Detection of 1 – 5 CFUs of *Salmonella* in 750 g Confectionary Samples After 18 Hours with Hygiena® Real-Time PCR Assays and Different DNA Isolation Options

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

INTRODUCTION:

In 2021, 60,050 confirmed cases of human salmonellosis were registered in the EU. This is an increase of more than 14% compared to 2020. Also, more sample units of milk, egg and meat products tested positive for *Salmonella* in 2021 than 2020. A fast and easy diagnostic tool for the detection of *Salmonella* in food is essential to minimize the impact on humans and the food market due to *Salmonella* outbreaks.

In order to react more quickly to salmonellosis outbreaks than ISO 6579 examination allows, it is common to use alternative PCR methods and pool samples in even larger batches. Therefore, it must be guaranteed that the presence and absence of *Salmonella* can also be analyzed in larger sample sizes at any time. And in case of material shortages, the possibility of replacing components with alternative products is essential.

For this reason, Hygiena® examined an enrichment medium, 3.5% milk, that can replace the ISO-recommended enrichment medium. In addition, Hygiena tested different DNA extraction methods as substitute options and solutions for individual requirements for automated and manual systems. Therefore, samples up to 750 g from matrices of the chocolate bar production process were tested with an enrichment in 3.5% or 0.1% milk and/or Buffered Peptone Water with an incubation time of 16 h at 37 ± 1 °C. In this unpaired study, three different DNA extraction methods for automated and manual use were examined in combination with the **food**proof® *Salmonella* Detection LyoKit (KIT230099, Hygiena Diagnostics GmbH). For the comparison of all results, the ISO 6579-1:2017/AMD 1:2020 method was conducted in parallel.

The successful *Salmonella* testing shows the possibility of replacing the standard enrichment medium with 3.5% milk and the option to find an optimized DNA extraction method for individual requirements to test samples up to 750 g of confectionary matrices with the necessary sensitivity.

Purpose:

The objective of this study was to detect 1 – 5 CFUs of *Salmonella* by challenging 750 g or 250 g samples of matrices from the production process of chocolate bars within 18 – 19 hours total, with an enrichment time of 16 hours, DNA extraction and real-time PCR analysis included. The qualitative results of the alternative method should be comparable to the reference method ISO 6579-1:2017/AMD 1:2020, regardless of enrichment media, extraction method or *Salmonella* strains.

Alternative Method – Kits:

1. **food**proof® Magnetic Preparation Kit I (KIT230180, Hygiena Diagnostics GmbH)
2. **food**proof® StarPrep® One Kit (KIT230175, Hygiena Diagnostics GmbH)
3. BAX® Lysis (ASY2011, Hygiena)
4. **food**proof® *Salmonella* Detection LyoKit (KIT230099, Hygiena Diagnostics GmbH)

METHOD:

For detection of *Salmonella* spp. in eight different matrices from the production process of chocolate bars, three heat- (*S.* Enteritidis and *S.* Montevideo) and dry-stressed (*S.* Nottingham for milk powder) strains were inoculated with a concentration of 1 – 5 CFU/test portion. The bacterial counts as well as the degree of damage were determined using the plate counting method on TSA and XLD agar.

For the enrichment, 750 g or 250 g (wheat flour) samples were diluted 1:10 in 0.1% fat-skimmed milk or 3.5% milk and for swabs, additionally in Buffered Peptone Water (BPW). Following the inoculation and enrichment media dilution, the samples were incubated for 16 hours at 37 ± 1 °C before testing using the alternative method and the reference method. Three different DNA extraction methods for automated (**food**proof Magnetic Preparation Kit I) and manual applications (BAX Lysis Buffer, **food**proof StarPrep One Kit) were conducted according to the respective manual with the procedures summarized in Table 1. The real-time PCR System **food**proof *Salmonella* Detection LyoKit (KIT230099) was used for detection. PCR was performed on LightCycler® 480 instrument version II (Roche® Diagnostics). Each matrix was examined with each procedure in five replicates.

For confirmation and comparison of the methods, all matrices were analyzed according to the ISO 6579-1, 2017-02.

Table 1. Enrichment/DNA Extraction/PCR Protocol Steps Evaluated in the Study

Protocol Steps		Automated Process	Manual Process BAX®	Manual Process foodproof®
Enrichment	Medium	0.1% skimmed fat milk	0.1% skimmed fat milk	0.1% skimmed fat milk
		3.5% milk	3.5% milk	3.5% milk
		BPW (only swabs)	BPW (only swabs)	BPW (only swabs)
	Dilution	1:10	1:10	1:10
	Time	16 hrs	16 hrs	16 hrs
	Temperature	37 ± 1 °C	37 ± 1 °C	37 ± 1 °C
Extraction	Kit	foodproof Magnetic Preparation Kit I	BAX Lysis Buffer	foodproof StarPrep One Kit
	Sample Volume Extraction	200 µL	5 µL	100 µL
	Protocol	Semi-automated DNA extraction with KingFisher™ Flex	According to manual	Standard
PCR	Kit	foodproof *Salmonella* Detection LyoKit	foodproof *Salmonella* Detection LyoKit	foodproof *Salmonella* Detection LyoKit
	Sample Volume PCR	25 µL (5 µL for cocoa powder)	25 µL (5 µL for cocoa powder)	25 µL (5 µL for cocoa powder)
Confirmation	Method	Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of *Salmonella* — Part 1: Detection of *Salmonella* spp.		
	ISO 6579 - 1:2017			

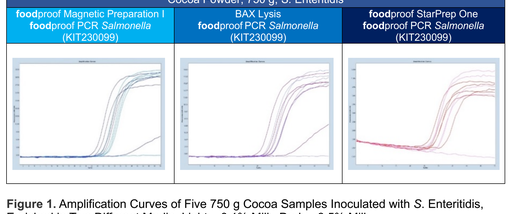

Figure 1. Amplification Curves of Five 750 g Cocoa Samples Inoculated with *S.* Enteritidis, Enriched in Two Different Media: Light – 0.1% Milk, Dark – 3.5% Milk

METHOD COMPARISON STUDY WITH 1 – 5 CFU/TEST PORTION:

For each of the eight matrices of the production process of chocolate bars, all five replicates with inoculation concentration of 1 – 5 CFU *Salmonella* Enteritidis per test portion generate the same results as the ISO method 6579-1:2017.

For the strains *S.* Montevideo and *S.* Nottingham, 99.2% of the samples agreed with the results of the ISO method, except for two negative deviations from 254 correctly analyzed samples: one DNA extraction conducted with the **food**proof Magnetic Preparation Kit and one with the BAX Lysis.

In total, 508 of 510 tested samples agreed with the results from the ISO 6579-1:2017/AMD 1:2020 method. To be able to substitute the enrichment medium in case of 0.1% milk or BPW material shortage, the results demonstrate that the higher fat content milk (3.5%) has no negative influence on the detection of *Salmonella*: With 3.5% milk, no deviation in comparison to the ISO method could be observed, regardless of the extraction method.

Overall, the **food**proof *Salmonella* Detection LyoKit can stably analyze contamination of 1 – 5 CFU of *Salmonella* in eight matrices from the production process of chocolate bars, regardless of strains, matrices, enrichment medium and DNA extraction methods, even with a sample size of 750 g.

SIGNIFICANCE:

These studies were able to confirm that the Hygiena alternative method for detecting *Salmonella* in 16 hours enrichment cultures of 0.1% and 3.5% milk does not result in a loss of sensitivity, even with a sample size of 750 g chocolate bar production matrices. The agreement of 99.5% between the results of the alternative method in comparison to the reference method ISO 6579-1:2017/AMD 1:2020, regardless of the DNA extraction methods and the enrichment medium, showed the stable correctness of results from the **food**proof *Salmonella* Detection LyoKit. This gives every user the opportunity to substitute the common enrichment medium with 3.5% milk and the flexibility to choose a DNA extraction method that meets individual requirements.

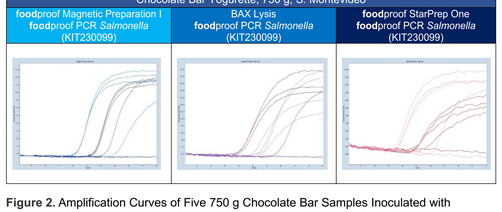

Figure 2. Amplification Curves of Five 750 g Chocolate Bar Samples Inoculated with *S.* Montevideo, Enriched in Two Different Media: Light – 0.1% Milk, Dark – 3.5% Milk

Table 2. Method Comparison – ISO 6579 versus Alternative Method with Different Options for Extraction: Spiking *S.* Enteritidis

Spiking of *Salmonella* Enteritidis 1-5 CFU/Sample Size				Enrichment											
				0.1% Milk				3.5% Milk				Buffered Peptone Water			
Manual Process foodproof StarPrep One Kit and foodproof *Salmonella* Detection LyoKit															
Matrix	Inoculation Level	Sample Size	Replicate	PA	NA	PD	ND	PA	NA	PD	ND	PA	NA	PD	ND
Wheat Flour	4.9 CFU	250 g	5	5	0	0	0	5	0	0	0				
Milk Powder	3.1 CFU	750 g	5	5	0	0	0	5	0	0	0				
Cocoa Powder	4.8 CFU	750 g	5	4	1	0	0	5	0	0	0				
Dark Chocolate	4.8 CFU	750 g	5	3	2	0	0	2	3	0	0				
Milk Chocolate	2.8 CFU	750 g	5	4	1	0	0	3	2	0	0				
kinderSchokolade®	4.0 CFU	750 g	5	4	1	0	0	5	0	0	0				
Yogurette	4.9 CFU	750 g	5	2	3	0	0	2	3	0	0				
Swabs (RomerLabs® 10004589)	4.7 CFU	1 swab	5	5	0	0	0	5	0	0	0	5	0	0	0
Manual Process BAX Lysis (KIT2006) and foodproof *Salmonella* Detection LyoKit															
Matrix	Inoculation Level	Sample Size	Replicate	PA	NA	PD	ND	PA	NA	PD	ND	PA	NA	PD	ND
Wheat Flour	4.9 CFU	250 g	5	5	0	0	0	5	0	0	0				
Milk Powder	3.1 CFU	750 g	5	5	0	0	0	5	0	0	0				
Cocoa Powder	4.8 CFU	750 g	5	4	1	0	0	5	0	0	0				
Dark Chocolate	4.8 CFU	750 g	5	3	2	0	0	2	3	0	0				
Milk Chocolate	2.8 CFU	750 g	5	4	1	0	0	3	2	0	0				
kinderSchokolade®	4.0 CFU	750 g	5	4	1	0	0	5	0	0	0				
Yogurette	4.9 CFU	750 g	5	2	3	0	0	2	3	0	0				
Swabs (RomerLabs® 10004589)	4.7 CFU	1 swab	5	5	0	0	0	5	0	0	0	5	0	0	0
Manual Process foodproof StarPrep One Kit and foodproof *Salmonella* Detection LyoKit															
Matrix	Inoculation Level	Sample Size	Replicate	PA	NA	PD	ND	PA	NA	PD	ND	PA	NA	PD	ND
Wheat Flour	4.9 CFU	250 g	5	5	0	0	0	5	0	0	0				
Milk Powder	3.1 CFU	750 g	5	5	0	0	0	5	0	0	0				
Cocoa Powder	4.8 CFU	750 g	5	4	1	0	0	5	0	0	0				
Dark Chocolate	4.8 CFU	750 g	5	3	2	0	0	2	3	0	0				
Milk Chocolate	2.8 CFU	750 g	5	4	1	0	0	3	2	0	0				
kinderSchokolade®	4.0 CFU	750 g	5	4	1	0	0	5	0	0	0				
Yogurette	4.9 CFU	750 g	5	2	3	0	0	2	3	0	0				
Swabs (RomerLabs® 10004589)	4.7 CFU	1 swab	5	5	0	0	0	5	0	0	0	5	0	0	0

Table 3. Method Comparison – ISO 6579 versus Alternative Method with Different Options for Extraction: Spiking *S.* Montevideo/*S.* Nottingham

Spiking of *Salmonella* Montevideo (Milk Powder: *Salmonella* Nottingham) 1-5 CFU/Sample Size				Enrichment											
				0.1% Milk				3.5% Milk				Buffered Peptone Water			
Automated Process foodproof Magnetic Preparation Kit I and foodproof *Salmonella* Detection LyoKit															
Matrix	Inoculation Level	Sample Size	Replicate	PA	NA	PD	ND	PA	NA	PD	ND	PA	NA	PD	ND
Wheat Flour	4.9 CFU	250 g	5	5	0	0	0	5	0	0	0				
Milk Powder	3.1 CFU	750 g	5	3	1	0	1	5	0	0	0				
Cocoa Powder	3.6 CFU	750 g	5	4	1	0	0	4	1	0	0				
Dark Chocolate	4.3 CFU	750 g	5	5	0	0	0	5	0	0	0				
Milk Chocolate	4.2 CFU	750 g	5	5	0	0	0	4	1	0	0				
kinderSchokolade®	1.6 CFU	750 g	5	3	2	0	0	2	3	0	0				
Yogurette	3.7 CFU	750 g	5	4	1	0	0	4	1	0	0				
Swabs (RomerLabs® 10004589)	4.9 CFU	1 swab	5	5	0	0	0	5	0	0	0	5	0	0	0
Manual Process BAX Lysis (KIT2006) and foodproof *Salmonella* Detection LyoKit															
Matrix	Inoculation Level	Sample Size	Replicate	PA	NA	PD	ND	PA	NA	PD	ND	PA	NA	PD	ND
Wheat Flour	4.9 CFU	250 g	5	5	0	0	0	5	0	0	0				
Milk Powder	3.1 CFU	750 g	5	3	1	0	1	5	0	0	0				
Cocoa Powder	3.6 CFU	750 g	5	3	1	0	1	4	1	0	0				
Dark Chocolate	4.3 CFU	750 g	5	5	0	0	0	5	0	0	0				
Milk Chocolate	4.2 CFU	750 g	5	5	0	0	0	4	1	0	0				
kinderSchokolade®	1.6 CFU	750 g	5	3	2	0	0	2	3	0	0				
Yogurette	3.7 CFU	750 g	5	4	1	0	0	4	1	0	0				
Swabs (RomerLabs® 10004589)	4.9 CFU	1 swab	5	5	0	0	0	5	0	0	0	5	0	0	0
Manual Process foodproof StarPrep One Kit and foodproof *Salmonella* Detection LyoKit															
Matrix	Inoculation Level	Sample Size	Replicate	PA	NA	PD	ND	PA	NA	PD	ND	PA	NA	PD	ND
Wheat Flour	4.9 CFU	250 g	5	5	0	0	0	5	0	0	0				
Milk Powder	3.1 CFU	750 g	5	4	1	0	0	5	0	0	0				
Cocoa Powder	3.6 CFU	750 g	5	4	1	0	0	4	1	0	0				
Dark Chocolate	4.3 CFU	750 g	5	5*	0	0	0	5	0	0	0				
Milk Chocolate	4.2 CFU	750 g	5	5	0	0	0	4	1	0	0				
kinderSchokolade®	1.6 CFU	750 g	5	3	2	0	0	2	3	0	0				
Yogurette	3.7 CFU	750 g	5	4	1	0	0	4	1	0	0				
Swabs (RomerLabs® 10004589)	4.9 CFU	1 swab	5	5	0	0	0	5	0	0	0	5	0	0	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; *with one sample a differential centrifugation step (2 min, 300 g) was conducted, after PCR inhibition occurs (internal amplification control channel was negative)