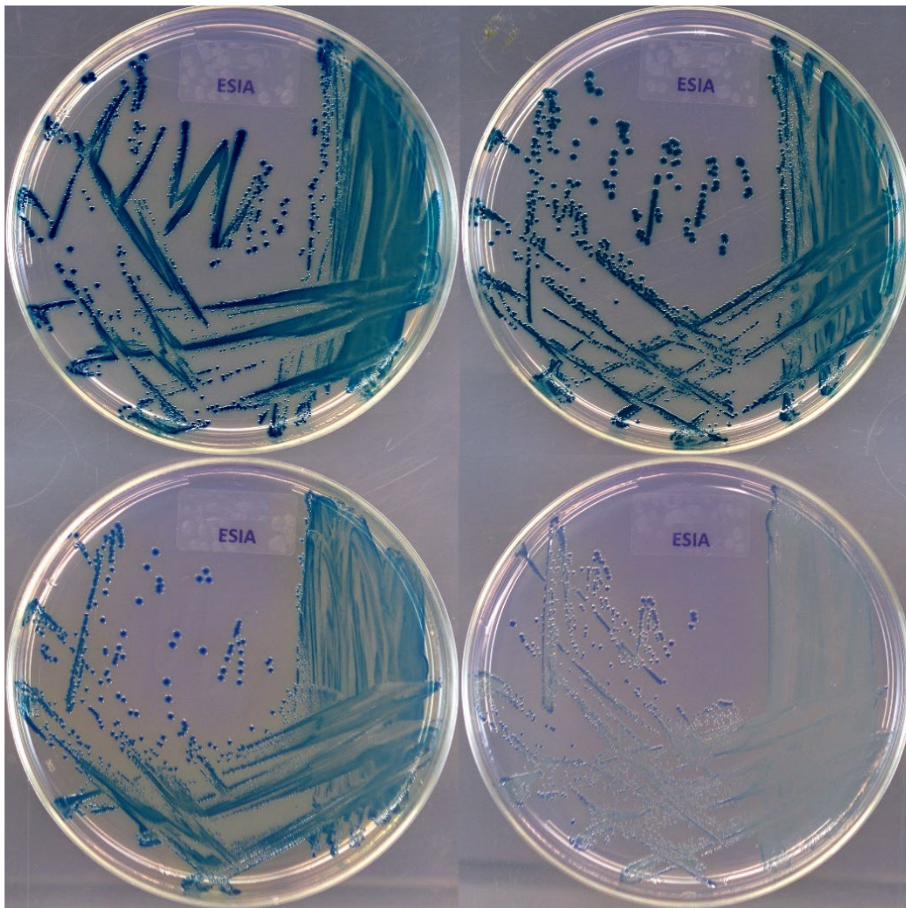
(e) *Cronobacter* mixed with background flora on R&F agar. The background flora changes the background color of the agar from red to yellow in most areas, and *Cronobacter* colonies appear green to black against yellow background.



(f) *Cronobacter* colonies on CCI agar on a light background. The colors can be dark green (upper left), green (upper right), pale green (bottom left) and yellow green (bottom right).

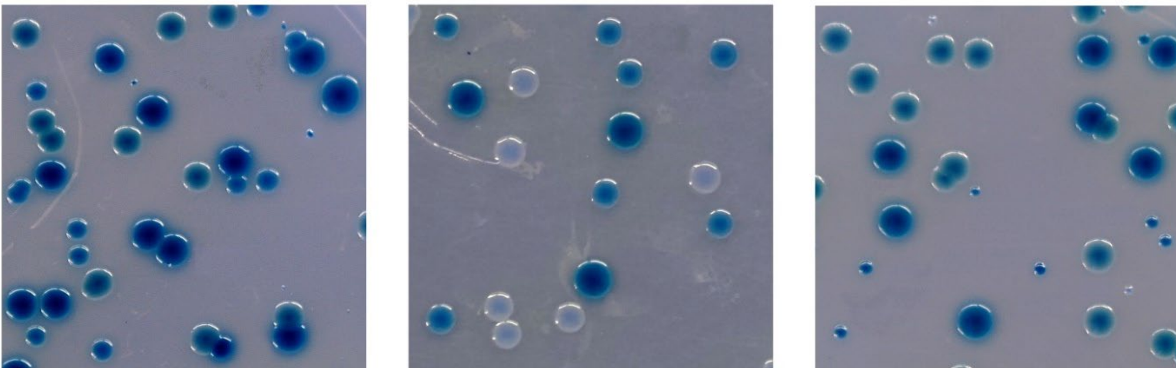


(g) Mixed *Cronobacter* colonies on CCI agar on a dark background**.** Rare strains can have very small colonies**.**



(h) *Cronobacter* colonies on ESIA agar on a dark background. The colors can be dark blue green (upper), blue green with isolated colonies more blue and heavy growth areas more green (bottom left). Some strains have very light green color and appear mostly blue (bottom right).

(i) Same two plates in the bottom of (h) on a light background. The colonies appear greener than those on a dark background.



(j) Mixed *Cronobacter* colonies on ESIA agar on a dark background. Rare strains have light blue color.

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

G. PCR confirmation

Prepare DNA from at least one isolated, suspect colony from each chromogenic agar plate if available or a TSA plate used for further purification. Plates can be kept at 4°C for not more than 24 h before DNA extraction. Transfer one colony to 150 µL of PCR grade dH_2O contained in a 1.5 mL plastic centrifuge tube and heat for 5 min in a boiling water bath or heating block set to 100°C. Chill the tubes in ice and centrifuge at 15,000 × *g* for 2 min. Use 1 µL of this lysed material as DNA template for the real-time PCR assay above (Section D). If a colony is picked from a chromogenic agar plate directly for PCR confirmation, subculture the colony on TSA in case VITEK 2 confirmation is needed for that colony.

H. Biochemical confirmation

With a sterile inoculating loop, pick at least one suspect *Cronobacter* colony from each chromogenic agar plate if available, further purify on TSA and confirm using VITEK 2 biochemical identification system according to the manufacturer's instructions.

When qPCR is used initially to confirm suspect isolates, secondary confirmation of the same colony by VITEK 2 is highly recommended. When VITEK 2 is used initially to confirm suspect isolates and yields positive identification of the *Cronobacter sakazakii* group, secondary confirmation of the same colony by qPCR is required. When VITEK 2 is used initially to confirm suspect isolates and yields negative identification of *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 other biochemical test kits.

I. 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 size Erlenmeyer flasks or similar size sample bags, respectively and proceed with sample preparation, culture isolation and identification. Calculate MPN of *Cronobacter* cells/g of sample based on the number of "tubes" at each dilution in which the presence of *Cronobacter* was confirmed.

J. Subtyping of *Cronobacter*

Confirmed *Cronobacter* isolates can be further characterized by whole genome sequencing using standardized GenomeTrakr methods and submit data to GenomeTrakr.

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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. Int. J. Syst. Evol. Microbiol. 64: 3402-3410

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Appendix

Table A. Inclusivity testing results for *Cronobacter*.

Original Lab	Original ID	Organism	Source	Country Origin	DFI[a]	R&F[b]	Real-Time PCR
UCD[c]/UZH[d]/NRC[e]	E265	*C. malonaticus*	milk powder	Malaysia	Positive	Positive	Positive
ILSI[f]	F6-036	*C. sakazakii*	Environment (Milk powder)	Malaysia	Positive	Positive	Positive
ILSI	F6-038	*C. sakazakii*	Environment (Milk powder)	Holland	Positive	Positive	Positive
ILSI	F6-040	*C. sakazakii*	Environment (Milk powder)	Holland	Positive	Positive	Positive
UCD/UZH /NRC	E464	*C. dublinensis*	Environment (Milk powder)	Zimbabwe	Positive	Positive	Positive
ATCC[g]; NCTC[h]	ATCC 29544; NCTC 11467	*C. sakazakii*	human (throat)	unknown	Positive	Positive	Positive
FDA[i]	607	*C. sakazakii*	unknown	unknown	Positive	Positive	Positive
UCD/UZH /NRC	E515	*C. dublinensis*	water	Switzerland	Positive	Positive	Positive
UCD/UZH /NRC	1330T	*C. condimenti*	spiced meat	Slovakia	Positive	Positive	Positive
ATCC	ATCC 12868	*C. sakazakii*	unknown	unknown	Positive	Positive	Positive
ATCC	ATCC 51329	*C. muytjensii*	unknown	unknown	Positive	Positive	Positive
HCSC[j]; FDA	SK90	*C. sakazakii*	clinical (children's hospital)	Canada	Positive	Positive	Positive
UCD/UZH /NRC	E632	*C. sakazakii*	food	USA	Positive	Positive	Positive
HCSC	HPB 2848	*C. sakazakii*	clinical	Canada	Positive	Positive	Positive
HCSC	HPB 2873	*C. sakazakii*	clinical	Canada	Positive	Positive	Positive

Original Lab	Original ID	Organism	Source	Country Origin	DFI[a]	R&F[b]	Real-Time PCR
HCSC	HPB 2874	*C. sakazakii*	clinical	Canada	Positive	Positive	Positive
UCD/UZH /NRC	H. Muytjens (Prague 72 26248)	*C. sakazakii*	unknown	Czech Republic	Positive	Positive	Positive
UCD/UZH /NRC	H. Muytjens 52	*C. malonaticus*	milk powder	Australia	Positive	Positive	Positive
UCD/UZH /NRC	H. Muytjens 58	*C. sakazakii*	milk powder	Belgium	Positive	Positive	Positive
UCD/UZH /NRC	H. Muytjens 15	*C. sakazakii*	milk powder	Denmark	Positive	Positive	Positive
UCD/UZH /NRC	H. Muytjens 8	*C. sakazakii*	milk powder	France	Positive	Positive	Positive
UCD/UZH /NRC	H. Muytjens 35	*C. sakazakii*	milk powder	Russia	Positive	Positive	Positive
UCD/UZH /NRC	H. Muytjens 26	*C. sakazakii*	milk powder	Russia	Positive	Positive	Positive
UCD/UZH /NRC	H. Muytjens (Nijmegen 15)	*C. sakazakii*	neonate	Holland	Positive	Positive	Positive
UCD/UZH /NRC	H. Muytjens (Nijmegen 21)	*C. sakazakii*	neonate	Holland	Positive	Positive	Positive
CDC[k]	CDC 5960-70	*C. dublinensis*	human (blood)	USA	Positive	Positive	Positive
CDC	CDC 3523-75	*C. malonaticus*	human (bone marrow)	USA	Positive	Positive	Positive
NCTC	NCTC 9238	*C. sakazakii*	human (abdominal pus)	UK	Positive	Positive	Positive
NCTC	NCTC 9529	*C. universalis*	water	UK	Positive	Positive	Positive
ATCC	ATCC BAA893	*C. sakazakii*	unknown	USA	Positive	Positive	Positive
ATCC	ATCC BAA894	*C. sakazakii*	unknown	USA	Positive	Positive	Positive
CDC	CDC 996-77	*C. sakazakii*	human (spinal fluid)	USA	Positive	Positive	Positive

Original Lab	Original ID	Organism	Source	Country Origin	DFI[a]	R&F[b]	Real-Time PCR
CDC	CDC 1058-77	*C. malonaticus*	human (breast abscess)	USA	Positive	Positive	Positive
CDC	CDC 407-77	*C. sakazakii*	human (sputum)	USA	Positive	Positive	Positive
CDC	CDC 3128-77	*C. sakazakii*	human (sputum)	USA	Positive	Positive	Positive
CDC	CDC 9369-75	*C. sakazakii*	unknown	USA	Positive	Positive	Positive
UZH	z3032	*C. turicensis*	neonate (meningitis)	Switzerland	Positive	Positive	Positive
HCSC ILSI	SK81 F6-023	*C. sakazakii*	human	Canada	Positive	Positive	Positive
ILSI; RADl	F6-029	*C. sakazakii*	neonate	Holland	Positive	Positive	Positive
ILSI	01-10-2001; F6-034	*C. sakazakii*	clinical	USA	Positive	Positive	Positive
ILSI	8397; F6-043	*C. sakazakii*	clinical	USA	Positive	Positive	Positive
CDC; ILSI	CDC 289-81; F6-049	*C. malonaticus*	clinical	USA	Positive	Positive	Positive
CDC; ILSI	CDC 1716-77; F6-052	*C. sakazakii*	human (blood)	USA	Positive	Positive	Positive
ILSI; RAD	F6-032; H. Muytjens 7	*C. sakazakii*	milk powder	Uruguay	Positive	Positive	Positive
UCD	CFS112	*C. sakazakii*	milk powder	Ireland	Positive	Positive	Positive
UCD	CFS349N	*C. sakazakii*	milk powder	New Zealand	Positive	Positive	Positive
UCD	CFS352N	*C. sakazakii*	milk powder	New Zealand	Positive	Positive	Positive
UCD	ES187	*C. dublinensis*	milk powder	Ireland	Positive	Positive	Positive
CDC	CDC 9363-75	*C. sakazakii*	stool	USA	Positive	Positive	Positive
CDC	CDC 4963-71	*C. sakazakii*	stool	USA	Positive	Positive	Positive
CDC	CDC 1895-73	*C. malonaticus*	human (faeces)	USA	Positive	Positive	Positive
RF[m]	ES626	*C. sakazakii*	rice flour	USA	Positive	Positive	Positive

[a] Positive of DFI shows green or light green colony as *Cronobacter*
[b] Positive of R&F shows blue-green-grey-black colony as *Cronobacter*
[c] UCD: S. Fanning, Centre for Food Safety, University College Dublin, Belfield, Dublin 4, Ireland
[d] UZH: R. Stefan, Institute for Food Safety, University of Zurich, Winterthurerstrasse 270, CH-8057, Switzerland
[e] NRC: Nestlé Research Centre, Vers-Chez-les-Blanc, Lausanne, CH-1000, Switzerland
[f] ILSI: R. Ivy, Food Safety Lab, Cornell University, 412 Stocking Hall, Ithaca, NY, USA
[g] ATCC: American Type Culture Collection, Manassas, VA, USA
[h] NCTC: National Collection of Type Cultures, London, UK
[i] FDA: R. Buchanan, FDA-CFSAN, College Park, MD, USA
[j] HCSC: F. Pagotto, Health Products and Food branch, Health Canada
[k] CDC: Center for Disease Control, Atlanta, GA, USA
[l] RAD: Department of Medical Microbiology, University of Nijmegen, Radboud, Netherlands
[m] RF: L. Restaino, R&F Laboratories, Downers Grove, IL, USA

Table B. Exclusivity testing results for *Cronobacter*.

Original Lab	Strain ID	Organism	Source	DFI[a]	R&F[b]	Real-Time PCR
ATCC[c]	13047	*Enterobacter cloacae*	spinal fluid	Negative	Negative	Negative
ATCC	13048	*Klebsiella aerogenes*	sputum	Negative	Negative	Negative
ATCC	13182	*Klebsiella oxytoca*	Pharyngeal tonsil	Negative	Negative	Negative
ATCC	13880	*Serratia marcescens*	pond water	Negative	Negative	Negative
ATCC	14485	*Streptococcus thermophilus*	unknown	No growth	No growth	Negative
ATCC	15469	*Edwardsiella tarda*	faeces	Negative	Negative	Negative
ATCC	23055	*Acinetobacter calcoaceticus*	unknown	Negative	Negative	Negative
ATCC	23216	*Leclercia adecarboxylata*	drinking water	Negative	Negative	Negative
ATCC	23373	*Enterobacter cloacae*	corn	Negative	Negative	Negative
ATCC	25408	*Citrobacter koseri*	throat	Negative	Negative	Negative
ATCC	25830	*Morganella morganii*	patient with summer diarrhea	Negative	Negative	Negative
ATCC	25922	*Escherichia coli*	clinical isolate	Negative	Negative	Negative
ATCC	27028	*Citrobacter koseri*	blood culture	Negative	Negative	Negative
ATCC	27982	*Phytobacter ursingii*	IV fluid	Negative	Negative	Negative
ATCC	49008	*Pantoea agglomerans*	foodstuff	Negative	Negative	Negative
ATCC	29013	*Klebsiella pneumoniae*	blood	Negative	Negative	Negative
ATCC	29944	*Providencia rettgeri*	unknown	Negative	Negative	Negative
ATCC	27853	*Pseudomonas aeruginosa*	unknown	Negative	Negative	Negative
ATCC	33110	*Kluyvera intermedia*	water	Positive	Negative	Negative
ATCC	33731	*Lelliottia amnigena*	unknown	Negative	Positive	Negative
ATCC	35030	*Enterobacter cloacae*	unknown	Negative	Negative	Negative
ATCC	13472	*Bacillus cereus*	unknown	No growth	No growth	Negative

Original Lab	Strain ID	Organism	Source	DFI[a]	R&F[b]	Real-Time PCR
ATCC	33105	*Serratia ficaria*	Calimyrna fig	Negative	Negative	Negative
ATCC	35314	*Enterobacter cancerogenus*	Blood	Negative	Positive	Negative
ATCC	33028	*Pluralibacter gergoviae*	urine	Negative	Negative	Negative
ATCC	33420	*Proteus vulgaris*	clinical isolate	Negative	Negative	Negative
ATCC	33650	*Escherichia hermanii*	human toe	Negative	Negative	Negative
ATCC	15246	*Alcalgenes faecalis*	unknown	Negative	Negative	Negative
ATCC	33832	*Pseudescherichia vulneris*	unknown	Negative	Negative	Negative
ATCC	29212	*Enterococcus faecalis*	unknown	No growth	No growth	Negative
ATCC	10054	*Micrococcus luteus*	unknown	No growth	No growth	Negative
ATCC	43864	*Citrobacter freundii*	unknown	Negative	Negative	Negative
ATCC	51713	*Buttiauzella noakiae*	unknown	Positive	Positive	Negative
ATCC	25741	*Pediococus acidilactici*	unknown	No growth	No growth	Negative
ATCC	49141	*Enterobacter ludwigii*	clinical	Negative	Negative	Negative
ATCC	8090	*Citrobacter freundii*	unknown	Negative	Negative	Negative
ATCC	9789	*Bacillus licheniformis*	milk	No growth	No growth	Negative
UZH/UCD/ NRC	E904; 05-01-120	*Enterobacter hormaechei*	milk powder	Negative	Positive	Negative
ATCC	35956	*Enterobacter asburiae*	human	Negative	Negative	Negative
ATCC	49163	*Enterobacter hormaechei*	blood	Negative	Positive	Negative
LMG[h]; UZH	LMG 23730	*Siccibacter turicensis*	fruit powder	Positive	Negative	Negative
LMG; UZH	LMG 23731	*Siccibacter turicensis*	fruit powder	Positive	Negative	Negative
LMG; UZH	LMG 23732	*Franconibacter helveticus*	fruit powder	Positive	Positive	Negative
LMG; UZH	LMG 23733	*Franconibacter helveticus*	fruit powder	Positive	Positive	Negative
LMG; UZH	LMG 24057	*Franconibacter pulveris*	fruit powder	Positive	Positive	Negative

Original Lab	Strain ID	Organism	Source	DFI[a]	R&F[b]	Real-Time PCR
LMG; UZH	LMG24058	*Franconibacter pulveris*	fruit powder	Positive	Negative	Negative
FDA[i]		*Salmonella Cubana*	milk	Negative	Negative	Negative
FDA	Yp 1313	*Yersinia pseudotuberculosis*	unknown	Negative	Negative	Negative
FDA	Ye 37	*Yersinia enterocolitica*	unknown	Negative	Negative	Negative
FDA	2457T	*Shigella flexneri*	clinical	Negative	Negative	Negative
FDA		*Shigella sonnei*	clinical	Negative	Negative	Negative
False-positive				8 / 51	8 / 51	0 / 51

[a] Positive of DFI shows green colony as Cronobacter
[b] Positive of R&F shows blue-green-black colony as Cronobacter
[c] ATCC: American Type Culture Collection, Manassas, VA, USA
[d] UZH: R. Stephan, Institute for Food Safety, University of Zurich, Winterthurerstrasse 270, CH-8057, Switzerland
[e] UCD: S. Fanning, Centre for Food Safety, University College Dublin, Belfield, Dublin 4, Ireland
[f] NRC: Nestlé Research Center, Vers-Chez-les-Blanc, Lausanne, CH-1000, Switzerland
[g] ILSI: R. Ivy, Food Safety Lab, Cornell University, 412 Stocking Hall, Ithaca, NY, USA
[h] LMG: BCCM/LMG Bacteria CollectionExternal Link Disclaimer, Ghent, Belgium
[i] FDA: K. Lampel, FDA-CFSAN, College Park, MD, USA