Validation of the Hygiena® foodproof® *Salmonella* plus *Cronobacter* Detection LyoKit Compared to ISO Reference Methods for Infant Cereals, Infant Formula With or Without Probiotics and Ingredients, and Production Environmental Samples

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BAX® System X5 | BAX® System Q7 | foodproof® | microproof®

INTRODUCTION:

Salmonella and *Cronobacter* species are gram-negative, rod-shaped, non-spore-forming bacteria belonging to the family of *Entero-bacteriaceae*. Infections of infants with these microorganisms are linked to the consumption of contaminated powdered infant formula. Because of their ability to adapt to dryness, *Salmonella* and *Cronobacter* species are able to survive in dry foods such as powdered infant formula, herbal teas and starches even throughout the desiccation or manufacturing process. Due to the robustness of these microbial species and the vulnerability of the target group, regulatory agencies require pathogenic screening for *Salmonella* spp. and *Cronobacter* spp. throughout the entire production process of powdered infant formula and in the final product.

To ensure product safety, Hygiena® offers the infant formula industry the foodproof® *Salmonella* plus *Cronobacter* Detection LyoKit, a real-time PCR test that detects both *Salmonella* and *Cronobacter* in a single enrichment and PCR reaction.

PURPOSE:

This study evaluated the foodproof S*almonella* plus Cronobacter Detection LyoKit combined with BAX® Prep Gram-Negative and foodproof StarPrep Three DNA extraction procedures in comparison to ISO 6579-1:2017 / Amd.1:2020 and ISO 22964:2017 standards according to the requirements of DIN EN ISO 16140-2:2016 and the AFNOR technical rules.

REGISTERED TRADEMARKS:

METHOD:

The method comparison part of this NF VALIDATION™ study according to ISO 16140-2 protocol including sensitivity, level of detection, and specificity studies was conducted by Adria Développement. For the sensitivity study, 141 (*Salmonella* target) and 137 (*Cronobacter* target) samples of probiotic and non-probiotic infant formula, ingredients (375 g) and production environmental samples (200 g and surfaces) were enriched in buffered peptone water (1:10 dilution; with 10 mg/L vancomycin for probiotic-containing samples) and incubated for 16 to 24 hours at 37 °C. Following incubation, DNA extraction was performed using the foodproof StarPrep Three Kit (single tube and 8-strip format) and the BAX Prep Gram-Negative Lysis Kit, followed by real-time PCR analysis. In addition, 60 samples per target were analyzed by the alternative and the reference methods to determine LOD_{50} and RLOD values. Specificity panels including 100 *Salmonella* and 50 *Cronobacter* strains and 30 non-target strains were evaluated to ensure inclusivity and exclusivity of PCR targets.

SENSITIVITY STUDY:

The sensitivity is the ability of the method to detect the analyte by either the reference or alternative method.

For the sensitivity study, 141 (*Salmonella* target) and 137 (*Cronobacter* target) uncontaminated and artificially contaminated samples of probiotic and non-probiotic infant formula, related ingredients (375 g) and production environmental samples (200 g and surfaces) were tested by the alternative and reference methods (ISO 6579-1:2017 / Amd.1:2020 and ISO 22964:2017). Artificial contamination of samples was carried out using a spiking or a seeding protocol after heat treatment and a maximum inoculation level of 5 CFU per test portion or 3 CFU per test portion depending on the protocol.

The observed values for the deviating results (ND-PD) and (ND+PD) are below the acceptability limits and were met for each individual category, for all combined categories, for all DNA extraction methods and real-time PCR cyclers, and for each target in the foodproof *Salmonella* plus *Cronobacter* Detection LyoKit.

Table 1a. *Salmonella* spp. - Overview of the Calculated Sensitivity Parameters

DNA extraction kit	real-time PCR cycler	PA	NA	PD	ND	FP	Se_{alt} (%)	Se_{ref} (%)	RT (%)	FPR (%)
foodproof StarPrep Three Kit (single tube or 8-strip)	BAX Q7	65	70	3	2	1	97.1	95.7	96.5	1.4
	LightCycler 480	65	71	3	2	0	97.1	95.7	96.5	0.0
BAX Prep Gram-Negative Lysis Kit	BAX Q7	65	70	3	2	1	97.1	95.7	96.5	1.4
	LightCycler 480	65	70	3	1	2	97.1	95.7	96.5	2.8

Table 1b. *Cronobacter* spp. - Overview of the Calculated Sensitivity Parameters

DNA extraction kit	real-time PCR cycler	PA	NA	PD	ND	FP	Se_{alt} (%)	Se_{ref} (%)	RT (%)	FPR (%)
foodproof StarPrep Three Kit (single tube or 8-strip)	BAX Q7	56	62	12	1	6	97.1	82.9	89.8	9.0
	LightCycler 480	54	62	12	3	6	94.3	82.9	88.3	9.0
BAX Prep Gram-Negative Lysis Kit	BAX Q7	55	64	12	2	4	95.7	82.9	89.1	6.0
	LightCycler 480	54	66	12	3	2	94.3	82.9	88.3	3.0

PA: number of positive results obtained with both the alternative and the reference method; **NA:** number of negative results obtained with both the alternative and the reference method; **ND:** number of obtained results that are negative with the alternative method and positive with the reference method; **PD:** number of obtained results that are positive with the alternative method and negative with the reference method; **FP:** number of false positives; **Se_{alt}:** sensitivity for the alternative method; **Se_{ref}:** sensitivity for the reference method; **RT:** relative trueness; **FPR:** false positive rate.

LEVEL OF DETECTION & RELATIVE LEVEL OF DETECTION STUDY:

The level of detection (LOD_{50}) is the smallest number of culturable microorganisms that can be detected 50% of the time in a given sample. The relative level of detection (RLOD) is defined as the ratio of the LOD of the alternative method and the LOD of the reference method.

For each of the two categories, one sample type per parameter was tested with three different levels of contamination. One set of samples included 5 uninoculated samples (0 CFU per test portion), 20 low level inoculated samples (0.1-1.2 CFU per test portion) to obtain fractional positive results, and 5 high level inoculated samples (1.1-4.5 CFU per test portion). For inoculation, a different target strain was used for each sample type.

The LOD_{50} for *Salmonella* detection and category 1 was calculated and is 0.5 CFU per test portion for both the reference and the alternative method. For *Cronobacter* detection the LOD_{50} varies from 0.5 to 0.9 CFU per test portion for the reference method and from 0.8 to 1.8 CFU per test portion for the alternative method. The RLOD values (using the confirmed alternative method results) for both targets of the foodproof *Salmonella* plus *Cronobacter* Detection LyoKit meet the acceptability limits, which are 1.5 for paired and 2.5 for unpaired studies, for all matrix/strain pairs, DNA extraction methods and real-time PCR instruments tested.

Table 2a. *Salmonella* spp. - LOD_{50} after Confirmation of the Alternative Method Results

Category	(Matrix / Strain) Pair	Level of Detection at 50% (CFU / test portion)				
		Reference Method ISO 6579-1	Alternative Method			
			StarPrep Three 8-Strip		BAX Prep Gram-Negative	
			BAX Q7	LC480	BAX Q7	LC480
Infant formula with or without probiotics and related ingredients	Maltodextrin / *S.* Cerro Ad2152	0.5 [0.3;0.9]	0.5 [0.3;0.9]	0.5 [0.3;0.9]	0.5 [0.3;0.9]	0.5 [0.3;0.9]
COMBINED		**0.5 [0.3;0.9]**	**0.5 [0.3;0.9]**	**0.5 [0.3;0.9]**	**0.5 [0.3;0.9]**	**0.5 [0.3;0.9]**

Table 2b. *Salmonella* spp. - RLOD after Confirmation of the Alternative Method Results

Category	(Matrix / Strain) Pair	Relative Level of Detection			
		Alternative Method			
		StarPrep Three 8-Strip		BAX Prep Gram-Negative	
		BAX Q7	LC480	BAX Q7	LC480
Infant formula with or without probiotics and related ingredients	Maltodextrin / *S.* Cerro Ad2152	1,000	1,000	1,000	1,000
Production environmental samples	Stainless steel surface / *S.* Anatum Ad2718 + *Citrobacter freundii* 39	1,000	1,000	1,000	1,000
COMBINED		**1,000**	**1,000**	**1,000**	**1,000**

Table 2c. *Cronobacter* spp. - LOD_{50} and RLOD after Confirmation of the Alternative Method Results

Category	(Matrix / Strain) Pair	Level of Detection at 50% (CFU / test portion)					Relative Level of Detection				
		Reference Method ISO 22964	Alternative Method				Alternative Method				
			StarPrep Three 8-Strip		BAX Prep Gram-Negative		StarPrep Three 8-Strip		BAX Prep Gram-Negative		
			BAX Q7	LC480	BAX Q7	LC480	BAX Q7	LC480	BAX Q7	LC480	
Infant formula with or without probiotics and ingredients	Infant formula with probiotics (375 g) / *C. sakazakii* Ad2413	0.5 [0.3 - 1.0]	0.8 [0.5 – 1.5]	0.8 [0.5 – 1.5]	0.8 [0.5 – 1.5]	0.8 [0.5 – 1.5]	1,355	1,355	1,355	1,355	
Production environmental samples	Dusts (200 g) / *C. mutjensii* E888	0.9 [0.5 – 1.8]	1.8 [0.9 – 3.5]	1.5 [0.8 – 3.0]	1.5 [0.8 – 3.0]	1.3 [0.7 – 2.6]	1,943	1,609	1,609	1,357	
COMBINED		**0.8 [0.5 – 1.1]**	**1.2 [0.7 – 1.8]**	**1.1 (0.7 – 1.7]**	**1.1 (0.7 – 1.7]**	**1.0 [0.7 – 1.6]**	**1,560**	**1,458**	**1,458**	**1,366**	

INCLUSIVITY & EXCLUSIVITY STUDY:

50 *Cronobacter* and 100 *Salmonella* target strains and 30 non-target strains per target were used for specificity testing. Target strains were freshly cultured overnight in BHI medium at 37 °C and inoculated into BPW supplemented with vancomycin (10 mg/L) at a level to achieve 10-100 CFU/225 mL. Following incubation for 16 h at 37 °C, the alternative method including confirmatory tests were performed. All non-target strains were grown overnight in BHI medium at 37 °C. Dilutions were done to achieve a concentration of 10^5 CFU/mL BPW broth. Following inoculation, exclusivity samples were incubated for 24 h at 37 °C prior to analysis using the alternative method.

The alternative protocol was applied using the foodproof StarPrep Three Kit (single tube or 8-strip format) and the BAX Prep Gram-Negative Lysis Kit for DNA extraction before performing PCR analysis on the BAX System Q7 and the LightCycler® 480 II instrument from Roche.

Data from the specificity study show that the alternative foodproof *Salmonella* plus *Cronobacter* Detection method is selective and specific for *Salmonella* spp. and *Cronobacter* spp.

SIGNIFICANCE:

This Hygiena foodproof method offers infant formula industries a rapid, reliable and easy-to-use PCR-based technology for the simultaneously detection of *Salmonella* spp. and *Cronobacter* spp. from one enrichment culture. By combining the detection of these two pathogens in only one real-time PCR reaction, testing time and costs are significantly reduced. Providing different opportunities for DNA extraction gives the end user a high degree of flexibility in selecting a DNA extraction method that meets their individual requirements.