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Application Note

Rapid Detection of Pathogens in Infant Food

Assurance® GDS detection of low-level *Cronobacter sakazakii* presence and *Salmonella* cross-contamination in infant formulas

Introduction

Over 90% of *Cronobacter sakazakii* infections in infants have been epidemiologically linked to powdered infant formulas[1]. This pathogen is responsible for severe meningitis in newborns and babies. Likewise, some reported *Salmonella* epidemics in infants have been related to *Salmonella*-contaminated infant formula. To avoid contaminated food products reaching the market, infant formula production has been stringently regulated[2].

The Assurance® GDS PickPen® technique improves pathogen detection by using immunomagnetic separation (IMS) to separate and concentrate the targeted pathogen in food and beverage samples, including infant formulas. By combining IMS with subsequent PCR detection using the GDS Rotor-Gene® thermocycler, Assurance® GDS significantly improves the assay's sensitivity and allows the detection of very low contaminant concentrations in a sample.

In this study, we evaluated the Assurance® GDS *Cronobacter* Tq II kit's capacity to detect very low CFU contamination levels of *Cronobacter sakazakii* ATCC®[3] 29004 in different infant formula food products. Moreover, infant formulas contaminated with a mix of *Cronobacter sakazakii* and *Salmonella enterica* subsp. *enterica* serovar Typhimurium NCTC 12023 (WDCM 00031) were analyzed with the Assurance® GDS *Cronobacter* Tq II and Assurance® GDS *Salmonella* Tq kits.

These analyses were performed according to the ISO 6579-1 and ISO 22964 standards[4].

The data presented in this application note relate only to the investigations conducted for this study. They are not part of the product validation for the Assurance® GDS *Cronobacter* Tq II and Assurance® GDS *Salmonella* Tq kits.

Materials

Equipment and consumables

Item	Cat. No.
DiluCult™ 1 gravimetric dilutor	**542760**
ESH sample homogenizer	**542765**
Assurance® GDS with RotorGene® and its software	**73070BC**
PickPen® II	**73091BC**
Assurance® GDS PickPen® II Tips	**73085BC**
Assurance® GDS adhesive film strips	**73026BC**

Media, fluids, and reagents

Item	Cat. No.
Trypcase Soy Broth in 10 mL tubes	**21410**
Buffered Peptone Water acc. ISO 6579, ISO 19250, ISO 21528, ISO 22964, ISO 6887, FDA-BAM and EP GranuCult®	**1072285000**
Assurance® GDS *Cronobacter* Tq II kit	**71012BC**
Assurance® GDS *Salmonella* Tq kit	**71008BC**

Matrices

Infant formulas satisfy infants' specific nutritional requirements during early development. Adapted infant formulas for specific needs are also on the market, for example lactose-free or probiotics-containing products. Therefore, this study investigated seven different infant formulas from different suppliers (**see tables 1 and 2**).

Microorganisms

Strain	ATCC or NCTC number	Cat. No.
Cronobacter sakazakii	ATCC® 29004	**In-house**
Salmonella enterica subsp. *enterica* serovar Typhimurium	NCTC 12023 (WDCM 00031)	**LENTICULE® CRM12023L-10EA**

Method

Inoculum preparation

A *Cronobacter sakazakii* liquid culture was prepared as follows: 1 mL of an in-house *Cronobacter sakazakii* cryo-suspension was resuspended in 10 mL trypcase soy broth (TSB). The culture was incubated for 18 hours at 35-37 °C.

For the *Salmonella* culture preparation, a lenticule disc was thawed in 10 mL of TSB, then incubated for 18 hours at 35-37 °C.

Serial dilutions and enumerations were performed until a concentration of <10 CFUs in 100 µL was reached.

Sample preparation

Two sample amounts were used for the investigations: 25 g (3 replicates) and 375 g (2 replicates) of infant formula. A non-inoculated, negative control was performed in parallel with 25 g of infant formula. For each replicate, 25 g or 375 g of infant formula sample was weighed with the DiluCult™ gravimetric dilutor in a stomacher bag, then diluted 1:10 in buffered peptone water and prewarmed to 35-37 °C, reaching a final volume of 225 mL or 3375 mL, respectively.

The bags containing the 25 g samples were then homogenized with the sample homogenizer and those containing 375 g samples were homogenized by hand.

Sample inoculation

Every sample bag, except for the negative controls, was inoculated with 100 µL of the appropriate dilution containing:

- <10 CFUs of *Cronobacter sakazakii*, or,
- <10 CFUs of *Cronobacter sakazakii* mixed with <10 CFUs *Salmonella enterica* subsp. *enterica* serovar Typhimurium

As pathogen concentration controls, five 90 mm trypcase soy agar plates were inoculated with 100 µL of the above dilutions.

Incubation temperature and time

The 25 g samples bags were incubated at 35-37 °C over 20 to 21 hours, the 375 g sample bags over 24 to 25 hours.

Pathogen detection by Assurance® GDS

300 µL of the enriched *Cronobacter sakazakii* sample and 1 mL of the *Salmonella* mixed assay were transferred to PCR plate wells containing the concentration reagent (magnetic beads). The Assurance® GDS *Cronobacter* Tq II and Assurance® GDS *Salmonella* Tq assays were performed according to the manufacturer's instructions for use.

Results

All non-inoculated samples (controls) were shown to be negative by the Assurance® GDS software. The enumeration controls on the 90 mm trypcase soy agar plates revealed average inoculation levels per sample of <5 CFUs for *Cronobacter sakazakii* and <8 CFUs for *Salmonella*.

Part 1 - Pure culture of *Cronobacter sakazakii* (<5 CFUs)

Standard infant formula and 5 other specialized formulas from different suppliers were inoculated with a pure culture of *Cronobacter sakazakii* <5 CFUs.

Sample	Negative control	*Cronobacter sakazakii* ATCC® 29004				
		25 g formula			375 g formula	
	25 g formula	R1	R2	R3	R1	R2
Standard formula	-	+	+	+	+	+
Formula with lecithin	-	+	+	+	+	+
Formula with starch	-	+	+	+	+	+
Formula with probiotics	-	+	+	+	+	+
Formula with galactooligosaccharides	-	+	+	+	+	+
Formula without lactose	-	+	+	+	+	+

Table 1: **Detection of Cronobacter sakazakii in 6 different infant formulas using the Assurance® GDS Cronobacter Tq II kit.** 3 replicates of the 25 g and 2 of the 375 g formula samples were performed and evaluated. (+) indicates that the targeted *Cronobacter sakazakii* strain was detected, (-) indicates no detection of the target strain, R=replicate.

The low-level presence of *Cronobacter sakazakii* in all the 25 g and 375 g samples of all 6 products was correctly detected after amplification with the Assurance® GDS *Cronobacter* Tq II kit. The supplements in the infant formulations, for example starch, did not impair the detection of low-level *Cronobacter sakazakii* contamination (see **table 1**).

Part 2 - Mixed culture of *Cronobacter sakazakii* (<5 CFUs) and *Salmonella* (<8 CFUs)

Standard and lactose-containing infant formula samples were inoculated with a mixed culture of *Cronobacter sakazakii* (<5 CFUs) and *Salmonella* (<8 CFUs).

Sample	Negative control	*Cronobacter sakazakii* ATCC® 29004					*Salmonella enterica* subsp. *enterica* serovar Typhimurium NCTC 12023				
		25 g formula			375 g formula		25 g formula			375 g formula	
	25 g formula	R1	R2	R3	R1	R2	R1	R2	R3	R1	R2
Standard formula	-	+	+	+	+	+	+	+	+	+	+
Formula with lactose	-	+	+	+	+	+	+	+	+	+	+

Table 2: Detection of Cronobacter sakazakii and Salmonella *enterica* subsp. *enterica* serovar Typhimurium in 2 different infant formulas using the Assurance® GDS Cronobacter Tq II and Assurance® GDS Salmonella Tq kits. 3 replicates of the 25 g and 2 of the 375 g formula samples were performed and evaluated. (+) indicates that the targeted strain was detected, (-) indicates no detection of the target strains, R=replicate.

The results show that Assurance® GDS correctly detected the *Cronobacter* sakazakii and *Salmonella enterica* subsp. *enterica* serovar Typhimurium contaminations in all the 25 g and 375 g samples of both tested products.

Conclusion

In this study we demonstrate the effectiveness of the Assurance® GDS *Cronobacter* Tq II and *Salmonella* Tq kits to detect the respective microorganisms in infant formulas. Both Assurance® GDS kits allowed successful detection of low contamination levels (<5 CFUs of *Cronobacter sakazakii*; <8 CFUs *Salmonella enterica* subsp. *enterica* serovar Typhimurium in 25 g or 375 g) in several infant formulas of different composition. Moreover, both organisms can be detected when simultaneously present in the food products.

Literature

1. Gautam Kalyantanda, Lyudmila Shumyak and Lennox Kenneth Archibald, *Cronobacter* Species Contamination of Powdered Infant Formula and the Implications for Neonatal Health, (2015), Frontiers in Pediatrics 3: 56.
2. Code of federal Regulations. Title 21: Food and Drugs. part 106—infant formula requirements pertaining to current good manufacturing practice, quality control procedures, quality factors, records and reports, and notifications
   Subpart B—Current Good Manufacturing Practice
3. ATCC®: American Type Culture Collection (ATCC) is a registered trademark of a nonprofit company.
4. ISO 6579-1 Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* - Part 1: Detection of *Salmonella* spp and ISO 22964 Microbiology of the food chain - Horizontal method for the detection of *Cronobacter* spp.

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