



**Alternative methods for agribusiness
Analytical performances certified**

**VALIDATION CERTIFICATE FOR ALTERNATIVE ANALYTICAL METHOD
ACCORDING TO STANDARD EN ISO 16140: 2003**

Certificate No.: AES 10/08 – 12/09

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The company **AES CHEMUNEX**
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is hereby authorized to refer to this **AFNOR Validation certificate** for the following alternative qualitative analysis method:

ADIAFOOD *Listeria monocytogenes*

Protocol reference: ADIAF-LM-Rev.0

SCOPE

All human food products and environmental samples.

RESTRICTIONS OF USE

None.

REFERENCE METHOD

EN ISO 11290-1 (1997) including **amendment A1** (2004): Food microbiology - Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 1: Detection method.

**Deputy General Manager
Jacques BESLIN**

A handwritten signature in black ink, appearing to be "Jacques Beslin", written over a horizontal line.

AFNOR Certification

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PRINCIPLE OF THE METHOD

The ADIAFOOD *Listeria monocytogenes* method is based on real-time Polymerase Chain Reaction (PCR) technology. The system is automatized and provides rapid detection of *Listeria monocytogenes* strains by specifically identifying the DNA sequence of this pathogen in a series of sequential steps that include: Sample Preparation and Enrichment, DNA Extraction, and Pathogen Detection. The kit is available in two formats, the microplate format and the strip tube format.

In the context of AFNOR Validation, all samples identified as positive by the alternative method must be confirmed by one of the following means:

- According to classical tests described in methods standardized by CEN or ISO (including a purification step). To obtain colonies proceed by carrying out a subculture of 100 μ l of the enriched Listerboost onto a selective medium for *Listeria*. Refer to the latest edition of the standard NF EN ISO 7218 for confirmation procedures that can be used in standard methods.
- By isolating 100 μ l of Listerboost enriched on ALOA™ agar.

In the event of discordant results (positive with alternative method, non-confirmed by means of options described above) the laboratory must follow the necessary steps to ensure validity of the result obtained.

For example by transferring 100 μ l of enriched Listerboost in 10 ml of Fraser broth and continuing the procedure by following the NF EN ISO 11290-1 the current standard.

Relative ACCURACY, relative SPECIFICITY and relative SENSITIVITY Comparison of performances of the alternative method and the reference method

In 2009 tests were carried out on 320 product samples, of which 77 were naturally contaminated, 80 artificially contaminated, and 163 non-contaminated, belonging to the following principal food product categories:

Dairy products, meat products, vegetables, seafood products and environmental samples.

All samples were analysed in **single** by the **two methods**.

Table of results (Cf. Table 1 of the EN ISO 16140 standard):

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 128 ⁽¹⁾	Positive deviation A+ / R- PD = 14 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 15 ⁽²⁾	Negative agreement A- / R- NA = 163 ⁽³⁾

(1) Confirmed positives

(2) Of which 4 samples presumed positive by the alternative method were negative after confirmation

(3) Of which 7 samples presumed positive by the alternative method were negative after confirmation

Percentages obtained compared to the reference method are as follows:

- Relative accuracy: **AC = 90.9%**
- Relative specificity: **SP = 92.1%**

NB: **relative specificity** below 100% results from a number of confirmed supplementary positives and not from false positives.

- Relative sensitivity: **SE = 89.5%**

Sensitivity was also recalculated taking into account all confirmed positives (including supplementary positives by alternative method):

Alternative method:	Reference method:
$(PA + PD) / (PA + PD + ND) = 90.4\%$	$(PA + ND) / (PA + PD + ND) = 91.1\%$

Analysis of discrepant results (according to appendix F of standard EN ISO 16140):

PD = 14 and ND = 15; so $Y = PD + ND = 29$

Test of Mc Nemar: $X^2 = d^2/Y$, with $d = |PD - ND|$; $X^2 = 0,034$; so $X^2 < 3,841$

Conclusion

The two methods are comparable for $\alpha = 0.05$.

Relative DETECTION LEVEL

Comparison of performances of the alternative method and the reference method

Tests were carried out in 2009, on the 5 combinations of food products/strains described in the table below.

Products were analysed **6 times** by the **2 methods** at **4 levels** of contamination.

Results obtained are as follows:

Matrix	Strain	Relative detection level (CFU/25g or 25 ml) With confidence interval (3) LOD ₅₀	
		Alternative method	Reference method
Potted meat	<i>Listeria monocytogenes</i> 1/2b	0.8 [0.5 – 1.3]	0.8 [0.4 – 1.4]
Raw milk	<i>Listeria monocytogenes</i> 1/2b	1.6 [1.1 – 2.4]	1.1 [0.8 – 1.7]
Smoked salmon	<i>Listeria monocytogenes</i> 4b	0.5 [0.4 – 0.6]	0.5 [0.4 – 0.6]
Celery	<i>Listeria monocytogenes</i> 1/2c	1.1 [0.7 – 1.9]	0.9 [0.6 – 1.3]
Water process	<i>Listeria monocytogenes</i> 3a	0.5 [0.3 – 1.0]	0.7 [0.4 – 1.2]

(3) **LOD₅₀**: estimation of level of contamination enabling positive detection by alternative method in 50% of cases.

"Hitchins A. Proposed Use of a 50% Limit of detection Value in Defining Uncertainty Limits in the Validation of Presence-Absence Microbial detection Methods, Draft 10th December, 2003"

Conclusion

The relative detection level of the alternative method is between 0.3 and 2.4 CFU/25 g.

The relative detection level of the reference method is between 0.4 and 1.7 CFU/25 g.

INCLUSIVITY / EXCLUSIVITY

Implementation of alternative method only

- 50 strains of *Listeria monocytogenes* were detected out of 50 tested.
- The study of 32 strains not belonging to the genus *Listeria monocytogenes* (from which 12 were not *Listeria* spp) did not detect the presence of any cross-reaction.

PRACTICABILITY

Implementation of alternative method only

- **Response time:**
 - **Positive** results are obtained in 3 days using the alternative method against 8 days using the reference method.
 - **Negative** results are obtained in 1 day using the alternative method against 5 days using the reference method.
 - In the case of results presumed positive using the alternative method, but given negative following confirmation, these negative results are obtained in 2 to 4 days.
- **Personnel training:** 1 day of training for an operator not introduced to the method.

INTER-LABORATORY STUDY

The inter-laboratory study was conducted in 2009 with 14 participating laboratories. The analyses were carried out on samples of pasteurized milk, artificially contaminated with a *Listeria monocytogenes* strain at the 3 following levels of contamination:

- 0 CFU/ 25 ml
- 3 CFU/ 25 ml
- 30 CFU/ 25 ml

The laboratories tested, using **both methods, 8 replicate samples for each level** of contamination, giving a total of 24 analyses for each participating laboratory.

The following results were obtained:

Contamin- ation level	Total number of samples	Number of samples analysed	Number of results processed*	Number of negative results		Number of positive results	
				REF	ALT	REF	ALT
0	112	112	104	104	104	0	0
1	112	112	104	5	4	99	100
2	112	112	104	0	1	104	103

* The results of one laboratory were excluded because of cross contamination of samples.

Calculations

- Relative accuracy = **97%**
- Specificity = **100%**
- Sensitivity = **98%**

Sensitivity was also recalculated taking into account all confirmed positive results (this includes supplementary positives with alternative method):

Alternative method:

$$(PA + PD) / (PA + PD + ND) = 98\%$$

Reference method:

$$(PA + ND) / (PA + PD + ND) = 98\%$$

Accordance, concordance and concordance odds ratio:

Accordance: percentage chance of finding the same result (i.e. both negative or both positive) from two identical test portions analysed in the same laboratory, under repeatability conditions (i.e. one operator using the same apparatus and same reagents within the shortest feasible time interval). The accordancy is the average (mean) of the probabilities that two replicates give the same result for each laboratory

Concordance: percentage chance of finding the same result for two identical samples analysed in two different laboratories. The concordance is the percentage of all pairings of duplicates giving the same result

Concordance odds ratio (COR): defined by the following formula:

$$\text{COR} = \frac{\text{accordance} \times (100 - \text{concordance})}{\text{concordance} \times (100 - \text{accordance})}$$

The following table indicates values for the **alternative method**:

Contamination level	Accordance	Concordance	COR
L0	100%	100%	1.00
L1	93%	92%	1.16
L2	98%	98%	1.00

The following table indicates values for the **reference method**:

Contamination level	Accordance	Concordance	COR
L0	100%	100%	1.00
L1	92%	92%	1.00
L2	100%	100%	1.00

Conclusion

Variability of the alternative method (accordance, concordance, concordance odds ratio) is equivalent to that of the reference method.

Please send any queries concerning the performance of the validated method to AFNOR Certification.

You may download a summary document on the preliminary and inter-laboratory studies on www.afnor-validation.com