NMKL - NordVal International
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NordVal International Certificate

Issued for:	**foodproof® *Listeria* plus *L. monocytogenes* Detection LyoKit - 5'Nuclease - for *Listeria* spp. and *Listeria monocytogenes* detection in food products and production environmental samples**
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First approval date:	02 November 2021
Renewal date:	23 October 2023
Valid until:	02 November 2025

foodproof® *Listeria* plus *L. monocytogenes* Detection LyoKit - 5'Nuclease

Manufactured and supplied by:

Hygiena Diagnostics GmbH
Hermannswerder 17
D-14473 Potsdam
Germany

fulfills the requirements of the NordVal validation protocol / ISO 16140-2. The reference method was EN ISO 11290-1:2017: Microbiology of the food chain - Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 1: Detection method.

NordVal International has reviewed the method and the validation study conducted by ADRIA Développement, France. The results of the validations document that the alternative method performs equivalently to the reference method for the detection of *Listeria* spp. and *Listeria monocytogenes* in food products and production environmental samples. NordVal International has concluded that it has been satisfactorily demonstrated that the method performance characteristic requirements have been fulfilled.

The production of the **food**proof® *Listeria plus L. monocytogenes* Detection LyoKit - 5'Nuclease - fulfills the requirements outlined by ISO 9001.

Date: 23. October 2023

Yours sincerely,

Hrólfur Sigurðsson

Hrólfur Sigurðsson
Chair of NordVal International

Eystein Oveland
NMKL Executive Director

PRINCIPLE OF THE METHOD

The method is based on an enrichment step, DNA extraction and real-time PCR detection using PCR instruments with a FAM, a VIC/Yakima Yellow or HEX and a ROX or Texas Red detection channel.

The following three enrichment / DNA extraction protocols were evaluated during the validation study. There is a declaration of equivalence between the **food**proof® StarPrep Two Kit and the **food**proof® StarPrep Two 8-Strip Kit:

		Protocol A	**Protocol B**	**Protocol C**
Enrichment step		Actero *Listeria* Enrichment Media (ALEM) 22 h ± 2 h 36°C ± 1°C (dilution 1:7)	Half Fraser (ISO) 25 h ± 1 h 30°C ± 1°C (dilution 1:10)	Half Fraser (ISO) 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 The presence of only typical colonies allows confirmation of the positive PCR result.		

Kits for DNA extraction:
- **food**proof® StarPrep Two Kit (order No. S 400 08.1)
- **food**proof® StarPrep Two 8-Strip Kit (order No. S 400 17 L)

Kit for PCR:
- **food**proof® *Listeria* plus *L. monocytogenes* Detection LyoKit (order No. R 602 51-1 or R 602 51-2)

The detection kit provides all reagents required for PCR.

FIELD OF APPLICATION

The **food**proof® *Listeria* plus *L. monocytogenes* LyoKit - 5' Nuclease – is applicable for detection of *Listeria* spp. and *Listeria monocytogenes* in food products and production environmental samples.

HISTORY

In 2023, the name of manufacturer and supplier was changed to Hygiena Diagnostics GmbH due to a rebranding.

METHOD PERFORMANCE CHARACTERISTICS

Selectivity studies

For *Listeria* spp., 50 strains belonging to *Listeria* genus including 20 *Listeria monocytogenes* and 30-non target strains were tested. All *Listeria* strains were detected by PCR and all the non-target strains gave negative PCR results.

For *Listeria monocytogenes,* 50 *Listeria monocytogenes* strains gave positive PCR results. 30 non-target strains, including 10 *Listeria* genus strains not belonging to *Listeria monocytogenes* were all found negative.

For inclusivity studies, the most challenging protocol was tested (Protocol A, shortest incubation time). For all tested target and non-target strains, the **food**proof® StarPrep Two 8-Strip Kit (procedure A STANDARD) was used for DNA extraction.

Sensitivity studies

The sensitivity is the ability of the method to detect the analyte.

A total of 404 samples were analysed using the three protocols. 60% of the samples tested were naturally contaminated. 108 samples were artificially contaminated using 44 different strains of *L. monocytogenes*, yielding fractional positive recovery. The results for the categories analysed for *Listeria* spp. and *Listeria monocytogenes*, respectively, using Protocol A, B and C are provided in **Table 1** and **Table 2**.

Table 1. Results of the sensitivity study for Listeria spp. using Protocols A, B and C

Matrices	PA	NA	PD	ND	FP	Sum	RT (%)	SE_{alt} (%)	SE_{ref} (%)	FPR(%)
Protocol A										
composite foods/ready to eat and ready to reheat	28	24	8	11	0	71	73.2	76.6	83.0	0
meat products	33	20	5	7	2	67	80.6	82.6	89.1	9.5
milk and dairy products	30	29	3	0	0	62	95.2	100.0	90.9	0
vegetables	37	31	3	4	5	80	90.0	88.9	93.3	14.30
seafood and fishery products	23	22	9	8	0	62	72.6	80.0	77.5	0
environmental samples	32	23	5	2	0	62	88.7	94.9	87.2	00
TOTAL	**183**	**149**	**33**	**32**	**7**	**404**	**83.4**	**86.4**	**86.8**	**4.5**
Protocol B										
composite foods/ready to eat and ready to reheat	39	31	0	0	1	71	100.0	100.0	100.0	3.1
meat products	41	25	1	0	0	67	98.5	100.0	97.6	0
milk and dairy products	30	31	0	0	1	62	100.0	100.0	100.0	3.1
vegetables	42	35	2	0	1	80	97.5	100.0	95.5	2.8
seafood and fishery products	31	29	0	0	2	62	100.0	100.0	100.0	6.5
environmental samples	34	28	0	0	0	62	100.0	100.0	100.0	0
TOTAL	**217**	**179**	**3**	**0**	**5**	**404**	**99.3**	**100.0**	**98.6**	**2.7**

Matrices	PA	NA	PD	ND	FP	Sum	RT (%)	SE_{alt} (%)	SE_{ref} (%)	FPR(%)
Protocol C										
composite foods/ready to eat and ready to reheat	39	30	0	0	2	71	100.0	100.0	100.0	6.3
meat products	40	24	2	1	0	67	95.5	97.7	95.3	0
milk and dairy products	29	28	1	0	4	62	96.8	96.8	96.8	12.9
vegetables	42	35	2	0	1	80	97.5	100.0	95.5	2.8
seafood and fishery products	31	27	3	0	1	62	95.2	100.0	91.2	3.6
environmental samples	34	27	0	0	1	62	100.0	100.0	100.0	3.6
TOTAL	**215**	**171**	**8**	**1**	**9**	**404**	**97.5**	**99.1**	**96.4**	**5.0**

PA = number of obtained results that are positive with both the alternative and the reference method

NA = number of obtained results that are negative 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 (possible false negative)

PD = number of obtained results that are positive with the alternative method and negative with the reference method (possible false positive)

FP = false positive result

RT = the relative trueness; the degree of correspondence between the response obtained by the alternative method and the reference method

SE = the sensitivity; the ability of the method to detect the analyte
(SEalt = sensitivity of the alternative method, SE_{ref} = sensitivity of the reference method)

FPR = False positive rate

Table 2. The results of the sensitivity study for Listeria monocytogenes using Protocols A, B and C

Matrices	PA	NA	PD	ND	FP	Sum	RT (%)	SE_{alt} (%)	SE_{ref} (%)	FPR(%)
Protocol A										
composite foods/ready to eat and ready to reheat	23	32	9	7	0	71	77.5	82.1	76.9	0
meat products	23	28	6	9	1	67	77.6	76.3	84.2	3.4
milk and dairy products	30	29	3	0	0	62	95.2	100.0	90.9	0
vegetables	28	42	3	5	2	80	88.8	83.8	91.9	4.7
seafood and fishery products	21	25	7	9	0	62	74.2	75.7	81.1	0
environmental samples	31	24	5	2	0	62	88.7	94.7	86.8	0
TOTAL	**156**	**180**	**33**	**32**	**3**	**404**	**83.7**	**85.1**	**85.1**	**1.6**
Protocol B										
composite	30	40	0	0	1	71	100.0	100.0	100.0	2.4

Matrices	PA	NA	PD	ND	FP	Sum	RT (%)	SE_{alt} (%)	SE_{ref} (%)	FPR(%)
foods/ready to eat and ready to reheat										
meat products	31	33	1	1	1	67	97.0	97.0	97.0	2.9
milk and 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
seafood and 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
TOTAL	**188**	**210**	**1**	**1**	**4**	**404**	**99.5**	**99.5**	**99.5**	**1.9**
Protocol C										
composite foods/ready to eat and 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	97.0	100.0	94.1	0
milk and 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 and fishery products	30	29	2	0	1	62	96.8	100.0	93.8	3.3
environmental samples	33	27	0	0	2	62	100.0	100.0	100.0	6.9
TOTAL	**188**	**200**	**5**	**0**	**11**	**404**	**98.5**	**99.5**	**97.4**	**5.2**

See abbreviation of PA, NA etc. under Table 1.

The observed values for the deviating results (ND-PD) and (ND+PD) are below the acceptability limits and were hence met for each individual category and for all the combined categories for all three protocols.

Level of Detection (LOD) and Relative Level of Detection (RLOD)

The level of detection (LOD_{50}) is the smallest number of culturable microorganisms that can be detected in the sample in 50% of occasions.
The relative level of detection is the ratio of the LOD of the alternative method and the LOD of the reference method.
Eight (matrix/strains) pairs were analysed by the alternative method for *Listeria* spp. detection and six (matrix/strain) pairs for *L. monocytogenes* by using the three protocols for the alternative method and the reference method.

The LOD_{50} of the alternative method varied from 0.4 to 1.0 CFU/test portion (Protocol A) and from 0.3 to 1.1 CFU/test portion (Protocols B and C) and the LOD_{50} of the reference method varied from 0.3 to 1.1 CFU/test portion.

The RLOD is close to 1.0 and is acceptable according to the criteria (acceptance level of 1.5 for the paired studies (Protocols B and C) and 2.5 for the unpaired study (Protocol A), respectively).

The LOD_{50} calculations per Category for Protocols A, B and C are given in **Tables 3 A**, **B** and **C**, respectively.

Table 3A. LOD_{50} results using Protocol A

Category	(Strain / matrix) pair	Level of detection at 50% (CFU / sample size) according to Wilrich & Wilrich[1]	
		Reference method	Alternative method
1	Deli salad / *L. monocytogenes* Ad494	0.7 [0.4 - 1.3]	0.9 [0.5 – 1.6]
2	Rillettes / *L. monocytogenes* Ad669	1.0 [0.6 - 1.7]	1.0 [0.6 – 1.7]
3	Raw milk / *L. monocytogenes* Ad618	1.1 [0.6 - 2.0]	0.8 [0.4 – 1.4]
	Ricotta / *L. ivanovii* Ad1737	0.7 [0.4 - 1.2]	0.5 [0.3 – 0.9]
4	Cantaloupe / *L. monocytogenes* Ad532	1.0 [0.6 - 1.6]	1.0 [0.6 – 1.7]
5	Smoked salmon / *L. monocytogenes* Ad670	0.6 [0.3 - 1.1]	0.4 [0.3 – 0.8]
	Frozen shrimps / *L. innocua* Ad1200	0.5 [0.3 - 1.9]	0.5 [0.3 – 0.9]
6	Process water / *L. monocytogenes* Ad551	0.3 [0.2 - 1.6]	0.6 [0.3 – 1.1]
Combined		**0.7 [0.6 – 0.9]**	**0.7 [0.6 – 0.9]**

[1] Wilrich C, Wilrich PT. Estimation of the POD function and the LOD of a qualitative microbiological measurement method. J AOAC Int. 2009; 92 (6): 1763-72.

Table 3B. LOD_{50} results using Protocol B

Category	(Strain / matrix) pair	Level of detection at 50% (CFU / sample size) according to Wilrich & Wilrich	
		Reference method	Alternative method
1	Deli salad / *L. monocytogenes* Ad494	0.7 [0.4 - 1.3]	0.7 [0.4 – 1.3]
2	Rillettes / *L. monocytogenes* Ad669	1.0 [0.6 - 1.7]	1.0 [0.6 – 1.7]
3	Raw milk / *L. monocytogenes* Ad618	1.1 [0.6 - 2.0]	1.1 [0.6 – 2.0]
	Ricotta / *L. ivanovii* Ad1737	0.7 [0.4 - 1.2]	0.7 [0.4 – 1.2]
4	Cantaloupe / *L. monocytogenes* Ad532	1.0 [0.6 - 1.6]	1.0 [0.6 – 1.6]
5	Smoked salmon / *L. monocytogenes* Ad670	0.6 [0.3 - 1.1]	0.6 [0.3 – 1.1]
	Frozen shrimps / *L. innocua* Ad1200	0.5 [0.3 - 1.9]	0.5 [0.3 – 1.9]
6	Process water / *L. monocytogenes* Ad551	0.3 [0.2 - 1.6]	0.3 [0.2 – 1.6]
Combined		**0.7 [0.6 – 0.9]**	**0.7 [0.6 - 0.9]**

Table 3C. LOD_{50} results using Protocol C

Category	(Strain / matrix) pair	Level of detection at 50% (CFU / sample size) according to Wilrich & Wilrich	
		Reference method	Alternative method
1	Deli salad / *L. monocytogenes* Ad494	0.7 [0.4 - 1.3]	0.7 [0.4 – 1.3]
2	Rillettes / *L. monocytogenes* Ad669	1.0 [0.6 - 1.7]	1.0 [0.6 – 1.7]
3	Raw milk / *L. monocytogenes* Ad618	1.1 [0.6 - 2.0]	1.1 [0.6 – 2.0]
	Ricotta / *L. ivanovii* Ad1737	0.7 [0.4 - 1.2]	0.7 [0.4 – 1.2]
4	Cantaloupe / *L. monocytogenes* Ad532	1.0 [0.6 - 1.6]	1.0 [0.6 – 1.6]
5	Smoked salmon / *L. monocytogenes* Ad670	0.6 [0.3 - 1.1]	0.6 [0.3 – 1.1]
	Frozen shrimps / *L. innocua* Ad1200	0.5 [0.3 - 1.9]	0.5 [0.3 – 1.9]
6	Process water / *L. monocytogenes* Ad551	0.3 [0.2 - 1.6]	0.3 [0.2 – 1.6]
Combined		**0.7 [0.6 – 0.9]**	**0.7 [0.6 – 0.9]**

INTERLABORATORY STUDY

16 collaborators were involved in the study. Each analyzed 24 blind coded cheese samples inoculated with *Listeria monocytogenes* using both the reference method and Protocol A but using either the **food**proof® StarPrep Two Kit or the **food**proof® StarPrep Two 8-Strip Kit depending on the material available in their laboratory.

The targeted inoculation levels were 0 CFU/25g, 1.5 CFU/25g and 6 CFU/25g.
Results from six collaborators were excluded for interpretation as either the analyses were carried out too late or due to the fact that a high number of positive PCR results were obtained for uninoculated samples, leaving valid results from 10 collaborators.

The positive results obtained by the reference method are provided in **Table 4**.

Table 4. Positive results by the reference method

Laboratories	Contamination level		
	L_0	L_1	L_2
D	0/8[a]	4/8[b]	8/8[c]
E	0/8	6/8	8/8
G	0/8	7/8	8/8
H	0/8	6/8	8/8
I	0/8	7/8	8/8
K	0/8	7/8	8/8
M	0/8	6/8	8/8
N	0/8	8/8	8/8
O	0/8	7/8	8/8
P	0/8	4/8	8/8
Total	***P_0=0/8***	***P_1=62/80***	***P_2=80/80***

L_0 = negative control
L_1 = 1.5 CFU/25g
L_2 = 6.0 CFU/25g
a Number of positive reference method results at level 0

b Number of positive reference method results at level 1
c Number of positive reference method results at level 2

The positive results obtained by the alternative method are provided in Table 5 (*Listeria* spp.) and Table 6 (*Listeria monocytogenes*).

Table 5. Positive results (before and after confirmation) by the alternative method – Listeria spp.

Laboratories	Contamination level					
	L_0		L_1		L_2	
	Screening	Confirmed	Screening	Confirmed	Screening	Confirmed
D	0/8[a]	0/8[b]	6/8[c]	6/8[d]	8/8[e]	8/8[f]
E	0/8	0/8	6/8	6/8	8/8	8/8
G	0/8	0/8	6/8	6/8	8/8	8/8
H	0/8	0/8	6/8	6/8	8/8	8/8
I	0/8	0/8	6/8	6/8	8/8	8/8
K	1/8	0/8	6/8	6/8	8/8	8/8
M	0/8	0/8	5/8	5/8	8/8	8/8
N	0/8	0/8	8/8	8/8	8/8	8/8
O	0/8	0/8	7/8	7/8	8/8	8/8
P	2/8	0/8	7/8	6/8	8/8	8/8
Total	P_0=3/80	CP_0=0/80	P_1=63/80	CP_1=62/80	P_2=80/80	CP_2=80/80

L_0 = negative control
L_1 = 1.5 CFU/25g
L_2 = 6.0 CFU/25g
a Number of positive alternative method results at level 0
b Number of confirmed positive alternative method results at level 0
c Number of positive alternative method results at level 1
d Number of confirmed positive alternative method results at level 1
e Number of positive alternative method results at level 2
f Number of confirmed positive alternative method results at level 2

Table 6. Positive results (before and after confirmation) by the alternative method – Listeria monocytogenes

Laboratories	Contamination level					
	L_0		L_1		L_2	
	Screening	Confirmed	Screening	Confirmed	Screening	Confirmed
D	0/8[a]	0/8[b]	6/8[c]	6/8[d]	8/8[e]	8/8[f]
E	0/8	0/8	5/8	5/8	8/8	8/8
G	0/8	0/8	6/8	6/8	8/8	8/8
H	0/8	0/8	6/8	6/8	8/8	8/8
I	0/8	0/8	6/8	6/8	8/8	8/8
K	1/8	0/8	6/8	6/8	8/8	8/8
M	0/8	0/8	5/8	5/8	8/8	8/8
N	0/8	0/8	8/8	8/8	8/8	8/8
O	0/8	0/8	7/8	7/8	8/8	8/8
P	2/8	0/8	7/8	6/8	8/8	8/8
Total	***P_0*=3/80**	***CP_0*=0/80**	***P_1*=62/80**	***CP_1*=61/80**	***P_2*=80/80**	***CP_2*=80/80**

L_0 = negative control

L_1 = 1.5 CFU/25g

L_2 = 6.0 CFU/25g

a Number of positive alternative method results at level 0
b Number of confirmed positive alternative method results at level 0
c Number of positive alternative method results at level 1
d Number of confirmed positive alternative method results at level 1
e Number of positive alternative method results at level 2
f Number of confirmed positive alternative method results at level 2

The specificity (%) of the reference method and the alternative method:

Specificity for the reference method $SP_{ref} = \left[1 - \frac{P_0}{N_-}\right] \cdot 100\% = 100\%$

Specificity for the alternative method $SP_{alt} = \left[1 - \frac{CP_0}{N_-}\right] \cdot 100\% = 100\%$

Where:

N_- is the total number of all L_0 tests

P_0 is the number of false positive obtained by the reference method

CP_0 is the number of false positive obtained by the alternative method

The relative trueness, the sensitivity of the alternative method and the reference method and the false positive rate of level 1 and 2 are provided in **Table 7**.

Table 7. Results of the Interlaboratory study

Level	* PA	NA	PD	ND	FP	Sum	RT (%)	SE_{alt} (%)	SE_{ref} (%)	FPR(%)
***Listeria* spp.**										
L_1 = 1.5 CFU/25g	48	4	14	14	1	80	65.0	81.6	81.6	25.0
L_2 = 6.0 CFU/25g	80	0	0	0	0	80	100.0	100.0	100.0	0
Listeria monocytogenes										
L_1 = 1.5 CFU/25g	48	5	13	14	1	80	66.3	81.3	82.7	20.0
L_2 = 6.0 CFU/25g	80	0	0	0	0	80	100.0	100.0	100.0	0

* See abbreviations under Table 1.

As the positive and the negative deviations are in the same magnitude, resulting in comparable sensitivity rates, it can be interpreted that the methods perform equivalently. When calculating the acceptability limit, AL, according to ISO 16140-2, it demonstrates that the acceptance criteria have been met.

CONCLUSION

The studies have shown that the alternative method fulfills the requirements of the NordVal International Protocol No. 1 / ISO 16140-2 and guarantees equivalent results to the reference method.