Automated DNA Isolation Combined with Real-Time PCR Enables an Easy-to-Handle Method for Simultaneous Detection of *Salmonella* and *Cronobacter* on Surfaces

Anne Rölfing, Alexandra Jahn, Rumeysa Göcen, Stefanie Wendrich, Patrice Chablain
Hygiena® Diagnostics GmbH, Hermannswerder 17, 14473 Potsdam, Germany

BAX® System Q7 | foodproof® | microproof®

INTRODUCTION:

Salmonella and *Cronobacter* species are gram-negative, rod-shaped, non-spore-forming bacteria belonging to the family of *Enterobacteriaceae*. *Salmonella* and *Cronobacter* species are responsible for a high lethality rate caused by the consumption of contaminated infant, follow-on or medical purpose formulae. These microorganisms can survive in dry foods and in the production environment because of their ability to adapt to dryness.
Due to this serious infection risk, it is essential to examine the environment of production sites with highly sensitive detection systems.

The foodproof® *Salmonella* plus *Cronobacter* Detection LyoKit has been designed for the robust, reliable and accurate detection and identification of *Salmonella* and *Cronobacter*, in one single real-time PCR test, confirmed by a AFNOR certification (QUA 18/12-12/24 and QUA 18/13-12/24) for manual extraction.

To better handle a large volume of samples, an automated extraction system, the foodproof® Magnetic Preparation Kit I, was examined for analyzing environmental samples, with a significantly reduced hands-on time.

PURPOSE:

This study analyzed the applicability of an automated DNA isolation system, the foodproof® Magnetic Preparation Kit I, in combination with the real-time PCR System, the foodproof® *Salmonella* plus *Cronobacter* LyoKit, for surface examinations. For results evaluation, the following methods were compared by running a paired study: the reference methods - the cultural method ISO 6579-1 (2017 - 07) “Microbiology of the food chain - Horizontal method for the detection, enumeration and serotype of *Salmonella* - Part I: Detection of *Salmonella* spp.”, and the culture method, ISO 22964 (2017 - 08) “Microbiology of the food chain - Horizontal method for the detection of *Cronobacter* spp.”.

REGISTERED TRADEMARKS/ GLOBAL CERTIFICATIONS:

DNA Extraction: foodproof® Magnetic Preparation Kit I (KIT230180)
PCR: foodproof® *Salmonella* plus *Cronobacter* Detection LyoKit (KIT230131)

METHOD:

Plastic surfaces were spiked in 10 replicates with low-level concentrations of *Cronobacter* (dry-stressed) or *Salmonella* (heat- and dry-stressed strains) with and without an excess of the respective other organism. For heat-stressed strains, the inoculation suspension was adjusted to 9 – 11 CFU per surface for fractional spiking. For dry-stressed strains, the inoculation concentration was approximately 1 CFU per surface. The inoculum in a volume of 100 µL was spiked directly onto the plastic surface in a square area of 10 cm by 10 cm (100 cm²). The heat-stressed strains were allowed to dry at room temperature under the sterile workbench for 16 - 24 hours (according to AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces, 2012). Under the same conditions, the drying time for dry-stressed strains was 15 min. The degree of injury from heat or dry stress was determined to be between 50% and 80 %.

To test the influence of high bacterial background on crosstalk between the detection channels, the surfaces were spiked in parallel with an excess of the respective other organism.
If *Salmonella* was spiked at a low level, *Cronobacter* was spiked two potencies higher and vice versa.

To simulate the environment of the production site, each surface was dusted with probiotic milk powder at the end of drying time. The surface was swabbed by using Letheen broth hydrated Sponge Sticks. After swabbing, the Sponge Sticks were stored in 10 ml Letheen Broth for 2 hrs at room temperature. Subsequently, the samples were enriched in 100 mL pre-warmed BPW for 16 hours at 36 °C. Following incubation, DNA extraction was conducted with the foodproof Magnetic Preparation Kit I on the KingFisher™ Flex and analyzed with the PCR Kit, foodproof® *Salmonella* plus *Cronobacter* LyoKit, on the LightCycler® 480. An amplification control and UNG treatment are included to avoid false-positive results.

For evaluation of the results, the study was conducted as a paired study following the ISO 6579-1:2017 and 22964:2017 methods. Colony confirmation was conducted using MALDI-TOF-MS.

Table 1: Inoculation Scheme for All Tests

Matrix	Strain 1	Inoculation	Strain 2	Inoculation
Sponge-Stick	*Salmonella* Abaetetuba	9 - 11 CFU / surface	/	/
	Salmonella Montevideo	9 - 11 CFU / surface	/	/
	Salmonella Nottingham	1 CFU / surface	/	/
	Cronobacter sakazakii	1 CFU / surface	/	/
	Salmonella Nottingham	1 - 5 CFU / surface	*Cronobacter sakazakii*	100 - 500 CFU / surface
	Cronobacter sakazakii	1 - 5 CFU / surface	*Salmonella* Nottingham	100 - 500 CFU / surface

RESULTS:

LOW-LEVEL SPIKING: For each strain, ten plastic surfaces (100 cm²) were inoculated either with 11.4 CFU *S.* Abaetetuba or 9.2 CFU *S.* Montevideo per surface. The inoculation level for S. Nottingham was 0.9 CFU per surface. For *S.* Abaetetuba, 6 of 10 replicates were positive for *Salmonella* (Table 2, Figure 1). Fractional spiking of *S.* Montevideo showed 3 positive samples. Low-level spiking of *S.* Nottingham resulted in 8 of 10 positives. In every test, the 10 replicates showed negative results in the *Cronobacter* detection channel. All positive and negative results of *S.* Abaetetuba, *S.* Montevideo and *S.* Nottingham were confirmed by the ISO 6579-1:2017 method.
For the dry-stressed strain, *C. sakazakii*, the inoculation was 1.0 CFU per surface. With this low-level spiking of *C. sakazakii*, 5 of 10 replicate positives could be generated (Table 3, Figure 2). The *Salmonella* detection channel was negative for all 10 replicates. All positive and negative results from the alternative method were confirmed by the reference method, ISO 22964:2017. Due to the fractional spiking and the 100% accordance with the reference results, it can be concluded that the reference and the alternative method have the same sensitivity.

Table 2: Results of *Salmonella* Abaetetuba Detection, Exemplary for *Salmonella* Detection, in Environmental Swab Samples with Alternative vs. Reference Methods

Salmonella Abaetetuba		foodproof® Magnetic Preparation Kit I (KIT230180) / foodproof® *Salmonella* plus *Cronobacter* Detection LyoKit (KIT230131)		ISO 6579-1 *Salmonella* Detection
Heat stress: 51.0 %		200 µL for Prep / 25 µL for PCR		RVS / MKTTn
Inoculation	Pos. Results PCR & ISO	Mean Cp Value	Mean Cp Value	
CFU/Sample	Pos/Rep	*Salmonella* Detection	*Cronobacter* Detection	XLD / Br. *Salm.* Agar
11.4	6/10	21.90	0.00	pos
		27.25	0.00	pos
		21.53	0.00	pos
		0.00	0.00	neg
		24.14	0.00	pos
		0.00	0.00	neg
		0.00	0.00	neg
		0.00	0.00	neg
		21.64	0.00	pos
		25.14	0.00	pos
0	0/1	0.00	0.00	neg

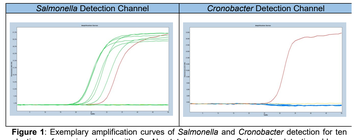

Figure 1: Exemplary amplification curves of *Salmonella* and *Cronobacter* detection for ten plastic surfaces inoculated with *S.* Abaetetuba: green – *Salmonella* detection, blue – *Cronobacter* detection, red – Positive Control of PCR, yellow – Negative Control of PCR

Table 3: Results of *Cronobacter sakazakii* Detection in Environmental Swab Samples with the Alternative vs. Reference Methods

Cronobacter sakazakii		foodproof® Magnetic Preparation Kit I (KIT230180) / foodproof® *Salmonella* plus *Cronobacter* Detection LyoKit (KIT230131)		ISO 22964 *Cronobacter* Detection
Dry stress: 60.0 %		200 µL for Prep / 25 µL for PCR		CSB
Inoculation	Pos. Results PCR & ISO	Mean Cp Value	Mean Cp Value	
CFU/Sample	Pos/Rep	*Salmonella* Detection	*Cronobacter* Detection	CCI
1.0	5/10	0.00	0.00	neg
		0.00	0.00	neg
		0.00	30.02	pos
		0.00	0.00	neg
		0.00	25.07	pos
		0.00	23.20	pos
		0.00	23.01	pos
		0.00	0.00	neg
		0.00	25.01	pos
		0.00	0.00	neg
0	0/1	0.00	0.00	neg

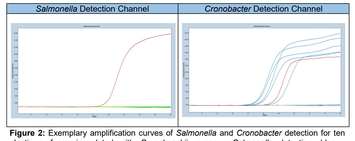

Figure 2: Exemplary amplification curves of *Salmonella* and *Cronobacter* detection for ten plastic surfaces inoculated with *C.* sakazakii: green - *Salmonella* detection, blue - *Cronobacter* detection, red - PC of PCR, yellow – Negative Control of PCR

Table 6: Inoculation Level per Sample and Positive Detection per Replicate for All Tests

Test Strain		Inoculation Level / Surface	Positive Detection / Replicate
S. Abaetetuba		11.4 CFU	6/10
S. Montevideo		9.2 CFU	3/10
S. Nottingham		0.9 CFU	8/10
C. sakazakii		1.0 CFU	5/10
Excess Testing	Low-level spiking: *S.* Nottingham	2.3 CFU	9/10
	High-level spiking: *C. sakazakii*	3.6 x 10⁴ CFU	10/10
	Low-level spiking: *C. sakazakii*	2.7 CFU	8/10
	High-level spiking: *S.* Nottingham	4.0 x 10² CFU	10/10

The total bacterial count of the uninoculated Sponge-Stick samples was determined before incubation. The mean value of total bacteria counts before spiking is 5.7 x 10² CFU/mL. This high excess of microbiological background concentration caused by the probiotic milk powder from the vacuum cleaner does not lead to a reduction in sensitivity or inhibit the assay.

EXCESS TESTING: To examine the influence of background flora, a 100 times higher concentration of background organism was spiked onto the surface. The additional low-level spiked (2.3 CFU/surface) *S.* Nottingham was detected in 9 of 10 replicates. The excess of *Cronobacter,* with 3.6 x 10² CFU per surface, generated Cp values under 20 in all 10 replicates (Table 4, Figure 3). The fractional spiked *C. sakazakii* (2.7 CFU/surface) resulted in 8 positives of 10 replicates (Table 5, Figure 4). The excess of *S.* Nottingham with 4.0 x 10² CFU per sample is clearly recognizable in 10 of 10 positive results. All results were confirmed by microbiological reference method ISO 6579 and ISO 22964. This demonstrates that neither background flora nor an excess of target analyte leads to false-positive or false-negative results.

Table 4: Results of Excess testing (low-level spiking *Salmonella* Nottingham with high-level spiking *Cronobacter sakazakii*) in environmental swab samples with alternative compared to reference method

Excess testing			foodproof® Magnetic Preparation Kit I (KIT230180) / foodproof® *Salmonella* plus *Cronobacter* Detection LyoKit (KIT230131)		ISO 6579-1 *Salmonella* Detection
			200 µl for Prep / 25 µl for PCR		RVS / MKTTn
Inoculation	Pos. Results PCR & ISO	Pos. Results PCR	Mean Cp Value	Mean Cp Value	
CFU/Sample	*Salmonella* Pos/Rep	*Cronobacter* Pos/Rep	*Salmonella* Detection	*Cronobacter* Detection	XLD / Br. *Salm.* Agar
Salmonella Nottingham: 2.3 *Cronobacter sakazakii*: 3.6 x 10²	9/10	10/10	23.10	20.59	pos
			22.31	19.42	pos
			22.32	19.12	pos
			23.18	19.08	pos
			22.42	19.05	pos
			22.37	18.73	pos
			22.81	19.64	pos
			0.00	19.87	neg
			23.06	20.14	pos
			23.43	20.72	pos
0	0/1	0/1	0.00	0.00	neg

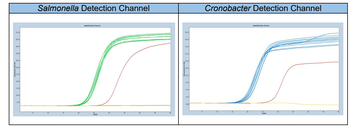

Figure 3: Exemplary amplification curves of *Salmonella* and *Cronobacter* detection for ten plastic surfaces inoculated with *S.* Nottingham (low-level spiking) and *C. sakazakii* (high-level spiking): green – *Salmonella* detection, blue – *Cronobacter* detection, red - Positive Control of PCR, yellow – Negative Control of PCR

Table 5: Results of Excess testing (low-level spiking *Cronobacter sakazakii* with high-level spiking *Salmonella* Nottingham) in environmental swab samples with alternative compared to reference method

Excess testing			foodproof® Magnetic Preparation Kit I (KIT230180) / foodproof® *Salmonella* plus *Cronobacter* Detection LyoKit (KIT230131)		ISO 22964 *Cronobacter* Detection
			200 µL for Prep / 25 µL for PCR		RVS / MKTTn
Inoculation	Pos. Results PCR	Pos. Results PCR & ISO	Mean Cp Value	Mean Cp Value	
CFU/Sample	*Salmonella* Pos/Rep	*Cronobacter* Pos/Rep	*Salmonella* Detection	*Cronobacter* Detection	XLD / Br. *Salm.* Agar
Cronobacter sakazakii: 2.7 *Salmonella* Nottingham: 4.0 x 10²	10/10	8/10	22.15	23.95	pos
			21.80	29.14	pos
			22.19	0.00	neg
			21.60	0.00	neg
			21.68	23.27	pos
			21.57	25.59	pos
			21.44	25.24	pos
			21.74	26.30	pos
			21.83	27.08	pos
			20.92	26.17	pos
0	0/1	0/1	0.00	0.00	neg

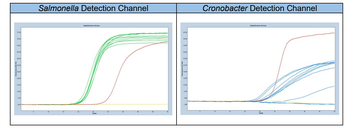

Figure 4: Exemplary amplification curves of *Salmonella* and *Cronobacter* detection for ten plastic surfaces inoculated with *C. sakazakii* (low-level spiking) and *S.* Nottingham (high-level spiking): green – *Salmonella* detection, blue – *Cronobacter* detection, red – Positive Control of PCR, yellow – Negative Control of PCR

SUMMARY:

Low concentrations of *Salmonella* and *Cronobacter* on surfaces were successfully analyzed after a 16 h incubation at 36 °C using an automated high-throughput PCR-based method. The results are in 100% accordance with the ISO reference methods. Neither crosstalk between the detection channels due to an excess of one target organism nor high probiotic background flora influenced the correct detection of the pathogenic organisms.

SIGNIFICANCE:

The foodproof Magnetic Preparation Kit I, in combination with the foodproof real-time PCR System for simultaneous detection of *Salmonella* and *Cronobacter,* is a rapid method for surface testing with the same sensitivity as the reference methods, with significantly less hands-on time and thus, enabling a large number of samples to be processed in parallel.