



**Alternative methods for agribusiness
Analytical performances certified**

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

Certificate No.: AES 10/03 - 09/00

**Validation date : 2000.09.27
Renewal date* : 2005.04.07
2008.06.30
Extension on* : 2006.09.15
End of validity : 2012.09.27**

** EN ISO 16140 protocol was implemented during the 2005 extension for preliminary study and during the 2006 renewal for interlaboratory study*

The company
(head office,
distribution
and production site)

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

ALOA ONE DAY™

Protocol reference: **520080: 30/06/08-S**

SCOPE

All foodstuffs for human consumption and environment samples

RESTRICTIONS OF USE

None

REFERENCE METHOD

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

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PRINCIPLE OF THE METHOD

The ALOA ONE DAY™ method comprises a chromogenic agar medium (ALOA) that detects all *Listeria* by the detection of β -glucosidase activity and distinguishes *L. monocytogenes* by the formation of a clear precipitation halo of phospholipids cleaved by its specific phospholipase.

After inoculation, the plates are incubated at 37°C and are read after 24 to 48 hours. *Listeria monocytogenes* strains form typical colonies within 24 hours.

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, ISO or AFNOR (including a purification step), starting from colonies isolated from ALOA
- With ALOA Confirmation™ technique (according to the instructions in the ALOA Confirmation™ technical data sheet)
- By implementing any other AFNOR VALIDATION certified method based on a principle different from the ALOA ONE DAY™ alternative method, respecting specifications in the test instructions.

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.

NOTE: History of the Validation

This method was initially validated in 2000 as reference ALOA/L.Monodisk.

In 2002, an extension of the AFNOR VALIDATION mark was attributed to the ALOA ONE DAY method, replacing the ALOA/L.Monodisk method.

In 2004, the renewal study considered the following aspects:

- Study of a fifth matrix (environment samples)
- Inclusion of the new standardised validation reference: EN ISO 16140 for the preliminary study
- Inclusion of amendment A1 to the reference standard EN ISO 11290-1

Starting in 2000, tests were conducted in anticipation of the future standard EN ISO 16140 and the 2004 renewal study involved the following aspects:

- Comparative study
 - re-processing of previously obtained data for categories of foodstuffs for human consumption
 - study of categories of environment samples
- Interlaboratory study: utilisation of prior results

In 2006, a new **extension** of the AFNOR VALIDATION label was attributed in order to take into account the new interlaboratory study conducted according to the protocol described in standard EN ISO 16140. The results of this new study were included in this attestation, replacing those obtained with the former study protocol.

In June 2008, the validation was renewed without the carrying out of complementary tests, since neither the SMS method, nor neither the method taken in reference, nor protocol of validation were modified.

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

In 2000 tests were carried out on **164 product samples**, of which 53 were naturally contaminated, and 111 non-contaminated, belonging to the following principal food product categories: meat products, seafood, plant products and dairy products.

Additional tests were carried out in 2004 and 2005 on:

- **92 samples** belonging to the categories of meat products, seafood, plant products and dairy products, of which 23 samples were naturally contaminated, 52 artificially contaminated and 17 non-contaminated.
- **61 environment samples** of which 4 samples were naturally contaminated, 26 artificially contaminated and 31 non-contaminated.

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

Two readings (at 24 and 48 hours) were taken. The results are listed in two different tables.

Table of results (Cf. Table 1 of the EN ISO 16140 standard) : **24 hour reading** (all matrices taken together)

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 156⁽¹⁾	Positive deviation A+ / R- PD = 1⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 1⁽²⁾	Negative agreement A- / R- NA = 159⁽³⁾

(1) Confirmed positive

(2), (3) Of which no sample presumed positive with the alternative method, negative after confirmation

Percentages obtained compared to the reference method are as follows :

- Relative accuracy (at 24 hours) **AC = 99.36%**
- Relative specificity (at 24 hours) **SP = 99.37%**

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

- Relative sensitivity (at 24 hours) **SE = 99.36%**

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

Alternative method :

Reference method :

$$(PA + PD) / (PA + PD + ND) = 99.4\%$$

$$(PA + ND) / (PA + PD + ND) = 99.4\%$$

Table of results (Cf. Table 1 of the EN ISO 16140 standard) : **48 hour reading** (all matrices taken together)

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 157⁽¹⁾	Positive deviation A+ / R- PD = 2⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 0⁽²⁾	Negative agreement A- / R- NA = 158⁽³⁾

(1) Confirmed positive

(2), (3) Of which no sample presumed positive with the alternative method, negative after confirmation

Percentages obtained compared to the reference method are as follows :

- Relative accuracy (at 48 hours) **AC = 99.36%**
- Relative specificity (at 48 hours) **SP = 98.75%**

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

- Relative sensitivity (at 48 hours) **SE = 100%**

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

Alternative method :

$$(PA + PD) / (PA + PD + ND) = 100\%$$

Reference method :

$$(PA + ND) / (PA + PD + ND) = 98.74\%$$

Conclusion

The performances of the ALOA ONE DAY method are equivalent to those of the reference method. The results obtained with the ALOA ONE DAY method with readings at 24 and 48 hours are equivalent.

Relative DETECTION LEVEL

Comparison of performances of the alternative method and the reference method

Tests were carried out in 2004 and 2005, on the 5 food products/strains combinations described in the following table.

These products were representative of the following food categories: meat products, seafood, plant products and dairy products and environment 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
Rillettes (minced pork)	<i>L. monocytogenes</i> LM1	0.5 [0.4 - 0.7]	0.5 [0.4 - 0.7]
Salmon	<i>L. monocytogenes</i> LM16	0.5 [0.4 - 0.7]	0.5 [0.4 - 0.7]
Green salad	<i>L. monocytogenes</i> LM42	0.4 [0.3 - 0.5]	0.4 [0.3 - 0.5]
Unpasteurised milk	<i>L. monocytogenes</i> LM17	0.5 [0.4 - 0.8]	0.5 [0.4 - 0.8]
Drag swabs	<i>L. monocytogenes</i> LM12	0.5 [0.4 - 0.7]	0.5 [0.4 - 0.7]

(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 limit of detection of the alternative method is between 0.3 and 0.8 CFU/25 g. It is identical to that of the reference method.

INCLUSIVITY / EXCLUSIVITY

Implementation of alternative method only

- 50 strains of *Listeria monocytogenes* were detected among 50 tested.
- The study of 30 non *Listeria monocytogenes* strains did not reveal the presence of cross reactions. It should be mentioned that certain colonies of *Listeria ivanovli* presented a typical appearance with a thin halo after the first 24 hours of incubation.

PRACTICABILITY

Implementation of alternative method only

Response time :

- **Positive** results are obtained in **4 to 8 days (in case of reading at 24 hours)** or in **5 to 9 days (in case of reading at 48 hours)** using the ALOA ONE DAY method (after confirmation of typical colonies isolated by ALOA ONE DAY, according to the reference method, including the purification step) against 7 to 11 days with the reference method.
- **Negative** results are obtained (absence of typical colonies) in **2 days (in cases of reading at 24 hours)** or in **3 days (in cases of reading at 48 hours)** using the ALOA ONE DAY method against 5 to 11 days with the reference method.
- In the case of results presumed positive using the alternative method, but rendered negative following confirmation, these negative results are obtained in **4 days (in cases of reading at 24 hours)** or in **5 days (in cases of reading at 48 hours)**.

INTER-LABORATORY STUDY

The inter-laboratory study was conducted in 2006 with 14 participating laboratories. The analyses were carried out on samples of pasteurized goat milk artificially contaminated with a *Listeria monocytogenes 4b* strain at the following 3 levels of contamination:

- 0
- slightly superior to relative detection level
- 10 times superior to previous level

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

The following results were obtained:

Contamination level	Total number of samples	Number of samples analysed*	Number of results exploited **	Number of negative results		Number of positive results	
				REF	ALT	REF	ALT
0	112	112	104	104	104	0	0
1	112	112	104	0	0	104	104
2	112	112	104	0	0	104	104

* One laboratory reported non-compliant manipulation and its results were not included

Calculations

- Relative accuracy: 100%
- Specificity: 100%
- Sensitivity: 100%

Interpretation

Results of the interlaboratory study are comparable to those obtained during the preliminary study.

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:

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

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

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

Conclusion

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

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

On request, AFNOR Certification will send you a summary document (in French) on the preliminary and collaborative studies.