Validation of PhageDx™ Cronobacter Assay for the Identification of Cronobacter spp. in Powdered Infant Formula: AOAC Performance Tested Method SM 051803

Steve Erickson¹, Jose Gil², Jessica Stach¹, Wendy Hahn¹, and Minh M. Nguyen¹

¹Laboratory Corporation of America®/MedTox, 402 County Road D West, St. Paul, MN 55112
²Laboratory of America Corporation®/National Genetics Institute, 2440 Sepulveda Blvd., Suite 235, Los Angeles, CA 90064

Corresponding author’s e-mail: Nguyem5@LabCorp.com

Abstract

Background: The PhageDx™ Cronobacter Assay is based on the infection of Cronobacter spp. by specific bacteriophages and expression of a luciferase reporter gene. Results are generated in as little as 18.5 h for powdered infant formula (PIF).

Objective: An AOAC Performance Tested Methods SM (PTM) study was conducted to validate the PhageDx™ Cronobacter Assay for the detection of Cronobacter in 10, 100 and 300 g milk- and soy-based PIF test portions.

Methods: The performance of the PhageDx™ method was compared to the ISO 22964:2006 Microbiology of the food chain – Horizontal method for the detection of Cronobacter spp. and U.S. FDA Bacteriological Analytical Manual (BAM) Ch. 29 Cronobacter: 2012. Inclusivity/exclusivity, product consistency and stability, and robustness testing also were conducted.
**Results:** There was no significant difference between the 10 g, 100 g, or 300 g test portions for the milk and soy PIF matrixes between the PhageDx™ *Cronobacter* Assay, the ISO 22964:2017 and the FDA BAM Ch. 29 *Cronobacter* 2012 methods. The reporter bacteriophages were specific for *Cronobacter* and infected 75 strains in inclusivity testing. They did not infect 35 non-*Cronobacter* bacteria in exclusivity testing. Robustness testing showed that the method performed well with specific deviations from the standard protocol. Consistency and stability testing demonstrated that the recombinant phage gave consistent results across three production lots and was stable when stored under appropriate conditions for at least three months.

**Conclusions and Highlights:** Work in the submitting and independent laboratories demonstrated that the PhageDx™ *Cronobacter* Assay meets the qualifications for PTM status.

**General Information**

*Cronobacter*, formerly classified under *Enterobacter*, are bacteria that are resistant to desiccation, heat, and ultraviolet radiation. In infants, particularly neonates, *Cronobacter* can cause sepsis or severe meningitis resulting in possible long-term neurological issues. It is estimated that the rate of infection for low birth weight infants, who are particularly susceptible, is 8.7 per 100,000 and the mortality rate for infants from *Cronobacter* meningitis can be as high as 40% (1). Nearly all cases of infant *Cronobacter* infections have been associated with the consumption of contaminated powdered infant formula (PIF) (4). As a result, the WHO and the FDA deemed *Cronobacter* a health hazard to neonates that consume PIF contaminated with *Cronobacter* and require end product testing for *Cronobacter* (n=30, c=0, 10 g sample) as a compliance requirement before placing PIF on either the US or EU market (2, 3).
Principle of the Method

The PhageDx™ Cronobacter Assay is based on the infection of Cronobacter spp. by bacteriophages and replication of the infecting bacteriophages within their specific hosts. Bacteriophages demonstrate a high specificity for their bacterial host and are capable of replicating within their host quickly to high numbers. The recombinant phages used in the PhageDx™ Cronobacter Assay also express a luciferase reporter during replication. The presence of Cronobacter spp. is determined by incubating the lysate with the appropriate luciferase substrate and detecting emitted light in a luminometer. An absence of detected light indicates that no Cronobacter are present in that sample. An advantage of this system is that only viable bacteria are detected as bacteriophage only replicate in living cells.

Scope of method

(a) Target organism. — Cronobacter spp.

(b) Matrix. — Powdered infant formula (milk-based), powdered infant formula (soy-based).

(c) Summary of Validated Performance Claims. — Performance equivalent to that of the U.S. FDA Bacteriological Analytical Manual (BAM) Ch. 29 Cronobacter (4) and ISO 22964:2006 or ISO 22964:2017 Microbiology of the food chain – Horizontal method for the detection of Cronobacter spp. (5, 6).

Definitions
(a) Probability of Detection (POD).—The proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration. POD is concentration dependent. Several POD measures can be calculated: POD$_R$ (reference method POD), POD$_C$ (confirmed candidate method POD), POD$_{CP}$ (candidate method presumptive result POD) and POD$_{CC}$ (candidate method confirmation result POD).

(b) Difference of Probabilities of Detection (dPOD).—Difference of probabilities of detection is the difference between any two POD values. If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

Materials and Methods

Test Kit Information

(a) Kit Name.—PhageDx™ Cronobacter Assay.

(b) Catalog Number.—5008.

(c) Ordering Information.—Not applicable. For internal use at Laboratory Corporation of America only.

Test Kit Components

(a) PhageDx™ Cronobacter recombinant phage.—Part. No. 3101, 12 tubes containing 100 µL phage solution.

(b) Lysis buffer.—Part. No. 3002, 12 tubes containing 100 µL Lysis buffer.
(c) **Assay buffer.** —Part. No. 3003, 12 tubes containing 500 µL Assay buffer.

(d) **Luciferase substrate.** —Part. No. 3004, 12 tubes containing 10 µl Luciferase substrate.

(e) **96-well break-apart plate.** —Part. No. 3103, one pouch containing break-apart plate (8 wells × 12 strips).

(f) **One package insert.** —Part. No. 3102.

Additional supplies and reagents

(a) **Sample bags.** —Recommended sample bags: Fisher Scientific, Cat. No. 14-955-187 (10 g); Fisher Scientific Cat. No. 01-812 (100 g); Fisher Scientific, Cat. No. 14-209-300 (300 g)

(b) **Microfuge tubes (1.5 mL).**

(c) **Sample bag and tube racks.**

(d) **Buffered Peptone Water (BPW)** Thermo Fisher Scientific, Cat. No. CM0509.

(e) **Sample pipettor (2-5 mL).**

(f) **Sterile, filtered pipette (2-5 mL).**

(g) **Adjustable single channel pipettor (10 µL–1 mL) and appropriate sterile tips.**

(h) **Appropriate personal protective equipment.** —See [https://www.cdc.gov/labs/pdf/CDC-BiosafetyMicrobiologicalBiomedicalLaboratories-2009-P.PDF](https://www.cdc.gov/labs/pdf/CDC-BiosafetyMicrobiologicalBiomedicalLaboratories-2009-P.PDF).

(i) **Thermo Scientific™ Oxoid™ Brilliance™ Cronobacter Sakazakii Agar** Thermo Fisher Scientific Cat. No. CM1055B

Apparatus
(a) Homogenizer.—Seward Stomacher® 400/3500 or similar.

(b) Air incubators capable of 37 ± 1°C.

(c) Promega GloMax® 96 or Navigator luminometer.

(e) Personal computer for luminometer control and data analysis.

Safety Precautions

(a) The PhageDx™ Cronobacter Assay involves the enrichment of samples which may contain human pathogenic Cronobacter and have the potential for contamination with subsequent handling of those samples. This method should be conducted by properly trained laboratory personnel in a suitable microbiology laboratory in accordance with “Biosafety in Microbiological and Biomedical Laboratories”, U.S. Department of Health and Human Services, https://www.cdc.gov/labs/pdf/CDC-BiosafetyMicrobiologicalBiomedicalLaboratories-2009-P.PDF. Care should be taken when handling the sample and reagents while performing the method.

(b) Materials and reagents provided in the PhageDx™ Cronobacter Assay are not considered hazardous if used according to the assay method. Please review the Material Safety Data Sheet prior to performing the assay.

(c) Follow all relevant guidelines and laboratory protocols while performing the assay and manufacturer’s equipment instructions.

General Preparation

(a) Prepare BPW media according to manufacturer’s instructions.
(b) Before using the reagents, flick or spin the tube to collect all of the solution at the bottom of the tube.

(c) Due to the short enrichment times, it is vital to maintain the temperature of the sample and BPW media used in the incubation.

(d) Before adding the pre-warmed BPW to the sample, confirm that the media and incubator are warmed to 37 ± 1°C.

(e) Do not allow the pre-warmed media to cool before adding to the sample.

(f) Maintain the media at 37 ± 1°C in an incubator or water bath if preparing multiple samples.

Sample Preparation

(a) Weigh 10 g, 100 g or 300 g of powdered infant formula and place into a sample bag.

(b) Add 90 mL (10 g test portion), 300 mL (100 g test portion) or 900 mL (300 g test portion) pre-warmed (37 ± 1°C) BPW to the sample.

(c) Homogenize sample in a Stomacher® 400 or Stomacher® 3500, depending on sample size, at the highest setting for 120 s (or equivalent homogenizer and setting).

(d) Loosely close the sample bag and place in a static air incubator at 37 ± 1°C for 16-18 h using a sample rack to keep the bags separate and allow for heat transfer.

(e) Remove the enriched samples from the incubator and mix thoroughly by hand for at least 30 s or stomach to ensure complete mixing. NOTE: Sample must be thoroughly mixed so the analyte is distributed evenly throughout the entire sample. We recommend vigorous shaking and massaging for at least 30 seconds. Immediately proceed to the next step after mixing is complete. If sample sits for 15 min or longer, mix sample again before proceeding to the next step.
(f) Using a pipettor with a sterile tip, transfer 1 mL of the sample to a sterile 1.5 mL microfuge tube.

(g) Mix contents in microfuge tube and dilute sample 1:10 in BPW (100 µl sample in 900 µl BPW).

(h) Using a single channel pipettor and clean tip for each sample, transfer 150 µl of diluted sample to 96-well plate.

(i) Using a single channel pipettor and clean tip for each sample, add 10 µL of the phage solution to the sample and gently mix by pipetting up and down.

(j) Cover plate with plate sealing tape and place the sample in the 37 ± 1°C incubator for 2 h.

(k) Remove one tube containing the lysis buffer, assay buffer, and substrate for each 8 well strip used and thaw to room temperature. Flick or spin the tubes to collect all of the solution at the bottom of the tubes.

(l) Prepare the luciferase substrate working solution by transferring the entire contents of assay buffer (0.5 ml) to the substrate tube (10 µL) and mix well. NOTE: Use within 1 h of preparation

(m) Using a clean tip for each sample, add 10 µL lysis buffer and mix thoroughly by gently pipetting up and down being careful not to introduce bubbles.

(n) Add 50 µL of the 1:50 luciferase substrate working solution to each well using a single channel pipettor and clean tip for each sample. Mix thoroughly by gently pipetting up and down being careful not to introduce bubbles.

(o) Once all of the samples have received the substrate, place the sample plate in the luminometer, close the lid and initiate the read program.

Interpretation and Test Result Report
(a) The luminometer program will display the results on the screen as relative light unit (RLU) values corresponding to the well positions of the break-away plate.

(b) Samples positive for *Cronobacter* spp. will have a reading value of 500 RLU or greater. Negative samples will be less than 500 RLU. Note samples that are positive.

(c) Once all of the samples have been run and analyzed, remove the plate from the luminometer and follow the manufacturer’s instructions for cleaning the instrument and shut down.

**Confirmation**

(a) Confirmation of *Cronobacter* spp. can be performed by streaking 24 h enriched cultures onto Oxoid™ Brilliance™ *Cronobacter Sakazakii* Agar. To prepare for the confirmation, allow the samples to continue enriching for a total of 24 h (or for an additional 6–8 h) at 37 ± 1°C. Remove 50 µL of the overnight culture and streak onto Oxoid™ Brilliance™ *Cronobacter Sakazakii* Agar and incubate plates for 24 h at 37 ± 1°C.

(b) Plates with colonies that appear blue-green and grow well are positive.

(c) Alternative confirmation methods are described in ISO 22964:2017 Microbiology of the food chain – Horizontal method for the detection of *Cronobacter* spp. (6) and U.S. FDA Bacteriological Analytical Manual (BAM) Ch. 29 *Cronobacter* (4).

**Validation Study**

This validation study was conducted under the AOAC Research Institute *Performance Tested Method*™ program and the AOAC INTERNATIONAL Methods Committee Guidelines for Validation of
Microbiological Methods for Food and Environmental Surfaces, Appendix J (7). Method developer studies were conducted in the laboratories of Laboratory Corporation of America Holdings, and included the inclusivity/exclusivity study, matrix studies for all claim matrixes, product consistency and stability studies and robustness testing. The Independent Laboratory Study was conducted by Q Laboratories, Inc., and included a matrix study for milk-based powdered infant formula.

Method Developer Studies

Inclusivity and Exclusivity Studies

Inclusivity strains (Cronobacter) were obtained from academic, governmental, and commercially available sources (Table 1). Each strain was grown overnight in tryptic soya broth (TSB) media at 37 ± 1°C until stationary phase. Cells were diluted to 100 CFU in 0.1 mL and mixed with recombinant phage for 2 h at 37 ± 1°C. Following infection, samples were mixed with lysis buffer and luciferase substrate and then read in a luminometer. Samples with RLU values greater than 150 were considered positive. Exclusivity strains were also obtained from commercially available sources and were grown to stationary phase overnight. Assays with exclusivity strains were done as with inclusivity strains except overnight cultures were assayed directly (Table 2).

Results. — The PhageDx™ Cronobacter Assay demonstrates 100% inclusivity with the 75 Cronobacter strains tested (Table 1). The PhageDx™ Assay also demonstrates exclusivity for 35/39 non-Cronobacter strains tested (Table 2). The 3 non-Cronobacter strains which were detected by the PhageDx™ Assay were from the closely related Enterobacter genus. That Cronobacter was formerly named Enterobacter indicates how closely related these two genera are, thus it is not entirely surprising that there may be
some cross reactivity with selected members of this family.

Product Consistency (lot-to-lot) and Stability Studies

Three separate production lots of PhageDx™ Cronobacter recombinant phage were prepared according to written manufacturing documents and tested according to quality control procedures. Quality control procedures verified that each lot when diluted to working concentration had the same titer, background and level of detection. Recombinant phage lots were aged between 1 and 3 months when assayed for stability.

Consistency and stability were done according to AOAC guidance, where a sample was inoculated with Cronobacter malonaticus ES686, a strain isolated from an ingredient in PIF, to give fractional positives. Ten replicates were run in the PhageDx™ Assay, and the RLU values analyzed. A set of stability studies was also conducted using the non-target bacterium Citrobacter koseri (ATCC 25408). Overnight cultures of C. koseri were used directly in the assay. Results are shown in Table 3.

Results.—The PhageDx™ Cronobacter recombinant phages can be manufactured consistently and are stable for at least 3 months when stored at 4°C. Manufactured lots were made on 2/10/2017, 4/28/2017 and 5/5/2017 according to written manufacturing documents. Working solutions of each lot produced similar results when tested according to QC control tests for bacteriophage concentration, background signal and limit of detection. Stability tests of each lot were performed to determine the shelf life of the recombinant phage. These tests demonstrated that lots produced 1 month prior to testing showed no significant difference from lots produced 3 months prior to testing. Additionally, no variation in exclusivity was observed with these three recombinant phage lots in tests with C. koseri.
Robustness Study

Three parameters were varied to demonstrate assay robustness: enrichment time (14 and 24 h), recombinant phage concentration (± 20%) and luciferase substrate amount (± 10%). Briefly, 10 g milk-based powdered infant formula samples were left unspiked or spiked with 0.2–2 CFU/10 g Cronobacter muytjensii FSL-F6-031 dried in powdered infant formula and stored at room temperature (20–25°C) for 2–4 weeks. The PhageDx™ Cronobacter Assay protocol was followed with the variations in enrichment time, recombinant phage concentration and substrate amount as indicated in Table 4. Samples with RLU values greater than 500 were considered positive. Samples were confirmed by allowing samples to enrich for a total of 24 ± 2 h and then plating on Oxoid™ Brilliance™ Cronobacter Sakazakii Agar. Plates were incubated at 37 ± 1°C for an additional 24 ± 2 h. The presence of blue-green colonies that grew well indicated positive samples. A summary of the testing is presented in Table 4.

Results.—Robustness testing of the PhageDx™ Cronobacter Assay demonstrated that variations in enrichment time, recombinant phage concentration and luciferase substrate amount do not alter the results compared to the standard protocol. Enrichment times of 14 and 24 h, recombinant phage volumes of 8 and 12 µL and luciferase substrate volumes of 45 and 55 µL produced identical results to the standard protocol of 16 h enrichment, 10 µL of recombinant phage and 50 µL of luciferase substrate in both uninoculated and low inoculum test samples (Table 4). These results indicate that these deviations from the PhageDx™ Cronobacter Assay protocol did not alter the final results.

Matrix Study
The matrix study compared the PhageDx™ Cronobacter (10 g test portions) to ISO 22964:2006 (10 g test portions) and the PhageDx™ Cronobacter (100 and 300 g test portions) to FDA BAM Ch. 29 Cronobacter:2012 (100 g test portions). The PhageDx™ Cronobacter 10 g portions were compared to ISO 22964:2006 using a paired study design. The PhageDx™ Cronobacter 100 and 300 g portions were compared to the FDA BAM Ch. 29 100 g portions using an unpaired study design. For each matrix and each comparison, the study included five replicate test portions of uninoculated matrix (0 CFU/test portion), 20 replicate test portions at a low level to yield fractionally positive results (0.2–2 CFU/test portion), and five replicate test portions at a high level to yield consistently positive results (2–10 CFU/test portion).

Both milk-based and soy-based PIF were purchased from local retail stores and prescreened for natural contamination using ISO 22964:2006 method. To prepare the inoculum, Cronobacter was grown in TSB for 18–24 h at 37 ± 1°C. The culture was diluted in BPW, reconstituted in PIF and placed into a speed vacuum for 4–8 h until sample was completely dried. After desiccation, the dried inoculum was diluted into PIF matrix used in each study to obtain a low level expected to yield fractional positive results and a high level expected to yield all positive results and allowed to sit for 2-4 weeks at room temperature (20-25°C) to allow for equilibration in the matrix. A bulk lot of the matrix was inoculated with the diluted inoculum prior to testing.

On the day of analysis, total aerobic count was determined according to FDA BAM Ch. 3 (8) and the level of Cronobacter in low level and high-level inoculum was determined by most probable number (MPN) analysis. For the paired samples, MPN analysis was determined using the ISO 22694:2006 method. For low-level inoculum, 5 test portions of 25 g, 5 test portions of 4 g, and 20 test portions of 10 g from the matrix study were analyzed. For the high-level inoculum, 5 test portions of 10 g from the matrix study, 5 test portions of 4 g, and 5 test portions of 1.5 g were analyzed.
For the unpaired samples, MPN analysis was determined using the FDA BAM Ch. 29 method. For low-level inoculum, 5 test portions of 200 g, 5 test portions of 50 g, and 20 test portions of 100 g from the matrix study were analyzed. For the high-level inoculum, 5 test portions of 100 g from the matrix study, 5 test portions of 50 g, and 5 test portions of 25 g were analyzed. The number of positives was used to calculate the MPN using the LCF MPN calculator provided by AOAC RI. (9).

**PhageDx™ Cronobacter Assay**

Test portions were processed according to directions for use. Briefly, 90 mL (10 g test portion), 300 mL (100 g test portion) or 900 mL (300 g test portion) of pre-warmed BPW (37 ± 1°C) was added to PIF test portions. Samples were homogenized and enriched at 37 ± 1°C for 16–18 h. Enriched samples were mixed thoroughly before taking aliquots for analysis. Samples were diluted 1:10 (100 µl sample: 900 µl BPW) in pre-warmed BPW (37 ± 1°C) and 150 µl of the diluted sample was transferred to a 96 well plate. Samples were then infected with recombinant phage for 2 h at 37 ± 1°C. Lysis buffer and luciferase substrate were added to the samples. Samples were then read on a luminometer. Readings of ≥ 500 RLU were considered positive. To confirm per PhageDx™ Cronobacter Assay, samples were allowed to enrich for a total of 24 ± 2 h at 37 ± 1°C. Enriched samples were mixed thoroughly before taking aliquots for analysis. Fifty (50) µL were struck onto Oxoid™ Brilliance™ Cronobacter Sakazakii Agar and incubated for 24 ± 2 h at 37 ± 1°C. The presence of colonies that grow well (1-3 mm) and appear blue-green indicate a positive sample. For confirmation per FDA BAM Ch. 29, sections E and F were performed (4). Briefly, from a 24 h enrichment, 2 X 40 mL aliquots were centrifuged at 3,000 x g for 10 minutes. The supernatant was discarded and the resultant pellets were resuspended in 200 µl of sterile phosphate buffered saline. One hundred (100) µl aliquots of the resuspended pellet were plated on two DFI
chromogenic agar and two R&F *Cronobacter* chromogenic agar plates. In addition, a loopful of each enrichment was struck onto two DFI chromogenic agar and two R&F *Cronobacter* chromogenic agar plates. All plates were incubated at 36 ± 1°C for 18–24 h. Presumptive positive colonies were confirmed by PCR as outlined in section F of BAM Ch. 29 (4).

ISO 22964:2006

ISO 22964:2006, the current version at time of testing, was used in the method developer laboratory for the matrix evaluation. Briefly, 90 mL of BPW was added to 10 g of PIF. The samples were incubated at 37 ± 1°C for 18 ± 2 h. Then 0.1 mL was transferred from the BPW culture to 10 mL of mLST/vancomycin medium and incubated at 44 ± 1°C for 24 ± 2 h. A loopful of mLST/vancomycin culture was struck onto *Enterobacter sakazakii* Isolation Agar and incubated at 44 ± 1°C for 24 ± 2 h. One to five presumptive positive colonies were then struck onto tryptic soya agar (TSA) plates and incubated at 25°C for 48 ± 4 h. Yellow pigmented colonies were chosen for further biochemical confirmation tests (5).

FDA BAM Chapter 29

For the FDA BAM Ch. 29 *Cronobacter* method, 900 mL of sterile BPW was added to 100 g PIF in sterile 2 L Erlenmeyer flasks and gently agitated by hand until PIF was uniformly suspended. Test samples were incubated at 36 ± 1°C for 24 ± 2 h. After enrichment, the samples were thoroughly mixed and 4 X 40 mL from each sample were transferred into 50 mL centrifuge tubes. The aliquots were centrifuged at 3,000 x g for 10 minutes and the supernatant was discarded. The resultant pellet was resuspended in 200 µL of phosphate buffered saline. Two aliquots were used for PCR to determine
presumptive positives and two aliquots were used for cultural confirmation if necessary. For the PCR screen, two aliquots were transferred to 1.5 mL microcentrifuge tubes and centrifuged at 3,000 \( x \) \( g \) for 5 minutes. The supernatant was discarded and the pellet was resuspended in 400 \( \mu L \) of PrepMan Ultra® sample preparation reagent and mixed by vortex at maximum speed until the pellet was completely resuspended. The samples were heated in a dry bath incubator at 100°C for 10 minutes, then cooled to room temperature. Once the samples reached room temperature, the samples were centrifuged for 2 minutes at 15,000 \( x \) \( g \) and a 50 \( \mu L \) aliquot of the supernatant was transferred to a new microcentrifuge tube for PCR analysis. For each sample, PCR analyses were performed with and without internal control (InC). The PCR reaction components and the PCR protocol was followed as outlined in the FDA BAM Chapter 29 reference method. Presumptive positives were confirmed using FDA BAM Ch. 29, sections E and F. Briefly, 100 \( \mu L \) aliquots of the resuspended pellet were plated on two DFI chromogenic agar and two R&F \textit{Cronobacter} chromogenic agar plates. In addition, a loopful of each enrichment was streaked onto two DFI chromogenic agar and two R&F \textit{Cronobacter} chromogenic agar plates. All plates were incubated at 36 ± 1°C for 18–24 h. Colonies were confirmed by PCR as outlined in section F of BAM Ch. 29 (4).

All test results were analyzed using POD statistical analysis to 95% confidence intervals (CI). POD analysis is described in the AOAC INTERNATIONAL guidelines in Appendix J. Data from the analysis are presented in Tables 5–8.

Results.—The method developer studies showed that there were no differences between the PhageDx™ Assay and the ISO 22964:2006 and the FDA BAM Ch. 29 \textit{Cronobacter} reference methods for all matrixes tested (Tables 5–8). All test portions that were presumptive positives by PhageDx™ \textit{Cronobacter} Assay were confirmed by their respective reference methods to contain \textit{Cronobacter}. There were no false negative results. The POD analyses indicated no significant differences exist between the
PhageDx™ Cronobacter Assay and the ISO 22964:2006 reference method in a paired study (Table 5).

There was no statistical difference between the number of PhageDx™ Cronobacter Assay presumptive positive results and the BAM Ch. 29 confirmation results (Table 6). Likewise, comparison of the PhageDx™ Assay presumptive results to either the BAM Ch 29 confirmation or the Oxoid™ Brilliance™ Cronobacter Sakazakii Agar plating confirmation were not significantly different (Tables 6 and 7). The comparison of the PhageDx™ Cronobacter Assay and the FDA BAM Ch. 29 unpaired study showed that the there was no statistical difference in the performance of the two methods (Table 8). The one exception was the 100 g milk based PhageDx™ Assay vs. BAM Ch. 29 Method Comparison (Table 8). The difference between the fractional positives was statistically significant, where the dPOD was 0.35, and the CI was (0.04, 0.58). The aerobic plate count of the PIF used in the study was 0 CFU/g, indicating that the PIF had no or very low levels of background flora present at the initiation of the enrichment process.

Independent Laboratory Validation Study

The independent laboratory evaluation included a matrix study for milk-based PIF comparing the PhageDx™ Cronobacter Assay to ISO 22964:2017 and FDA BAM Chapter 29 reference methods (6, 4). For the method comparison to ISO 22964:2017, 30 paired 10 g test portions were evaluated. For the method comparison to FDA BAM Chapter 29, 100 g and 300 g test portions of the PhageDx™ Cronobacter Assay were compared to 100 g test portions of the reference method. Within each sample set, there were 5 uninoculated samples (0 CFU/ test portion), 20 low-level inoculated samples (0.2-2 CFU/ test portion), and 5 high-level inoculated samples (2-10 CFU/ test portion). The low inoculation level was designed to produce fractional positive results, those in which the candidate or reference method produced 5–15 positive results (25–75%).
The PIF was purchased from a local distributor, prescreened for natural contamination of the analyte following ISO 22964:2017, and analyzed for total aerobic count by FDA BAM Chapter 3.

Following the screening, the matrix was inoculated with a strain of *Cronobacter* species. For the validation, a lyophilized culture was used to inoculate the PIF. The lyophilized culture was prepared by transferring a single *Cronobacter sakazakii* colony from tryptic soy agar with 5% sheep blood into brain heart infusion (BHI) broth and incubating the culture at 35 ± 2 °C for 18–24 hours. Following incubation, the culture was diluted in a sterile cryoprotectant, reconstituted nonfat dry milk (NFDM), and placed onto a freeze dry system for 48–72 hours. After removing the culture from the freeze dry system, the lyophilized culture was diluted in NFDM to a low level expected to yield fractional positive results and a high level expected to yield all positive results. A bulk lot of the matrix was inoculated. After inoculation, the matrix was held for 2 weeks at room temperature (24 ± 2°C) to allow for equilibration of the organism in the matrix.

Total aerobic count was determined according to FDA BAM Ch. 3. The level of *Cronobacter* in the low-level inoculum and high-level inoculum was determined by MPN on the day of analysis. For the paired sample analysis, the low-level MPN was determined by evaluating 5 x 25 g test portions, the 20 x 10 g test portions from the study, and 5 x 4 g test portions. The level of *Cronobacter* in the high-level inoculum was determined by evaluating the 5 x 10 g test portions from the study, 5 x 4 g test portions, and 5 x 1.5 g test portions.

For the unpaired analysis, the low-level MPN was determined by evaluating 5 x 200 g test portions, the 20 x 100 g reference method test portions from the study, and 5 x 50 g test portions. The level of *Cronobacter* in the high-level inoculum was determined by evaluating the 5 x 100 g reference method test portions from the study, 5 x 50 g test portions, and 5 x 25 g test portions. Each test portion was
enriched with BPW and analyzed by the reference method procedure. The number of positives from the 3 test levels was used to calculate the MPN using the LCF MPN calculator (version 1.6)(9).

ISO 22964:2017

For ISO 22964:2017, 10 g PIF test portions were enriched with 90 mL of BPW (ISO formulation) and incubated at 37 ± 1°C for 18 ± 2 hours. Following incubation, 0.1 mL of primary enrichment was transferred into 10 mL of Cronobacter selective broth (CSB) and incubated at 41.5 ± 1 °C for 24 ± 2 hours. Following incubation, a loopful of the CSB was struck to Chromogenic Cronobacter Isolation (CCI) agar and incubated at 41.5 ± 1 °C for 24 ± 2 hours. Following incubation of the CCI plates, one to five typical Cronobacter species colonies (medium sized colonies, 1 mm to 3 mm, blue-green to blue) were transferred to tryptone soya agar (TSA) and incubated at 35 ± 1°C for 18 to 24 hours. After incubation, an oxidase test was conducted on a typical colony (yellow-pigmented, 1 mm to 3 mm) and final biochemical confirmation was performed by using the VITEK® 2 GN Biochemical Identification card following AOAC Official Method 2011.17 (10).

FDA/BAM Chapter 29

For BAM Ch. 29, 100 g PIF test portions were added to 2 L Erlenmeyer flasks, enriched with 900 mL of pre-warmed (37°C) BPW, and incubated at 37 ± 1°C for 24 ± 2 hours. Following incubation, 4 x 40 mL aliquots were transferred to 4 x 50 mL conical vials. The aliquots were centrifuged at 3,000 x g for 10 minutes. For each conical tube, the supernatants were aspirated and the lipid precipitate was removed using sterile cotton swabs. The remaining pellet was re-suspended by adding 200 μL of phosphate
buffered saline and mixing the suspension by vortex at max speed for 20 seconds. For each sample, two of the aliquots were used for PCR screening of Cronobacter and two of the aliquots were used for cultural confirmation.

For the PCR screening, two aliquots were transferred to separate 1.5 mL microcentrifuge tubes and centrifuged at 3,000 x g for 5 minutes. The supernatant and lipid layer were removed and the pellet was re-suspended by adding 400 µL of PrepMan Ultra® sample preparation reagent and mixing by vortex at max speed until suspension was achieved. The samples were heat treated in a dry bath incubator at 100°C for 10 minutes, then cooled to room temperature. Once the samples reached room temperature, the samples were centrifuged for 2 minutes at 15,000 x g and a 50 µL aliquot of the supernatant was transferred to a new microcentrifuge tube for PCR analysis. For each sample, PCR analyses were performed with and without internal control (InC). The PCR reaction components and PCR protocol were followed as outlined in the FDA BAM Chapter 29 reference method.

Regardless of the presumptive PCR result, 100 µL of suspended cells from each sample was struck onto two DFI chromogenic agar plates and two R&F agar plates. DFI chromogenic agar plates and R&F agar plates were incubated at 36 ± 1°C for 18-24 hours. Following incubation typical Cronobacter colonies from DFI chromogenic agar (weak to dark green, brownish colonies, or green centered colonies with a white to yellow border) and R&F agar plates (blue to black or blue to grey colonies with a red background) were biochemically confirmed by VITEK® 2 GN Biochemical Identification card (AOAC Official Method 2011.17) and PCR analysis (10).

Results.—For all three levels, the POD analyses between the PhageDx™ Cronobacter Assay and the reference methods indicated that there was no statistically significant difference at the 5% level between the number of positive results obtained by the methods (Tables 5–8). For all
three levels, the POD analyses between presumptive results of the PhageDx™ *Cronobacter*
Assay and confirmed results indicated that there was no statistically significant difference at the
5% level for all test portions analyzed (Tables 5–8). The aerobic plate count of the PIF used in
the study was 40 CFU/g, indicating that the PIF had approximately 400 CFU (10 g), 4,000 CFU
(100 g), or 12,000 CFU (300 g) of background flora present at the initiation of the enrichment
process.

**Discussion**

The results of this validation study show that the PhageDx™ *Cronobacter* Assay is an effective
alternative to the ISO 22964:2006/2017 for the detection of *Cronobacter* in 10 g of milk- and soy- based
powdered infant formula and FDA BAM Ch. 29 for the detection of *Cronobacter* in 100 g or 300 g of milk-
and soy-based powdered infant formula. In inclusivity and exclusivity testing, the method was shown to
be specific for *Cronobacter*, correctly identifying all 75 *Cronobacter* target strains and 35 non-target
strains. The PhageDx™ *Cronobacter* Assay displayed cross reactivity with some closely related strains of
*Enterobacter*. *Cronobacter* was formerly categorized in the genus *Enterobacter*. This indicates how
closely related these two genera are, thus it is not entirely surprising that there may be some cross
reactivity with selected members of this family.

The recombinant phage can be produced consistently and is stable for 3 months when stored
appropriately. Robustness testing of the PhageDx™ *Cronobacter* Assay indicated that the method works
well when the assay parameters (enrichment time, recombinant phage concentration and substrate
amount) were varied from the stated protocol. Method developer studies demonstrated that the
performance of the PhageDx™ *Cronobacter* Assay was not statistically different from that of ISO 22964.
for 10 g test sample or FDA BAM Ch. 29 for 100 g and 300 g test samples. One exception was the comparison of the PhageDx™ and FDA BAM Ch. 29 for the 100 g milk based fractional positives data which was statistically significant (Table 8). One possible explanation is that this could be a result of skewed sample inoculation (PhageDx™ = 12, FDA BAM = 5). Alternatively, the PhageDx™ Assay may be more sensitive and was able to detect a greater number of presumptive positives than the FDA BAM Ch. 29 presumptive positive PCR method. However, since no false positives or false negatives were found in the study, it suggests that this result is likely a product of one or more of these factors.

Independent laboratory testing demonstrated that the PhageDx™ Cronobacter Assay was able to detect *Cronobacter* at low levels in 10 g, 100 g, and 300 g of powdered infant formula, which also contained approximately 40 CFU/g background flora, and an alternative confirmation procedure was shown to be identical to the reference method confirmation procedures.

The PhageDx™ Cronobacter Assay also has a number of advantages over the ISO 22964 and FDA BAM Ch. 29 reference methods. In addition to being a specific assay, the results are easy to interpret as an RLU endpoint is used to determine the outcome of the assay. This is in contrast to the ISO method where interpretation of reagent color changes is required or the FDA BAM method where PCR amplification plots may have to be assessed. With the PhageDx™ Assay, test samples with an RLU of 500 or greater are considered positive. Another advantage is that PhageDx™ provides a presumptive positive result in as little as 18.5 h compared to >24 h in the case of FDA BAM and >60 h in the case of ISO method. PhageDx™ is also a simple test that involves only five basic steps: enrichment, dilution, infection, substrate addition, and signal readout. Finally, PhageDx™ Assay is rapid method that offers a considerable cost and time savings alternative compared to the ISO 22964 and FDA BAM Ch. 29 reference methods.

**Conclusion**
Results of this validation study support the claim that the PhageDx™ Cronobacter Assay is a specific, sensitive, fast, and simple method for the detection of Cronobacter in powdered infant formula and is statistically comparable to the ISO:22964:2006/2017 and FDA BAM Ch. 29 Cronobacter methods. By using a luciferase-expressing recombinant bacteriophage, the assay was able to detect a single, viable bacterium after a 16 h enrichment and a 2 h infection. The PhageDx™ Cronobacter Assay thus offers shorter time-to-results compared with the other validated Cronobacter detection assays. The PhageDx™ Cronobacter Assay provides powdered infant formula manufacturers with an alternative method for conducting required regulatory testing that is easier to use and potentially more cost effective than current validated methods for Cronobacter detection.

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Submitting Company

Laboratory Corporation of America®
531 South Spring Street, Burlington, NC 27215

Independent Laboratory

Kiel Fisher, Leo Horine, James Agin, and Pat Bird
Q Laboratories Inc., Cincinnati, OH, USA 45204
Reviewer(s)

Yi Chen
U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, 5100 Paint Branch Pkwy, College Park, MD 20740

Joseph Odumeru
University of Guelph, Ontario, CANADA

Wayne Ziemer
Consultant 1301 Kristen Lane, Loganville, GA 30052

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Table 1. Inclusivity List: *Cronobacter*

| No. | *Cronobacter* strain        | Source       | Origin             | PhageDx™ result |
|-----|----------------------------|--------------|--------------------|-----------------|
| 1   | *Cronobacter sakazakii* ATCC® BAA-894 | Food, PIF   | Positive           |
| 2   | *Cronobacter sakazakii* ATCC12868 | Unknown      | Positive           |
| 3   | *Cronobacter sakazakii* ATCC29004 | Unknown      | Positive           |
| 4   | *Cronobacter sakazakii* ATCC 29544 | Clinical     | Positive           |
| 5   | *Cronobacter sakazakii* FDA® E54963-71 | Clinical     | Positive           |
| 6   | *Cronobacter sakazakii* FDA 255N | Clinical     | Positive           |
| 7   | *Cronobacter sakazakii* FDA CQ31 | PIF Environment | Positive         |
| 8   | *Cronobacter sakazakii* FDA CQ126 | PIF Environment | Positive         |
| 9   | *Cronobacter sakazakii* FDA E788 | Clinical     | Positive           |
| 10  | *Cronobacter sakazakii* FDA CQ123 | PIF Environment | Positive         |
| 11  | *Cronobacter sakazakii* FDA CQ92 | PIF Environment | Positive         |
| 12  | *Cronobacter sakazakii* FDA 2/4/2011 | PIF        | Positive           |
| 13  | *Cronobacter sakazakii* FDA E.sak713 | Food, PIF   | Positive           |
| 14  | *Cronobacter sakazakii* FDA LR834 | Environment Dairy | Positive      |
| 15  | *Cronobacter sakazakii* FDA 2193-02 | Clinical     | Positive           |
| 16  | *Cronobacter sakazakii* FDA LR835 | Environment Dairy | Positive      |
| 17  | *Cronobacter sakazakii* FDA ES9369-75 | Clinical     | Positive           |
| 18  | *Cronobacter sakazakii* FDA ES626 | Food, Rice flour | Positive        |
| 19  | *Cronobacter sakazakii* FDA 708  | Clinical     | Positive           |
| 20  | *Cronobacter sakazakii* FDA ES1059-71 | Clinical     | Positive           |
| 21  | *Cronobacter sakazakii* FDA ES718 | Clinical     | Positive           |
| 22  | *Cronobacter sakazakii* FDA ES717 | Food, PIF    | Positive           |
| 23  | *Cronobacter sakazakii* FDA 2154 | Clinical     | Positive           |
| 24  | *Cronobacter sakazakii* FDA 607A | Clinical     | Positive           |
| 25  | *Cronobacter sakazakii* FDA 2148 | Clinical     | Positive           |
| 26  | *Cronobacter sakazakii* FDA 2150 | Clinical     | Positive           |
| 27  | *Cronobacter sakazakii* FDA GK792.3 | PIF Environment | Positive      |
| 28  | *Cronobacter sakazakii* FDA GK799 | PIF Environment | Positive         |
| 29  | *Cronobacter sakazakii* FDA GK794 | PIF Environment | Positive         |
| 30  | *Cronobacter sakazakii* FDA GK800 | PIF Environment | Positive         |
| 31  | *Cronobacter sakazakii* FDA GK801.1 | PIF Environment | Positive         |
| 32  | *Cronobacter sakazakii* FDA GK797 | PIF Environment | Positive         |
| 33  | *Cronobacter sakazakii* FDA GK952 | PIF Environment | Positive         |
| 34  | *Cronobacter sakazakii* FDA LR702 | Food, PIF    | Positive           |
| 35  | *Cronobacter sakazakii* FDA LR703 | Food, Hi PDI flour | Positive        |
| No. | Species                          | Identifier     | Environment          | Result |
|-----|---------------------------------|----------------|----------------------|--------|
| 36  | *Cronobacter sakazakii*         | FDA LR704      | Food, Hi PDI flour   | Positive |
| 37  | *Cronobacter sakazakii*         | FDA LR705      | Food, Organic Soy Powder | Positive |
| 38  | *Cronobacter sakazakii*         | FSL\textsuperscript{d} F6-0023 | Clinical | Positive |
| 39  | *Cronobacter sakazakii*         | FSL F6-024     | Infant Formula       | Positive |
| 40  | *Cronobacter sakazakii*         | FSL F6-025     | Enviromental         | Positive |
| 41  | *Cronobacter sakazakii*         | FSL F6-027     | Enviromental         | Positive |
| 42  | *Cronobacter sakazakii*         | FSL F6-028     | Clinical             | Positive |
| 43  | *Cronobacter sakazakii*         | FSL F6-0029    | Clinical             | Positive |
| 44  | *Cronobacter sakazakii*         | FSL F6-0034    | Clinical             | Positive |
| 45  | *Cronobacter sakazakii*         | FSL F6-035     | Clinical             | Positive |
| 46  | *Cronobacter sakazakii*         | FSL F6-0036    | Enviromental         | Positive |
| 47  | *Cronobacter sakazakii*         | FSL F6-037     | Enviromental         | Positive |
| 48  | *Cronobacter sakazakii*         | FSL F6-0038    | Enviromental         | Positive |
| 49  | *Cronobacter sakazakii*         | FSL F6-039     | Enviromental         | Positive |
| 50  | *Cronobacter sakazakii*         | FSL F6-0040    | Environmental        | Positive |
| 51  | *Cronobacter sakazakii*         | FSL F6-041     | Environmental        | Positive |
| 52  | *Cronobacter sakazakii*         | FSL F6-042     | Infant Formula       | Positive |
| 53  | *Cronobacter sakazakii*         | FSL F6-0043    | Clinical             | Positive |
| 54  | *Cronobacter sakazakii*         | FSL F6-044     | Food                 | Positive |
| 55  | *Cronobacter sakazakii*         | FSL F6-045     | Food                 | Positive |
| 56  | *Cronobacter sakazakii*         | FSL F6-046     | Infant Formula       | Positive |
| 57  | *Cronobacter sakazakii*         | FSL F6-047     | Infant Formula       | Positive |
| 58  | *Cronobacter sakazakii*         | FSL F6-048     | Infant Formula       | Positive |
| 59  | *Cronobacter sakazakii*         | FSL F6-0049    | Clinical             | Positive |
| 60  | *Cronobacter sakazakii*         | FSL F6-050     | Clinical             | Positive |
| 61  | *Cronobacter muytjensii*        | ATCC 51329     | Unknown              | Positive |
| 62  | *Cronobacter malonaticus*       | FDA C1825      | Clinical             | Positive |
| 63  | *Cronobacter malonaticus*       | FDA E5686      | Food, PIF ingredient | Positive |
| 64  | *Cronobacter malonaticus*       | FDA E1895-73   | Clinical             | Positive |
| 65  | *Cronobacter malonaticus*       | FDA E0939A-75  | Clinical             | Positive |
| 66  | *Cronobacter malonaticus*       | FSL F6-030     | Infant Formula       | Positive |
| 67  | *Cronobacter malonaticus*       | FSL F6-0052    | Clinical             | Positive |
| 68  | *Cronobacter malonaticus*       | E265\textsuperscript{c} | Unknown | Positive |
| 69  | *Cronobacter muytjensii*        | FSL F6-031     | Infant Formula       | Positive |
| 70  | *Cronobacter turicensis*        | FDA Z3032      | Clinical             | Positive |
| 71  | *Cronobacter dublinensis*       | FDA 5960-70    | Clinical             | Positive |
| 72  | *Cronobacter malonaticus*       | CDC\textsuperscript{a} 3523-75 | Unknown | Positive |
| 73  | *Cronobacter dublinensis*       | E464\textsuperscript{c} | Unknown | Positive |
| 74  | *Cronobacter dublinensis*       | E515\textsuperscript{c} | Unknown | Positive |
| Cronobacter genospecies | NCTC\(^{9}\) 9529 | Unknown | Positive |
|------------------------|-------------------|---------|----------|
| \(^{a}\)American Type Culture Collection, Manassas, VA. | | | |
| \(^{b}\)Unknown: No information is available on the origin of the strain. | | | |
| \(^{c}\)U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, ND. | | | |
| \(^{d}\)Cornell Food Safety Laboratory, Cornell University, Ithaca, NY. | | | |
| \(^{e}\)Centers for Disease Control and Prevention, Atlanta, GA. | | | |
| \(^{f}\)National Collection of Type Cultures, Porton Dwn, Salisbury, UK. | | | |
Table 2. Exclusivity List

| No. | Strain                                    | Source       | Origin                  | PhageDx™ result |
|-----|-------------------------------------------|--------------|-------------------------|-----------------|
| 1   | *Hafnia alveii*                           | ATCC° 13337  | Unknown                 | Negative        |
| 2   | *Shigella flexneri*                       | ATCC 12022   | Unknown                 | Negative        |
| 3   | *Proteus mirabilis*                       | ATCC 43071   | Clinical, Toe           | Negative        |
| 4   | *Edwardsiella tarda*                      | ATCC 15947   | Stool                   | Negative        |
| 5   | *Escherichia hermanni*                    | ATCC 33650   | Clinical, Toe           | Negative        |
| 6   | *Staphylococcus aureus*                   | ATCC 27660   | Unknown                 | Negative        |
| 7   | *Staphylococcus aureus*                   | ATCC 6538    | Human lesion            | Negative        |
| 8   | *Staphylococcus aureus*                   | ATCC 25923   | Clinical                | Negative        |
| 9   | *Enterobacter cloacae, subsp cloacae*      | ATCC 13047   | Spinal Fluid            | Positive        |
| 10  | *Serratia marcescens*                     | ATCC 13880   | Pond water              | Negative        |
| 11  | *Acinetobacter calcoaceticus*             | ATCC 23055   | Unknown                 | Negative        |
| 12  | *Morganella morganii* : subsp. Maorganii M11 | ATCC 25830   | Clinical                | Negative        |
| 13  | *Pseudomonas aeruginosa; Strain Boston 41401* | ATCC 27853   | Blood Culture           | Negative        |
| 14  | *Proteus vulgaris*                        | ATCC 33420   | Clinical                | Negative        |
| 15  | *Enterococcus faecalis*                   | ATCC 29212   | Urine                   | Negative        |
| 16  | *Enterobacter aerogenes*                  | ATCC 13048   | Sputum                  | Positive        |
| 17  | *Staphylococcus epidermidis*              | ATCC 14990   | Nose                    | Negative        |
| 18  | *Staphylococcus aureus*                   | ATCC 29213   | Wound                   | Negative        |
| 19  | *Citrobacter freundii*                    | ATCC 8090    | Unknown                 | Negative        |
| 20  | *Shigella sonnei*                         | ATCC 9290    | Unknown                 | Negative        |
| 21  | *Klebsiella pneumoniae*                   | ATCC 4352    | Cow’s milk              | Negative        |
| 22  | *Salmonella enterica, serovar Choleraesuis* | ATCC 12011   | Unknown                 | Negative        |
| 23  | *Escherichia fergusonii*                  | ATCC 35469   | Human feces             | Negative        |
| 24  | *Yersinia enterocolitica*                 | ATCC 23715   | Human blood             | Negative        |
| 25  | *Escherichia coli*                        | ATCC 13706   | Unknown                 | Negative        |
| 26  | *Escherichia coli*                        | ATCC 9637    | Unknown                 | Negative        |
| 27  | *Escherichia coli*                        | ATCC 4157    | Unknown                 | Negative        |
| 28  | *Escherichia coli*                        | ATCC 51813   | Food                    | Negative        |
| 29  | *Escherichia coli*                        | ATCC 35421   | Unknown                 | Negative        |
| 30  | *Escherichia coli*                        | ATCC 8739    | Feces                   | Negative        |
| 31  | *Escherichia coli*                        | ATCC 35218   | Canine                  | Negative        |
| 32  | *Escherichia coli*                        | ATCC 11775   | Urine                   | Negative        |
|   | Species                                      | ATCC    | Collection   | Result   |
|---|----------------------------------------------|---------|--------------|----------|
| 33 | Escherichia coli                             | ATCC 25922 | Clinical     | Negative |
| 34 | Enterobacter asburiae                        | FSL c F6-0026 | Environmental | Positive |
| 35 | Salmonella enterica, serovar Anatum         | ATCC9270   | Pork Liver   | Negative |
| 36 | Citrobacter koseri                          | ATCC 25408 | Throat       | Negative |
| 37 | Citrobacter braakii                         | ATCC 51113 | Snake        | Negative |
| 38 | Pluralibacter gergoviae                      | ATCC 33028 | Urine        | Negative |

*a* American Type Culture Collection, Manassas, VA.

*b* Unknown: No information is available on the origin of the strain.

*b* Cornell Food Safety Laboratory, Cornell University, Ithaca, NY.
Table 3. Stability and Consistency (lot-to-lot) of PhageDx™ Cronobacter Recombinant Phage – POD Comparison

| Phage lot # | Lot age, months | N\(^a\) | x\(^b\) | POD\(_A\)^c | 95% CI | Phage lot # | Lot age, months | N | x | POD\(_B\)^d | 95% CI | dPOD\(_{AB}\) | 95% CI\(^f\) |
|-------------|-----------------|---------|--------|-------------|--------|-------------|-----------------|---|---|-------------|--------|-------------|--------|
| Cronobacter malonaticus (target) | | | | | | | | | | | | | |
| 0217\(^g\) | 2 | 10 | 6 | 0.6 | 0.31, 0.83 | 0517\(^i\) | 1 | 10 | 6 | 0.6 | 0.31, 0.83 | 0.0 | -0.37, 0.37 |
| 0417\(^h\) | 3 | 10 | 5 | 0.5 | 0.24, 0.76 | 0517 | 1 | 10 | 6 | 0.6 | 0.31, 0.83 | -0.10 | -0.45, 0.29 |
| 0417 | 3 | 10 | 5 | 0.5 | 0.24, 0.76 | 0217 | 2 | 10 | 6 | 0.6 | 0.31, 0.83 | -0.10 | -0.45, 0.29 |
| Citrobacter koseri (non-target) | | | | | | | | | | | | | |
| 0217 | 2 | 10 | 0 | 0.0 | 0.0, 0.28 | 0517 | 1 | 10 | 0 | 0.0 | 0.0, 0.28 | 0.0 | -0.28, 0.28 |
| 0417 | 3 | 10 | 0 | 0.0 | 0.0, 0.28 | 0517 | 1 | 10 | 0 | 0.0 | 0.0, 0.28 | 0.0 | -0.28, 0.28 |
| 0417 | 3 | 10 | 0 | 0.0 | 0.0, 0.28 | 0217 | 2 | 10 | 0 | 0.0 | 0.0, 0.28 | 0.0 | -0.28, 0.28 |

\(^a\)N = Number of test portions.
\(^b\)x = Number of positive test portions.
\(^c\)POD\(_A\) = Positive outcomes divided by the total number of trials first member of pair.
\(^d\)POD\(_B\) = Positive outcomes divided by the total number of trials second member of pair.
\(^e\)dPOD\(_{AB}\) = Difference in POD between the paired comparison.
\(^f\)95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
\(^g\)Lot 0217 was produced 2/10/2017.
\(^h\)Lot 0417 was produced 4/28/17.
\(^i\)Lot 0517 was produced 5/5/2017.
Table 4. Robustness study: Impact of Varying Enrichment time, Phage Concentration, Luciferase Substrate Concentration on PhageDx™

Cronobacter Assay Results – POD Comparison

| Test Condition | Enrichment time | Volume phage | Volume substrate | N | POD<sup>T</sup> | 95% CI | POD<sup>N</sup> | 95% CI | dPOD<sup>TN</sup> | 95% CI |
|----------------|-----------------|--------------|-----------------|---|----------------|--------|----------------|--------|---------------------|--------|
| Milk based powdered infant formula – spiked with Cronobacter muytjensii (target) | | | | | | | | | | |
| 1              | 14 h            | 8 µL         | 45              | 10| 5              | 0.24, 0.76 | 5              | 0.24, 0.76 | 0.0 | -0.37, 0.37 |
| 2              | 14 h            | 8 µL         | 55              | 10| 5              | 0.24, 0.76 | 5              | 0.24, 0.76 | 0.0 | -0.37, 0.37 |
| 3              | 14 h            | 12 µL        | 45              | 10| 5              | 0.24, 0.76 | 5              | 0.24, 0.76 | 0.0 | -0.37, 0.37 |
| 4              | 14 h            | 12 µL        | 55              | 10| 5              | 0.24, 0.76 | 5              | 0.24, 0.76 | 0.0 | -0.37, 0.37 |
| 5              | 24 h            | 8 µL         | 45              | 10| 5              | 0.24, 0.76 | 5              | 0.24, 0.76 | 0.0 | -0.37, 0.37 |
| 6              | 24 h            | 8 µL         | 55              | 10| 5              | 0.24, 0.76 | 5              | 0.24, 0.76 | 0.0 | -0.37, 0.37 |
| 7              | 24 h            | 12 µL        | 45              | 10| 5              | 0.24, 0.76 | 5              | 0.24, 0.76 | 0.0 | -0.37, 0.37 |
| 8              | 24 h            | 12 µL        | 55              | 10| 5              | 0.24, 0.76 | 5              | 0.24, 0.76 | 0.0 | -0.37, 0.37 |
| Milk based powdered infant formula – unspiked (non-target) | | | | | | | | | | |
| 1              | 14 h            | 8 µL         | 45              | 10| 0              | 0.00, 0.28  | 0              | 0.00, 0.28 | 0.0 | -0.25, 0.25 |
| 2              | 14 h            | 8 µL         | 55              | 10| 0              | 0.00, 0.28  | 0              | 0.00, 0.28 | 0.0 | -0.25, 0.25 |
| 3              | 14 h            | 12 µL        | 45              | 10| 0              | 0.00, 0.28  | 0              | 0.00, 0.28 | 0.0 | -0.25, 0.25 |
| 4              | 14 h            | 12 µL        | 55              | 10| 0              | 0.00, 0.28  | 0              | 0.00, 0.28 | 0.0 | -0.25, 0.25 |
| 5              | 24 h            | 8 µL         | 45              | 10| 0              | 0.00, 0.28  | 0              | 0.00, 0.28 | 0.0 | -0.25, 0.25 |
| 6              | 24 h            | 8 µL         | 55              | 10| 0              | 0.00, 0.28  | 0              | 0.00, 0.28 | 0.0 | -0.25, 0.25 |
| 7              | 24 h            | 12 µL        | 45              | 10| 0              | 0.00, 0.28  | 0              | 0.00, 0.28 | 0.0 | -0.25, 0.25 |
| 8              | 24 h            | 12 µL        | 55              | 10| 0              | 0.00, 0.28  | 0              | 0.00, 0.28 | 0.0 | -0.25, 0.25 |
### Table 5. PhageDx™ Cronobacter Assay vs. ISO 22964 Method Comparison Results

| Matrix                            | Strain                      | MPN\(^a\)/test portion | N\(^c\) | PhageDx™ Cronobacter Result | ISO 22964 |
|----------------------------------|-----------------------------|-------------------------|--------|-----------------------------|----------|
| Powdered infant formula (10g)    | C. _muytjensii_ FSL-F6-031 | N/A\(^i\)               | 5      | 0                           | 0        |
| (milk based)                     |                             | 0.46 (0.26, 0.72)       | 20     | 0.30                        | 0.00     |
|                                  |                             | 1.74 (0.77, 4.03)       | 5      | 0.80                        | 0.00     |
| Powdered infant formula (10g)    | C. _malonaticus_ ES686      | N/A\(^i\)               | 5      | 0                           | 0        |
| (soy based)                      |                             | 0.78 (0.46, 1.27)       | 20     | 0.50                        | 0.00     |
|                                  |                             | 4.03 (2.14, 11.5)       | 5      | 1.00                        | 0.00     |
| Powdered infant formula (10g)    | C. _sakazakii_ ATCC 29544   | N/A\(^i\)               | 5      | 0                           | 0        |
| (milk based)                     |                             | 0.49 (0.25, 0.85)       | 20     | 0.35                        | 0.00     |
|                                  |                             | 1.61 (0.75, 3.44)       | 5      | 1.00                        | 0.00     |

\(^a\)Matrix study is paired.  
\(^b\)MPN = Most Probable Number is based on the POD of reference method test portions using the Least Cost Formulations MPN calculator, with 95% confidence interval.  
\(^c\)N = Number of test portions.  
\(^d\)x = Number of positive test portions.  
\(^e\)POD\(_{CP}\) = Candidate method presumptive positive outcomes confirmed positive.  
\(^f\)POD\(_{CC}\) = Reference method confirmed positive outcomes divided by the total number of trials.  
\(^g\)dPOD\(_{CP}\) = Difference between the candidate method and reference method POD values.  
\(^h\)95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.  
\(^i\)N/A = Not applicable.  
\(^j\)Matrix tested by the independent laboratory.
### Table 6. PhageDx™ Cronobacter Assay Presumptive vs. Confirmed (per BAM Ch. 29) Results – POD Result

| Matrix tested by the independent laboratory. |

| Matrix | Strain | MPN\(^a\)/test portion | N\(^b\) | X\(^c\) | POD\(^{d}\) \(95\%\ CI\) | X | POD\(^{e}\) \(95\%\ CI\) | dPOD\(^{f}\) | 95% CI \(^g\) |
|--------|--------|--------------------------|--------|--------|--------------------------|----|--------------------------|----------------|----------------|
| Powdered infant formula (100g) (milk based) | *C. muytjensii* FSL-F6-031 | 0.32 (0.13, 0.56) | 5 | 0 | 0.00 (0.00, 0.43) | 0 | 0.00 (0.00, 0.43) | 0.00 | -0.47, 0.47 |
| | | 3.04 (1.50, 6.17) | 5 | 4 | 0.80 (0.38, 1.00) | 4 | 0.80 (0.38, 1.00) | 0.00 | -0.47, 0.47 |
| Powdered infant formula (300g) (milk based) | *C. muytjensii* FSL-F6-031 | 0.32 (0.13, 0.56) | 20 | 11 | 0.55 (0.34, 0.74) | 11 | 0.55 (0.34, 0.74) | 0.00 | -0.13, 0.13 |
| | | 3.04 (1.50, 6.17) | 5 | 5 | 1.00 (0.57, 1.00) | 5 | 1.00 (0.57, 1.00) | 0.00 | -0.47, 0.47 |
| Powdered infant formula (100g) (soy based) | *C. malonaticus* ES686 | 0.70 (0.40, 1.34) | 20 | 9 | 0.45 (0.26, 0.66) | 9 | 0.45 (0.26, 0.66) | 0.00 | -0.13, 0.13 |
| | | 2.40 (1.19, 4.86) | 5 | 5 | 1.00 (0.57, 1.00) | 5 | 1.00 (0.57, 1.00) | 0.00 | -0.47, 0.47 |
| Powdered infant formula (300g) (soy based) | *C. malonaticus* ES686 | 0.70 (0.40, 1.34) | 20 | 9 | 0.45 (0.26, 0.66) | 9 | 0.45 (0.26, 0.66) | 0.00 | -0.13, 0.13 |
| | | 2.40 (1.19, 4.86) | 5 | 5 | 1.00 (0.57, 1.00) | 5 | 1.00 (0.57, 1.00) | 0.00 | -0.47, 0.47 |
| Powdered infant formula (100g) (milk based) | *C. sakazakii* ATCC 29544 | 1.05 (0.64, 1.71) | 5 | 0 | 0.00 (0.00, 0.43) | 0 | 0.00 (0.00, 0.43) | 0.00 | -0.47, 0.47 |
| | | 2.28 (1.11, 4.70) | 5 | 5 | 1.00 (0.57, 1.00) | 5 | 1.00 (0.57, 1.00) | 0.00 | -0.47, 0.47 |
| Powdered infant formula (300g) (milk based) | *C. sakazakii* ATCC 29544 | 1.05 (0.64, 1.71) | 20 | 14 | 0.70 (0.48, 0.85) | 14 | 0.70 (0.48, 0.85) | 0.00 | -0.13, 0.13 |
| | | 2.28 (1.11, 4.70) | 5 | 5 | 1.00 (0.57, 1.00) | 5 | 1.00 (0.57, 1.00) | 0.00 | -0.47, 0.47 |

\(^a\)MPN = Most Probable Number is based on the POD of reference method test portions using the Least Cost Formulations MPN calculator, with 95% confidence interval.

\(^b\)N = Number of test portions.

\(^c\)X = Number of positive test portions.

\(^d\)POD\(_{CP}\) = Candidate method presumptive positive outcomes divided by the total number of trials.

\(^e\)POD\(_{CC}\) = Candidate method confirmed positive (per BAM Ch. 29) outcomes divided by the total number of trials.

\(^f\)dPOD\(_{CP}\) = Difference between the candidate method presumptive result and candidate method confirmed result POD values.

\(^g\)95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

\(^h\)N/A = Not applicable.
Table 7. PhageDx™ Cronobacter Assay Presumptive vs. Confirmed (per PhageDx™ confirmation procedure) Results – POD Result

| Matrix                                | Strain                  | MPN\(^a\)/test portion | N\(^b\) | PhageDx™ Presumptive Result | PhageDx™ Confirmed Result | dPOD\(^c\)         | 95% CI         |
|---------------------------------------|-------------------------|-------------------------|--------|-----------------------------|---------------------------|---------------------|----------------|
| Powdered infant formula (100g)        | *C. muytjensii* FSL-F6-031 | N/A\(^h\) | 5 | 0.00, 0.43                  | 0 | 0.00, 0.43                  | 0 | -0.47, 0.47   |
| (milk based)                          |                         | 0.32 (0.13, 0.56)       | 20 | 0.60, 0.78                  | 12 | 0.39, 0.78                  | 0 | -0.13, 0.13   |
| Powd...                                |                         | 3.04 (1.50, 6.17)       | 5 | 0.80, 1.00                  | 4 | 0.38, 1.00                  | 0 | -0.47, 0.47   |
| Powdered infant formula (100g)        | *C. muytjensii* FSL-F6-031 | N/A\(^h\) | 5 | 0.00, 0.43                  | 0 | 0.00, 0.43                  | 0 | -0.47, 0.47   |
| (soy based)                           |                         | 0.32 (0.13, 0.56)       | 20 | 0.55, 0.74                  | 11 | 0.34, 0.74                  | 0 | -0.13, 0.13   |
| Powd...                                |                         | 3.04 (1.50, 6.17)       | 5 | 1.00, 1.00                  | 5 | 0.57, 1.00                  | 0 | -0.47, 0.47   |
| Powdered infant formula (300g)        | *C. malonaticus* ES686  | N/A\(^h\) | 5 | 0.00, 0.43                  | 0 | 0.00, 0.43                  | 0 | -0.47, 0.47   |
| (soy based)                           |                         | 0.70 (0.40, 1.34)       | 20 | 0.45, 0.66                  | 9 | 0.26, 0.66                  | 0 | -0.13, 0.13   |
| Powd...                                |                         | 2.40 (1.19, 4.86)       | 5 | 1.00, 1.00                  | 5 | 0.57, 1.00                  | 0 | -0.47, 0.47   |
| Powdered infant formula (100g)        | *C. malonaticus* ES686  | N/A\(^h\) | 5 | 0.00, 0.43                  | 0 | 0.00, 0.43                  | 0 | -0.47, 0.47   |
| (milk based)                          |                         | 0.70 (0.40, 1.34)       | 20 | 0.45, 0.66                  | 9 | 0.26, 0.66                  | 0 | -0.13, 0.13   |
| Powd...                                |                         | 2.40 (1.19, 4.86)       | 5 | 1.00, 1.00                  | 5 | 0.57, 1.00                  | 0 | -0.47, 0.47   |
| Powdered infant formula (300g)        | *C. sakazakii* ATCC 29544 | N/A\(^h\) | 5 | 0.00, 0.43                  | 0 | 0.00, 0.43                  | 0 | -0.47, 0.47   |
| (milk based)                          |                         | 1.05 (0.64, 1.71)       | 20 | 0.60, 0.78                  | 12 | 0.39, 0.78                  | 0 | -0.13, 0.13   |
| Powd...                                |                         | 2.28 (1.11, 4.70)       | 5 | 1.00, 1.00                  | 5 | 0.57, 1.00                  | 0 | -0.47, 0.47   |
| Powdered infant formula (300g)        | *C. sakazakii* ATCC 29544 | N/A\(^h\) | 5 | 0.00, 0.43                  | 0 | 0.00, 0.43                  | 0 | -0.47, 0.47   |
| (soy based)                           |                         | 1.05 (0.64, 1.71)       | 20 | 0.70, 0.85                  | 14 | 0.48, 0.85                  | 0 | -0.13, 0.13   |
| Powd...                                |                         | 2.28 (1.11, 4.70)       | 5 | 1.00, 1.00                  | 5 | 0.57, 1.00                  | 0 | -0.47, 0.47   |

\(^a\)MPN = Most Probable Number is based on the POD of reference method test portions using the Least Cost Formulations MPN calculator, with 95% confidence interval.

\(^b\)N = Number of test portions.

\(^c\)x = Number of positive test portions.

\(^d\)POD\(_CP\) = Candidate method presumptive positive outcomes divided by the total number of trials.

\(^e\)POD\(_CC\) = Candidate method confirmed positive (per PhageDx™ confirmation procedure) outcomes divided by the total number of trials.

\(^f\)dPOD\(_CP\) = Difference between the candidate method presumptive result and candidate method confirmed result POD values.

\(^g\)95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

\(^h\)N/A = Not applicable.

\(^i\)Matrix tested by the independent laboratory.
| Matrix                     | Strain                     | MPN²/test portion | X³ | POD⁴,⁵ | 95% CI      | POD⁶,⁷ | 95% CI      | dPOD⁸ | 95% CI      |
|---------------------------|----------------------------|-------------------|----|--------|-------------|--------|-------------|-------|-------------|
| Powdered infant formula (100g) (milk based) | *C. muytjensii* FSL-F6-031 | N/A① | 5   | 0      | 0.00, 0.43  | 0      | 0.00, 0.43  | 0     | -0.43, 0.43 |
|                           |                            | 0.32 (0.13, 0.56) | 20  | 12     | 0.60, 0.78  | 5      | 0.25, 0.47  | 0.35  | 0.04, 0.58④|
|                           |                            | 3.04 (1.50, 6.17) | 5   | 4      | 0.80, 1.00  | 5      | 1.00, 1.00  | -0.20 | -0.62, 0.28 |
| Powdered infant formula (300g) (milk based) | *C. muytjensii* FSL-F6-031 | N/A① | 5   | 0      | 0.00, 0.43  | 0      | 0.00, 0.43  | 0     | -0.43, 0.43 |
|                           |                            | 0.32 (0.13, 0.56) | 20  | 11     | 0.55, 0.74  | 5      | 0.25, 0.47  | 0.30  | 0.00, 0.54  |
|                           |                            | 3.04 (1.50, 6.17) | 5   | 5      | 1.00, 1.00  | 5      | 1.00, 1.00  | 0.00  | -0.43, 0.43 |
| Powdered infant formula (100g) (soy based) | *C. malonaticus* ES686 | N/A① | 5   | 0      | 0.00, 0.43  | 0      | 0.00, 0.43  | 0     | -0.43, 0.43 |
|                           |                            | 0.70 (0.40, 1.34) | 20  | 9      | 0.45, 0.66  | 11     | 0.55, 0.75  | -0.10 | -0.37, 0.19 |
|                           |                            | 2.40 (1.19, 4.86) | 5   | 5      | 1.00, 1.00  | 5      | 1.00, 1.00  | 0.00  | -0.43, 0.43 |
| Powdered infant formula (300g) (soy based) | *C. malonaticus* ES686 | N/A① | 5   | 0      | 0.00, 0.43  | 0      | 0.00, 0.43  | 0     | -0.43, 0.43 |
|                           |                            | 0.70 (0.40, 1.34) | 20  | 9      | 0.45, 0.66  | 11     | 0.55, 0.75  | -0.10 | -0.37, 0.19 |
|                           |                            | 2.40 (1.19, 4.86) | 5   | 5      | 1.00, 1.00  | 5      | 1.00, 1.00  | 0.00  | -0.43, 0.43 |
| Powdered infant formula (100g) (milk based) | *C. sakazakii* ATCC 29544 | N/A① | 5   | 0      | 0.00, 0.43  | 0      | 0.00, 0.43  | 0     | -0.43, 0.43 |
|                           |                            | 1.05 (0.64, 1.71) | 20  | 12     | 0.60, 0.78  | 12     | 0.55, 0.78  | 0.00  | -0.28, 0.28 |
|                           |                            | 2.28 (1.11, 4.70) | 5   | 5      | 1.00, 1.00  | 5      | 1.00, 1.00  | 0.00  | -0.43, 0.43 |
| Powdered infant formula (300g) (milk based) | *C. sakazakii* ATCC 29544 | N/A① | 5   | 0      | 0.00, 0.43  | 0      | 0.00, 0.43  | 0     | -0.43, 0.43 |
|                           |                            | 1.05 (0.64, 1.71) | 20  | 14     | 0.70, 0.85  | 12     | 0.60, 0.78  | 0.10  | -0.18, 0.36 |
|                           |                            | 2.28 (1.11, 4.70) | 5   | 5      | 1.00, 1.00  | 5      | 1.00, 1.00  | 0.00  | -0.43, 0.43 |

① Matrix test portion for the PhageDx™ *Cronobacter* method is listed. Portions were compared to BAM Ch. 29 100 g test portions.

② MPN = Most Probable Number is based on the POD of reference method test portions using the Least Cost Formulations MPN calculator, with 95% confidence interval.

③ N = Number of test portions.

④ X = Number of positive test portions.

⑤ PODc = Candidate method presumptive positive outcomes confirmed positive were identical using both confirmation procedures, hence one result is reported here.

⑥ PODr = Reference method confirmed positive outcomes divided by the total number of trials.

⑦ dPOD = Difference between the candidate method and reference method POD values.

⑧ 95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

⑨ N/A = Not applicable.

⑩ Matrix tested by the independent laboratory.

⑪ Difference in number of positive samples between methods in unpaired test contributed to statistical difference. No false positives or false negatives were observed.