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

Validation date:	**03.12.2009**
Extension date:	**02.12.2010**
Renewal date:	**29.11.2013**
End of validity:	**03.12.2017**

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is hereby authorized to refer to this **NF VALIDATION certificate** for the following alternative qualitative analysis method:

ADIAFOOD *Listeria monocytogenes*

Protocol reference: ADIAF- LM- D and ADIAFD - LM - A

SCOPE

All human food products and environmental samples.

RESTRICTIONS

None.

REFERENCE METHOD

EN ISO 11290-1 (February 1997) and its **amendment A1** (February 2005): Food microbiology - Horizontal method for the detection and enumeration *of Listeria monocytogenes* - Part 1: Detection method.

Managing Director
Florence MÉAUX

CERTIFICATION DE PRODUITS INDUSTRIELS
ACCRÉDITATION N° 5-0030
PORTÉE DISPONIBLE SUR WWW.COFRAC.FR

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.

Both formats of the kit (microplate and strip tube) are included in the validated scope of the alternative method. It includes too the use of Stratagene cyclers MX3000P and MX3005P associated with the Sentinel software.

In the context of NF 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 from colonies (including a purification step). To obtain colonies proceed by carrying out a subculture of 100 ll of the enriched Listerboost onto a selective medium for *Listeria*. Refer to the latest edition of the standard EN ISO 7218 for confirmation procedures that can be used in standard methods.
- By isolating 100µl of Listerboost enriched on ALOA™ agar.

In the event of discordant results (presumptive 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 µl of enriched Listerboost in 10 ml of Fraser broth and continuing the procedure by following the EN ISO 11290-1 the current standard.

NOTE (History of validation)

In September 2010, the NF VALIDATION technical committee validated the modification of extraction supports which are used to implement the method AdiaFood *Listeria monocytogenes*. **A colouring agent**, for reaction control, was added into extraction microplates. The method protocol and reagents remain the same. No additional tests were done.

In December 2010, an extension study allowed to validate the use of **SENTINEL software version 2.1** with the method AdiaFood *Listeria monocytogenes*. Internal tests showed that the results obtained with new version were equivalent to those obtained with the version 2.0 previously validated. Results are not detailed in this certificate.

This certificate was re-issued on October 2012 in order to update the protocol reference following the validation of minor changes without any impact on the following validation results.

In November 2013, the certification was renewed without conducting any validation study. The method AdiaFood *Listeria monocytogenes* did not change since the last validation study. The reference method and the validation protocol are still the same.

In January 2014, the certification has been granted to the company bioMérieux SA without any change on the alternative method.

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

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

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 product/strain" described in the table below.

These products were belonging to the following food product categories: Dairy products, meat products, vegetables, seafood products and environmental samples.

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	*Listeria monocytogenes* 1/2b	0.8 [0.5 – 1.3]	0.8 [0.4 – 1.4]
Raw milk	*Listeria monocytogenes* 1/2b	1.6 [1.1 – 2.4]	1.1 [0.8 – 1.7]
Smoked salmon	*Listeria monocytogenes* 4b	0.5 [0.4 – 0.6]	0.5 [0.4 – 0.6]
Celery	*Listeria monocytogenes* 1/2c	1.1 [0.7 – 1.9]	0.9 [0.6 – 1.3]
Water process	*Listeria monocytogenes* 3a	0.5 [0.3 – 1.0]	0.7 [0.4 – 1.2]

(3) **LOD_{50}**: estimation of level of contamination enabling positive detection by alternative method in 50% of cases. *FDA. 2006. Final Report and Executive Summaries from the AOAC International Presidential Task Force on Best Practices in Microbiological Methodology. Appendix K. Statistics Working Group Tholen, D. W., D. S. Paulson, B. Jarvis, D. M. Mettler, B. Lombard, K. Newton, M. A. Mozola, and A. D. Hitchins.) Report Part 4a - LOD50.*

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 positive presumptive results presumed using the alternative method, but found 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:

Contami-nation 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 accordance 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:
COR= accordance x (100 - concordance) / concordance x (100 – accordance)

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

Contamination level	Accordance	Concordance	COR
L0	100%	100%	1.0
L1	93%	92%	1.2
L2	98%	98%	1.0

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

Contamination level	Accordance	Concordance	COR
L0	100%	100%	1.0
L1	92%	92%	1.0
L2	100%	100%	1.0

Conclusion

The 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