ISO Validation for Pathogen Detection in Food and Environmental Samples Utilizing Hygiena's foodproof® *Listeria* plus *Listeria monocytogenes* Multiplex PCR Assay

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One Health Diagnostics™

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INTRODUCTION:

Consuming food contaminated with *Listeria monocytogenes* can cause Listeriosis, a serious infection with a high mortality rate. *L. monocytogenes* is often found in samples that also contain other *Listeria* species. Therefore, the presence of members of the genus *Listeria* is often used as an indicator for conditions that allow the presence and growth of *L. monocytogenes*. Many ready-to-eat products and food production environments are monitored utilizing *Listeria* spp. as an indicator.

Most of the lately described species, however, differ substantially from the species known before 2009, making them unsuitable as indicator organisms for the pathogenic *L. monocytogenes*. Based on phenotypic and genomic characteristics, *L. monocytogenes* belongs to the *Listeria* "sensu stricto" group of species, including *L. innocua, L. ivanovii, L. marthii, L. seeligeri* and *L. welshimeri* in addition to *L. monocytogenes.*

The **food**proof® *Listeria* plus *L. monocytogenes* Detection LyoKit® detects all six *Listeria* "sensu stricto" species in one detection channel. *L. monocytogenes* is additionally identified in a second detection channel. The reaction mix is pre-filled and lyophilized. An internal amplification control prevents false negative results due to inhibition. The LyoKit has been NordVal validated according to ISO 16140-2.

PURPOSE:

This study evaluated the **food**proof *Listeria* plus *L. monocytogenes* Detection LyoKit for detection and discrimination between *Listeria monocytogenes* and food relevant *Listeria* species in comparison to ISO 11290-1:2017 reference method according to the requirements of DIN EN ISO 16140 2:2016 and the NordVal International validation protocol.

METHOD:

Sensitivity, level of detection (LOD), and specificity studies were conducted by Adria Développement for the method comparison part of this NordVal International validation study. For sensitivity and LOD studies, naturally, as well as artificially contaminated samples of five food categories and environmental samples were processed according to three protocols: samples were incubated in Half-Fraser broth (dilution 1:10) for 24 h and 46 h at 30 °C and in Actero™ *Listeria* Enrichment Broth (dilution 1:7) for 20 h at 36 °C. After incubation, DNA extraction was performed with the **food**proof StarPrep Two Kit in single tube or 8-strip format followed by real-time PCR analysis. Specificity testing comprised per target 50 strains for inclusivity and 30 strains for exclusivity.

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PROTOCOLS:

Table 1. Enrichment / DNA Extraction Protocols Evaluated in the Validation Study

		Protocol A	Protocol B	Protocol C
Enrichment step		Actero Listeria Enrichment Media 22 h ± 2 h 36 °C ± 1 °C (dilution 1:7)	Half Fraser 25 h ± 1 h 30 °C ± 1 °C (dilution 1:10)	Half Fraser 48 h ± 2 h 30 °C ± 1 °C (dilution 1:10)
Extraction	Kit	**food**proof StarPrep Two 8-Strip Kit	**food**proof StarPrep Two Kit	**food**proof StarPrep Two Kit
	Protocol	Procedure A STANDARD	Procedure A STANDARD	Procedure B RAPID
	Enrichment volume	800 µl	800 µl	200 µl
	PCR	5 µl	5 µl	5 µl
Confirmation		Streaking 10 µl onto O&A and Palcam plates. The presence of only typical colonies allows confirmation of the positive PCR result.		

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 the ratio of the LOD of the alternative method and the LOD of the reference method.

For each of the six categories, at least one sample type was tested with three different levels of contamination. One set of samples included 5 uninoculated samples (0 CFU/test portion), 20 low level inoculated samples (0.2-2 CFU/test portion) to obtain fractional positive results, and 5 high level inoculated samples (2-5 CFU/test portion). For inoculation, a different strain of *Listeria* was used for each sample type.

The LOD_{50} varied from 0.3 to 1.1 CFU/test portion for the reference method, from 0.4 to 1.0 CFU/ test portion for the alternative method (Protocol A) and from 0.3 to 1.1 CFU / test portion (Protocols B and C). The RLOD values (using the confirmed alternative method results) for both targets of the **food**proof *Listeria* plus *L. monocytogenes* Detection LyoKit meet the acceptability limit of 2.5 for unpaired studies (Protocol A) and 1.5 for paired studies (Protocols B and C) for all matrix/strain pairs tested.

Table 2. LOD_{50} and RLOD after Confirmation of the Alternative Method Results

Category	(Matrix / strain) pair	Level of Detection at 50% (CFU/sample size)			Relative Level of Detection (RLOD)		
		Reference method	Alternative method		Alternative method		
			Protocol A	Protocol B	Protocol C	Protocol A	Protocol B	Protocol C
Composite foods / Ready-to-eat & ready-to-reheat	Deli salad / *L. monocytogenes* Ad494	0.7 [0.4 - 1.3]	0.9 [0.5 – 1.6]	0.7 [0.4 – 1.3]	0.7 [0.4 – 1.3]	1.313	1.000	1.000
Meat products	Rillettes / *L. monocytogenes* Ad669	1.0 [0.6 - 1.7]	1.0 [0.6 – 1.7]	1.0 [0.6 – 1.7]	1.0 [0.6 – 1.7]	1.000	1.000	1.000
Milk & dairy products	Raw milk / *L. monocytogenes* Ad618	1.1 [0.6 - 2.0]	0.8 [0.4 – 1.4]	1.1 [0.6 – 2.0]	1.1 [0.6 – 2.0]	0.711	1.000	1.000
	Ricotta / *L. ivanovii* Ad1737	0.7 [0.4 - 1.2]	0.5 [0.3 – 0.9]	0.7 [0.4 – 1.2]	0.7 [0.4 – 1.2]	0.721	1.000	1.000
Vegetables	Cantaloupe / *L. monocytogenes* Ad532	1.0 [0.6 - 1.6]	1.0 [0.6 – 1.7]	1.0 [0.6 – 1.6]	1.0 [0.6 – 1.6]	1.037	1.000	1.000
Seafood & fishery products	Smoked salmon / *L. monocytogenes* Ad670	0.6 [0.3 - 1.1]	0.4 [0.3 – 0.8]	0.6 [0.3 – 1.1]	0.6 [0.3 – 1.1]	0.678	1.000	1.000
	Frozen shrimps / *L. innocua* Ad1200	0.5 [0.3 - 1.9]	0.5 [0.3 – 0.9]	0.5 [0.3 – 1.9]	0.5 [0.3 – 1.9]	1.179	1.000	1.000
Environmental samples	Process water / *L. monocytogenes* Ad551	0.3 [0.2 - 1.6]	0.6 [0.3 – 1.1]	0.3 [0.2 – 1.6]	0.3 [0.2 – 1.6]	2.055	1.000	1.000
	COMBINED	**0.7 [0.6 – 0.9]**	**0.7 [0.6 – 0.9]**	**0.7 [0.6 - 0.9]**	**0.7 [0.6 – 0.9]**	**1.004**	**1.000**	**1.000**

SENSITIVITY STUDY:

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

Five food categories and environmental samples were included in this study. A total number of 404 naturally and artificially contaminated samples were analyzed by both the alternative and reference method (ISO 11290-1:2017). 54-60% were naturally contaminated depending on the target (*Listeria* spp., *L. monocytogenes*) and the protocol tested. Artificial contamination of samples was performed using a seeding protocol and an average inoculation level of maximum 4.8 CFU/test portion.

The observed values for the deviating results (ND-PD) and (ND+PD) are below the acceptability limits and were therefore met for each individual category and for all the combined categories for all three protocols and for both targets in the **food**proof *Listeria* plus *L. monocytogenes* Detection LyoKit.

Table 3. *L. monocytogenes* - Overview of the Calculated Sensitivity Parameters per Category and Protocol

Category	PA	NA	PD	ND	FP	Total	SE alt (%)	SE ref (%)	RT (%)	FPR (%)
Protocol A										
Composite foods / Ready-to-eat & ready-to-reheat	23	32	9	7	0	71	82.1	76.9	77.5	0.0
Meat products	23	28	6	9	1	67	76.3	84.2	77.6	3.4
Milk & dairy products	30	29	3	0	0	62	100.0	90.9	95.2	0.0
Vegetables	28	42	3	5	2	80	83.8	91.9	88.8	4.7
Seafood & fishery products	21	25	7	9	0	62	75.7	81.1	74.2	0.0
Environmental samples	31	24	5	2	0	62	94.7	86.8	88.7	0.0
ALL CATEGORIES	**156**	**180**	**33**	**32**	**3**	**404**	**85.1**	**85.1**	**83.7**	**1.6**
Protocol B										
Composite foods / Ready-to-eat & ready-to-reheat	30	40	0	0	1	71	100.0	100.0	100.0	2.4
Meat products	31	33	1	1	1	67	97.0	97.0	97.0	2.9
Milk & dairy products	30	31	0	0	1	62	100.0	100.0	100.0	3.1
Vegetables	34	46	0	0	0	80	100.0	100.0	100.0	0.0
Seafood & fishery products	30	31	0	0	1	62	100.0	100.0	100.0	3.1
Environmental samples	33	29	0	0	0	62	100.0	100.0	100.0	0.0
ALL CATEGORIES	**188**	**210**	**1**	**1**	**4**	**404**	**99.5**	**99.5**	**99.5**	**1.9**
Protocol C										
Composite foods / Ready-to-eat & ready-to-reheat	30	38	0	0	3	71	100.0	100.0	100.0	7.3
Meat products	32	33	2	0	0	67	100.0	94.1	97.0	0.0
Milk & dairy products	29	28	1	0	4	62	96.8	96.8	96.8	12.9
Vegetables	34	45	0	0	1	80	100.0	100.0	100.0	2.2
Seafood & fishery products	30	29	2	0	1	62	100.0	93.8	96.8	3.3
Environmental samples	33	27	0	0	2	62	100.0	100.0	100.0	6.9
ALL CATEGORIES	**188**	**200**	**5**	**0**	**11**	**404**	**99.5**	**97.4**	**98.5**	**5.2**

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; **RT:** relative trueness; **SE alt:** sensitivity for the alternative method; **SE ref:** sensitivity for the reference method; **FPR:** false positive rate.

INCLUSIVITY & EXCLUSIVITY STUDY:

For *Listeria* spp. testing, 50 strains belonging to the *Listeria* Genus including 20 *Listeria monocytogenes* and 30-non target strains were tested. For *Listeria monocytogenes*, 50 target strains and 30 non-target strains including 10 *Listeria* spp. strains not belonging to *Listeria monocytogenes* were analyzed. Strains used for inclusivity tests were grown overnight in BHI medium at 37 °C. The most challenging protocol A with the shortest incubation time was tested. Dilutions were done in order to inoculate 10-100 cells/150 mL Actero *Listeria* enrichment media. Following inoculation and incubation for 20 h at 36 ± 1 °C, the alternative protocol was applied using the **food**proof StarPrep Two 8-Strip Kit for DNA extraction before performing the PCR with the CFX96 real-time PCR system from BIO-RAD and the confirmatory tests. Strains used for exclusivity tests were grown overnight in BHI at 37 °C and inoculated into BPW to achieve a concentration of 10^5 CFU/mL. Following inoculation, the samples were incubated for 24 h at 37 ± 1 °C before testing using the alternative method.

Data from the inclusivity and exclusivity study show that the alternative **food**proof *Listeria* plus *L. monocytogenes* Detection LyoKit method is selective and specific for *L. monocytogenes* and *Listeria* spp.

SIGNIFICANCE:

These data demonstrate that the evaluated multiplex real-time PCR assay is a rapid and reliable alternative method for the detection and discrimination of *Listeria* spp. and *L. monocytogenes*. Based on these results and a collaborative study, the assay received a NordVal validation certificate (NordVal No. 054). The validation of the **food**proof *Listeria* multiplex assay provides many industries with the ability to quickly screen for *Listeria* and then immediately identify if corrective actions need to occur for *Listeria monocytogenes* in a single test. This improves operational efficiencies throughout food production and testing laboratories.