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NordVal International Certificate

Issued for:	**Listeria Velox**
NordVal No:	058
First approval date:	10 February 2023
Valid until:	10 February 2025

Listeria Velox

Manufactured and supplied by:

DNA Diagnostic
Voldbjergvej 14
8240 Risskov.
Denmark

NordVal International has reviewed the method validation documentation. The validation was conducted by ISI Food Protection and ADRIA according to ISO 16140-2. The reference method was ISO 11290-1:2017: Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of Listeria spp.

NordVal International concludes that it has been satisfactorily demonstrated that the data and interpretations comply with the EN ISO 16140-2:2016 requirements and demonstrate comparable performance of the alternative method Listeria Velox: A fast qPCR test that detects *Listeria monocytogenes* and Listeria spp. including *Listeria monocytogenes*., to the ISO 11290-1:2017: Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of Listeria spp. in raw meat and ready to-cook meat products, raw poultry and ready to-cook poultry products, raw and ready-to-cook fish and seafood (unprocessed), ready-to-eat and ready-to-reheat meat products, poultry products and fishery products and environmental samples.

The production of the kits fulfils the requirements outlined in ISO 9001.

Date: 10 February 2023

Yours sincerely,

Hilde Skår Norli
Chair of NordVal International

Eystein Oveland
NMKL Secretary General

PRINCIPLE OF THE METHOD

Listeria Velox is a fast, simple, and reliable method for detection of *Listeria monocytogenes* and Listeria species in 25 g samples. Using an 18 hours-enrichment step, a simple and easy extraction protocol followed by a fast, sensitive, and highly specific qPCR reaction, the entire protocol from sample to result can be carried out in 20 hours. Listeria Velox is a total solution optimal for high-throughput applications.

FIELD OF APPLICATION

The Listeria Velox (Cat No. LV384D, LV384T) is designed for horizontal testing of food matrices and environmental samples. Accordingly, the comparison studies were performed with 5 test categories as summarized in **Table 1**.

Table 1. Matrix category.

Matrix category	Matrix type	Matrix items (examples)
Raw meat and ready-to-cook meat products (except poultry) Sample size: max. 25g Enrichment time: minimum 18h	Fresh meats (unprocessed)	Meat cuts
		Carpaccio's
		Minced meat, meat preparations
		Carcasses, rinsates, swabs
	Ready-to-cook (processed)	Frozen burger patties, marinated meat
Raw poultry and ready-to-cook poultry products. Sample size: max. 25g Enrichment time: minimum 18h	Fresh meats (unprocessed)	Carcasses, rinsates, swabs
		Meat cuts
		Minced meat, meat preparations
	Ready-to-cook products (processed)	Seasoned chicken breasts
Raw and ready-to-cook fish and seafood (unprocessed) Sample size: max. 25g Enrichment time: minimum 18h	Fish (unprocessed)	Fish
	Shellfish (unprocessed)	Oyster, clam, scallop, mussel
	Crustaceans (unprocessed)	Shrimp, crab, crab meat, lobster
	Ready to cook fish/seafood (processed)	Frozen fish sticks
Ready-to-eat and ready-to reheat meat products, poultry products and fishery products. Sample size: max. 25g Enrichment time: minimum 18h	Cooked meats	Cooked ham/turkey/shrimps
	Fermented or dried meats	Salami, chicken nugget, fermented herring
	Raw cured (smoked) meats	Prosciutto, smoked turkey, smoked salmon
	Canned meats (ambient stable)	Corned beef, canned duck pâté, canned crab
Environmental sample Enrichment time: minimum 18h	Surfaces	Sponge, swab
	Cleaning and process water	Process water

METHOD COMPARISON STUDY

Selectivity study

The selectivity is a measure of the inclusivity and the exclusivity, where the inclusivity is the detection of the target microorganism from a wide range of strains, and the exclusivity is the lack of interference from a relevant range of non-target microorganisms.

The selectivity study tested the selectivity of the Listeria Velox qPCR.

The inclusivity study tested 60 strains of Listeria of which 40 strains was *Listeria monocytogenes* and 20 strains was Listeria spp. other than L. monocytogenes.

The exclusivity study tested 32 strains of non-Listeria, including bacteria from the same bacterial order as Listeria (Bacillales), Bacillus and Staphylococcus.

All 60 strains of Listeria gave Ct-values of HEX signal corresponding to: “detected L. spp.” and are therefore all true positive regarding the HEX signal.

1 strain of *Listeria monocytogenes* gave Ct-value of Cy5 signal corresponding to: “not detected L. monocytogenes” and was therefore false negative. This strain, *Listeria monocytogenes* 4a (NTCT5214) was the only false negative *Listeria monocytogenes* in the inclusivity study.

20 strains of Listeria spp. other than L. monocytogenes gave Ct-values of Cy5 signal corresponding to: “not detected L. monocytogenes” and are therefore true negative.

All 32 strains of non-Listeria gave Ct-values of HEX signal corresponding to: “not detected L. spp.” and Ct-values of Cy5 signal corresponding to: “not detected L. monocytogenes” and therefore all strains are true negative.

Sensitivity study

The sensitivity is the ability of the method correctly to detect the analyte. The sensitivity study results are presented in **Table 2**. Since matrix 2 and 3 were naturally contaminated the comparisons were counted for Listeria species and *Listeria monocytogenes*, because both bacteria can be present in naturally contaminated samples. Only values for Listeria species were calculated as a part of the sum. Values for *Listeria monocytogenes* were not calculated as a part of the sum.

The alternative method, Listeria Velox had sensitivity ≥85% for 14 out of 16 serotypes (in 15 matrices).

The reference method, ISO11290-1:2017 had sensitivity ≥67% for 14 out of 16 serotypes (in 15 matrices).

Relative accuracy was ≥90% for 12 out of 16 serotypes (in 15 matrices).

False positive rate for Listeria Velox method was 0% for 10 out of 16 serotypes (in 15 matrices).

Regarding Acceptability Limit, all values are below the limit of five and therefore within the acceptability limit.

Table 2. Sensitivity study

										Sensitivity Listeria Velox	Sensitivity ISO 11290-1:2017	Relative Accuracy	False positive rate for Listeria Velox	Accceptability Limit	Accceptability Limit with 5 matrix categories
Matrix category	Matrix type	Serotype	PA	NA	PD	ND	FP	FN	Sum N	SE Alternative [%]	SE reference [%]	AC [%]	FPR [%]	AL	AL
1	1	*L. monocytogenes 1/2b*	12	8	0	0	1	n.d.	20	100	100	100	13		
1	2	*Listeria* species	15	5	0	0	0	0	20	100	100	100	0		
1	2	*Listeria monocytogenes*	0	20	0	0	13	0	20	#DIV/0!	#DIV/0!	100	65		
1	3	*Listeria* species	15	4	0	1	0	2	20	94	100	95	0		
1	3	*Listeria monocytogenes*	2	16	2	0	0	2	20	100	50	90	0		
Sum 1			**42**	**17**	**0**	**1**	**1**	**2**	**60**	**98**	**100**	**98**	**4**	**1**	**5**
2	4	*L. monocytogenes 4b*	6	10	4	0	1	n.d.	20	100	60	80	10		
2	5	*L. monocytogenes 3a*	4	14	2	0	0	n.a.d. 1	20	100	67	90	0		
2	6	*L. selligeri*	15	1	4	0	0	n.d.	20	100	79	80	0		
Sum 2			**25**	**25**	**10**	**0**	**1**	**1**	**60**	**100**	**69**	**83**	**3**	**-10**	**5**
3	7	*L. monocytogenes 1/2a*	13	7	0	0	0	n.d.	20	100	100	100	0		
3	8	*L. monocytogenes 2c*	10	8	2	0	0	0	20	100	83	90	0		
3	9	*L.* species	9	9	0	2	0	0	20	82	100	90	0		
Sum 3			**32**	**24**	**2**	**2**	**0**	**0**	**60**	**94**	**94**	**93**	**0**	**0**	**5**
4	10	*L. monocytogenes 1/2c*	7	6	7	0	2	n.d.	20	100	50	65	33		
4	11	*L. ivanovii*	0	8	12	0	1	n.d.	20	100	0	40	13		
4	12	*L. grayii*	8	7	0	5	5	n.d.	20	62	100	75	71		
Sum 4			**15**	**21**	**19**	**5**	**8**	**0**	**60**	**87**	**50**	**60**	**39**	**-14**	**5**
5	13	*L. innocua*	13	5	0	2	0	n.d.	20	87	100	90	0		
5	14	*L. weishimeri*	16	3	1	0	0	n.d.	20	100	94	95	0		
5	15	*L. monocytogenes 7*	11	7	0	2	1	n.d.	20	85	100	90	14		
Sum 5			**40**	**15**	**1**	**4**	**1**	**0**	**60**	**90**	**98**	**92**	**5**	**3**	**5**
Total sum			**154**	**102**	**32**	**12**	**11**	**3**	**300**	**94**	**82**	**85**	**10**		

Level of Detection (LOD_{50}) and Relative Level of Detection (RLOD)

The limit of detection for 50% fractional positives (LOD50%) and relative level of detection (RLOD) were calculated based on inoculation level and number of positive results (**Table 3**).

Table 3. Inoculation level and number of positive results detected with ISO 11290-1:2017 and Listeria Velox method.

Category	Matrix	Inoculation level		Number of inoculated samples	ISO 11290-1:2017	Listeria Velox
		cfu/sample	cfu/g		Number of positive results	Number of positive results
1	1	0.9	0.036	20	12	13
2	4	0.7	0.028	20	6	10
2	5	0.8	0.032	20	4	6
2	6	0.7	0.028	20	15	19
3	7	1.4	0.056	20	13	13
3	8	0.8	0.032	20	10	12
3	9	0.7	0.028	20	11	2
4	10	1.0	0.040	20	7	16
4	11	0.6	0.024	20	0	13
4	12	0.5	0.020	20	13	13

Category	Matrix	Inoculation level		Number of inoculated samples	ISO 11290-1:2017	Listeria Velox
		cfu/sample	cfu/g		Number of positive results	Number of positive results
5	13	1.0	0.040	20	15	13
5	14	1.3	0.052	20	16	17
5	15	1.2	0.048	20	13	12

The detection limits for the five categories and 13 artificially contaminated matrices are given in **Table 4**.

Table 4. Detection limits for 13 artificially contaminated matrices.

Category	Matrix	Detection limit [cfu / 25 g]	Lower confidence limit [cfu / 25 g]	Upper confidence limit [cfu / 25 g]
		$d_{0.5}$	$d_{0.5,Lower}$	$d_{0.5,Upper}$
1	1	0.594	0.333	1.062
2	4	0.700	0.367	1.334
2	5	1.555	0.684	3.533
2	6	0.162	0.084	0.310
3	7	0.924	0.517	1.652
3	8	0.605	0.333	1.100
3	9	4.605	1.119	18.955
4	10	0.431	0.247	0.751
4	11	0.396	0.222	0.708
4	12	0.330	0.185	0.590
5	13	0.660	0.369	1.180
5	14	0.475	0.271	0.832
5	15	0.908	0.499	1.650
Combined results		0.637	0.539	0.753

The detection limits were calculated using the excel sheet “PODLOD_ver10a” an excel program for the estimation of the POD function and the LOD of the qualitative microbiological measurement method according to Wilrich, C., and P.Th. Wilrich.

https://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/professoren/wilrich/index.html

For unpaired study data, the AL of RLOD is set at 2.5. **Table 5** shows that all categories have RLOD below 2.5 and therefore the Listeria Velox is shown to perform satisfactorily.

Table 5. Relative detection limits for each artificially contaminated matrix.

Category	Matrix	RLOD	RLODL	RLODU
1	1	0.895	0.436	1.838
2	4	0.540	0.227	1.285
3	7	0.879	0.428	1.805
4	10	0.200	0.083	0.481
5	13	1.086	0.532	2.216
Combined		0.645	0.460	0.903

RLOD values were calculated using the excel sheet "RLOD_ver3" an excel program for the estimation RLOD of two qualitative microbiological measurement methods according to Margaritescu, I., and Wilrich, P.-Th.

https://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/professoren/wilrich/index.html

INTERLABORATORY STUDY

14 collaborators participated to the study. Ground beef meat was inoculated with *Listeria monocytogenes* Ad1206 strain isolated from ground beef.

24 blind coded samples (25 g) for detection of Listeria spp. and *Listeria monocytogenes* by the Velox Listeria method.

24 blind coded samples (25 g) for detection of Listeria spp. and *Listeria monocytogenes* by ISO 11290-1 reference method.

The targeted inoculation levels were the following:

Level 0: 0 CFU/25g
Level 1: 1 CFU/25g, inoculation level providing fractional positive recovery data;
Level 2: 8 CFU/25g.

Of the 14 received data sets, three collaborators (A2, C1 and C2) were excluded due to contamination of Level 0. The ISO 11290-1 method and the Velox method were performed on two different sites for 3 sets of data (collaborators D and E). The results obtained are provided in **Table 6** (reference method, Listeria spp. detection), **Table 7** (reference method, *Listeria monocytogenes* detection), **Table 8** (alternative method, Listeria spp. detection) and **Table 9** (alternative method, *Listeria monocytogenes* detection).

Table 6. Positive results by the reference method - Listeria spp. Detection.

Collaborator	Contamination level		
	L0	L1	L2
A1	0	7	8
A3	1	8	8
B1	0	7	8
B2	1	7	8
C3	0	7	8
E1	0	7	8

Collaborator	Contamination level		
	L0	L1	L2
E2	0	6	8
E3	1	6	8
F1	0	5	8
F2	0	8	8
G1	0	6	8
TOTAL	$P_0 = 3$	$P_1 = 74$	$P_2 = 88$

Table 7 - Positive results by the reference method - *Listeria monocytogenes* detection

Collaborator	Contamination level		
	L0	L1	L2
A1	0	7	8
A3	0	8	8
B1	0	7	8
B2	0	7	8
C3	0	7	8
E1	0	7	8
E2	0	6	8
E3	0	6	8
F1	0	5	8
F2	0	8	8
G1	0	6	8
TOTAL	$P_0 = 0$	$P_1 = 74$	$P_2 = 88$

Table 8. Positive results (before and after confirmation) by the alternative method - Listeria spp. detection

Collaborator	Contamination level								
	L0			L1			L2		
	Before confirmation	*Confirmation*	*After confirmation*	*Before confirmation*	*Confirmation*	*After confirmation*	*Before confirmation*	*Confirmation*	*After confirmation*
A1	0	0	0	6	6	6	8	8	8
A3	0	0	0	6	7	6	8	8	8
B1	0	0	0	6	6	6	8	8	8
B2	1	0	0	7	7	7	8	8	8
C3	0	0	0	6	6	6	8	8	8
D1	0	0	0	7	7	7	8	8	8

Collaborator	Contamination level								
	L0			L1			L2		
	Before confirmation	*Confirmation*	*After confirmation*	*Before confirmation*	*Confirmation*	*After confirmation*	*Before confirmation*	*Confirmation*	*After confirmation*
D2	0	0	0	7	7	7	8	8	8
D3	0	0	0	8	8	8	8	8	8
F1	1	0	0	8	8	8	8	8	8
F2	2	1	1	5	5	5	8	8	8
G1	1	0	0	7	7	7	8	8	8
TOTAL	**P_0 = 5**	**C_0 = 1**	**CP_0 = 1**	**P_1 = 73**	**C_1 = 74**	**CP_1 = 73**	**P_2 = 88**	**C_2 = 88**	**CP_2 = 88**

Table 9. Positive results (before and after confirmation) by the alternative method - *Listeria monocytogenes* detection

Collaborator	Contamination level								
	L0			L1			L2		
	Before confirmation	*Confirmation*	*After confirmation*	*Before confirmation*	*Confirmation*	*After confirmation*	*Before confirmation*	*Confirmation*	*After confirmation*
A1	0	0	0	6	6	6	8	8	8
A3	1	0	0	6	7	6	8	8	8
B1	0	0	0	6	6	6	8	8	8
B2	0	0	0	7	7	7	8	8	8
C3	2	0	0	6	6	6	8	8	8
D1	0	0	0	7	7	7	8	8	8
D2	0	0	0	6	6	6	8	8	8
D3	0	0	0	8	8	8	8	8	8
F1	0	0	0	8	8	8	8	8	8
F2	1	0	0	5	5	5	8	8	8
G1	0	0	0	7	7	7	8	8	8
TOTAL	**P_0 = 4**	**C_0 = 0**	**CP_0 = 0**	**P_1 = 72**	**C_1 = 73**	**CP_1 = 72**	**P_2 = 88**	**C_2 = 88**	**CP_2 = 88**

SPECIFICITY

The Specificity percentage (SP) of the reference method and of the alternative method, using the data after confirmation, based on the results of level L0 are presented in **Table 10** and **Table 11**.

Table 10. Specificity percentage - Listeria spp. detection

Specificity for the reference method	$SP_{ref} = \left(1 - \left(\frac{P_0}{N_-}\right)\right) x\ 100\ \% =$	96,6 %
Specificity for the alternative method	$SP_{alt} = \left(1 - \left(\frac{CP_0}{N_-}\right)\right) x\ 100\ \% =$	97,7%

N: number of all L0 tests. P_0 = total number of false-positive results obtained with the blank samples before confirmation. CP_0 = total number of false-positive results obtained with the blank samples.

Table 11. Specificity percentage - *Listeria monocytogenes* detection

Specificity for the reference method	$SP_{ref} = \left(1 - \left(\frac{P_0}{N_-}\right)\right) x\ 100\ \% =$	100,0 %
Specificity for the alternative method	$SP_{alt} = \left(1 - \left(\frac{CP_0}{N_-}\right)\right) x\ 100\ \% =$	100,0 %

N: number of all L0 tests. P_0 = total number of false-positive results obtained with the blank samples before confirmation. CP_0 = total number of false-positive results obtained with the blank samples.

SENSITIVITY

The values for sensitivity, relative trueness and false positive ratio percentage for the alternative method and the reference method are presented in **Table 12**.

Table 12. Sensitivity, relative trueness, and false positive ratio percentages

Calculation	Equation	Level 1	
		***Listeria* spp.**	***Listeria monocytogenes***
Sensitivity for the alternative method	$SE_{alt} = \frac{(PA+PD)}{(PA+PD+ND)} x\ 100\% =$	83,0%	81,1%
Sensitivity for the reference method	$SE_{ref} = \frac{(PA+ND)}{(PA+PD+ND)} x\ 100\% =$	84,1%	83,8%
Relative trueness	$RT = \frac{(PA+NA)}{N} x\ 100\% =$	67,0%	65,9%
False positive ratio for the alternative method	$FPR = \frac{FP}{NA} x\ 100\% =$	/	/

CONCLUSION

The completed studies demonstrate that the alternative method fulfils the requirements of the NordVal International Protocol No. 1 / ISO 16140-2 and provides equivalent results to the reference method.