



**Alternative methods for agribusiness
Analytical performances certified**

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

Certificate No.: BRD 07/16 – 01/09

**Validation date: 26.01.2009
End of validity: 26.01.2013**

The company
(head office, distributor,
and production site)

Bio-Rad
3, Boulevard Raymond Poincaré
92430 MARNES LA COQUETTE
FRANCE

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

AL – detection method

Protocol reference: AL short protocol / Agar – V2
356-3695 / 356-4041
356-4042 / 356-4043

SCOPE

All human food products and environmental samples.

RESTRICTIONS OF USE

None.

REFERENCE METHOD

EN ISO 11290-1 (1997) including amendment A1 (2004): 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 "JBESLIN", written over a horizontal line.

AFNOR Certification

11, rue Francis de Pressensé – 93571 La Plaine Saint-Denis Cedex - France
Phone +33 (0)1 41 62 80 00 – Fax +33 (0)1 49 17 90 00
certification@afnor.com - www.afnor-validation.com

PRINCIPLE OF THE METHOD

The detection A.L. method is a chromogenic agar medium, that permits the simultaneous detection of *Listeria* genus by formation of a blue to blue-green colour (β -D-glucosidase activity) and to differentiate the *Listeria monocytogenes* by production of an opaque halo around the colony (phospholipase C activity). The final result is obtained after 24 hours (\pm 2hours) (possible reading after 48 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 (only one colony confirmation is required):

- According to classical tests described in methods standardized by CEN or ISO (including a purification step).
- Using nucleic probes as described in EN ISO 7218 standard (including or not the purification step).
- A colony isolated on A.L. agar may be confirmed by means of spot sub-culture on RAPID'L. *Mono* agar, without previous purification.
- Using any other AFNOR VALIDATION certified method, the principle of which is different from the AL enumeration method. The protocol of the second validated method shall be followed entirely. All steps that are before the step from which the confirmation is done shall be common to both methods.

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.

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

In 2008 tests were carried out on 339 product samples, of which 81 were naturally contaminated, 77 artificially contaminated, and 181 non-contaminated, belonging to the following principal food product categories:

Meat products, dairy products (including raw milk cheese), vegetables, seafood products (including smoked fish) and environmental samples.

All samples were analysed in single by the two methods.

Incubation of AL Agar medium during 22 hours at 37°C 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 = 155 ⁽¹⁾	Positive deviation A+ / R- PD = 1 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 2 ⁽²⁾	Negative agreement A- / R- NA = 181 ⁽³⁾

(1) Confirmed positives

(2) Of which none sample presumed positive by the alternative method was negative after confirmation

(3) Of which none samples presumed positive by the alternative method were negative after confirmation

Incubation of AL Agar medium during 48 hours at 37°C

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 = 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 = 181 ⁽³⁾

(1) Confirmed positives

(2) Of which none sample presumed positive by the alternative method was negative after confirmation

(3) Of which none samples presumed positive by the alternative method were negative after confirmation

Percentages obtained compared to the reference method are as follows:

Incubation of AL Agar medium	Relative accuracy AC	Relative specificity SP	Relative sensitivity SE
during 22 hours at 37°C	99.1%	99.5%	98.7%
during 48 hours at 37°C	99.4%	99.5%	99.4%

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):

Incubation of AL Agar medium	Alternative method: (PA + PD) / (PA + PD + ND)	Reference method: (PA + ND) / (PA + PD + ND)
during 22 hours at 37°C	98.7%	99.4%
during 48 hours at 37°C	99.4%	99.4%

Analysis of discrepant results (according to appendix F of standard EN ISO 16140):Incubation of AL Agar medium during 22 hours at 37°C

PD = 1, ND = 2; so Y = PD + ND = 3; Y ≤ 6: No statistical test available.

Incubation of AL Agar medium during 48 hours at 37°C

PD = 1, ND = 1; so Y = PD + ND = 2; Y ≤ 6: No statistical test available.

Conclusion

The two methods are equivalent.

Relative DETECTION LEVEL

Comparison of performances of the alternative method and the reference method

Tests were carried out in 2008, on 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>L. monocytogenes</i> 1/2b	0.7 [0.4 – 1.1]	0.7 [0.4 – 1.1]
Raw milk	<i>L. monocytogenes</i> 4b	0.7 [0.4 – 1.1]	0.7 [0.4 – 1.1]
Mix of raw vegetable	<i>L. monocytogenes</i> 4b	0.4 [0.2 – 0.5]	0.4 [0.2 – 0.5]
Smoked salmon	<i>L. monocytogenes</i> 1/2a	0.4 [0.2 – 0.6]	0.4 [0.2 – 0.6]
Water process	<i>L. monocytogenes</i> 1/2c	0.4 [0.2 – 0.6]	0.4 [0.2 – 0.6]

(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 levels of the alternative method and of the reference method are identical and ranged between 0.2 and 1.1 CFU/25g.

INCLUSIVITY / EXCLUSIVITY

Implementation of alternative method only

- 60 strains of *Listeria monocytogenes* were detected out of 60 tested.
- The study of 18 strains not belonging to the *Listeria* genus did not detect the presence of cross-reactions.

The study of 19 strains of *Listeria* not *monocytogenes* showed that *Listeria ivanovii* had characteristic aspect before 24 hours of incubation, with halos littlest.

PRACTICABILITY

Implementation of alternative method only

- **Response time:**
 - **Positive** results are obtained in 3 to 4 days using the alternative method (after confirmation on Rapid'L.mono Agar medium) or 9 days (after confirmation according to classical tests of the reference method) against 5 to 11 days using the reference method.
 - **Negative** results are obtained in 2 days using the alternative method against 5 days using 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 2 to 9 days.

INTER-LABORATORY STUDY

The inter-laboratory study was conducted in 2008 with 16 participating laboratories. The analyses were carried out on samples of pasteurized milk, artificially contaminated with a *Listeria monocytogenes* strain at the 4 following 3 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	128	128	112	112	112	0	0
1	128	128	112	0	0	112	112
2	128	128	112	0	0	112	112

* Les résultats de 2 laboratoires n'ont pas été exploités : l'un pour réception hors délai des échantillons et l'autre pour cause de non contamination des échantillons par le laboratoire expert.

Calculations

- Relative accuracy : **AC = 100%**
- Specificity : **SP = 100%**
- Sensitivity : **SE = 100%**

Interpretation

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

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

$$\text{Alternative method :} \\ (PA + PD) / (PA + PD + ND) = 100\%$$

$$\text{Reference method :} \\ (PA + ND) / (PA + PD + ND) = 100\%$$

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:

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

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

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

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

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