



Alternative methods for agribusiness
Analytical performances certified

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

Certificate No.: BRD 07/04 – 09/98

Validation date: 15.09.1998
Extension dates: 07.09.1999
25.09.2008
Renewal dates : 28.11.2002
15.09.2006*
End of validity: 15.09.2010

* EN ISO 16140 protocol was used in 2006 during the 2nd renewal

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

RAPID'L. MONO
Detection

Protocol reference : RAPID'L.mono (356-3694 / 355-5294) – V11
Rhamnose test (355-3669) – V0

SCOPE

All human food products and environmental samples.

RESTRICTIONS OF USE

None.

REFERENCE METHOD

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

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PRINCIPLE OF THE METHOD

The method enables plating and colony isolation on a single medium (RAPID'L. *Mono*) after a primary sample enrichment.

The RAPID'L. *Mono* is an agar plate for specific detection of *Listeria monocytogenes* by chromogenic detection of PIPLC activity (Phosphatidyl Inositol Phospholipase C) in *L. monocytogenes* and *L. ivanovii*, which form blue colonies and in the other *Listeria* species which form white colonies.

Xylose fermentation is used to differentiate *L. ivanovii* (xylose +ve: forms a yellow halo around the colony) from *L. monocytogenes* (xylose -ve: no halo around the colony).

In the context of AFNOR Validation, all samples identified as positive by the RAPID'L. *mono* test must be confirmed by one of the following means :

Confirmation of positive results for *Listeria monocytogenes* :

1. According to classical tests described in methods standardized by CEN or ISO starting from isolated RAPID'L. *mono* colonies, or
2. using nucleic probes as described in EN ISO 7218 standard (including or not the purification step), or
3. By implementing a spot sub-culture of an isolated colony on an Ottaviani & Agosti Agar *Listeria* plate, directly from RAPID'L. *mono* (respecting specifications in the manufacturer's test instructions), or
4. By carrying out Rhamnose Test, or
5. By implementing any other AFNOR validated method based on a principle different from the RAPID'L. *mono* test (respecting specifications in the manufacturer's test instructions).

Confirmation of positive results for *Listeria* other than *Listeria monocytogenes* :

- According to classical tests described in methods standardized by CEN or ISO starting from isolated RAPID'L. *mono* colonies, or
- using nucleic probes as described in EN ISO 7218 standard (including or not the purification step), or
- By using a miniaturised gallery of identification based on isolated colonies on RAPID'L. *mono*, or
- By implementing any other AFNOR validated method based on a principle different from the RAPID'L. *mono* test, (respecting specifications in the manufacturer's 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 : VALIDATION HISTORY

Renewal study of 2006 : Since the previous Validation, the formula of RAPID'L. *mono* has been changed, and the use of plates has been extended to all *Listeria spp.* The reference method has been changed (addition of amendment A1) and the protocol described in standard EN ISO 16140 has been applied.

The study has been almost entirely redone, except for a few results taken from the previous accuracy and exclusivity studies.

Extension study of 2008 : New assay permitted to validate a new test of confirmation Rhamnose test. The results obtained were in accordance with those expected.

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

Response of *Listeria monocytogenes* (after 22 hours of incubation)

In 2002 and 2006 tests were carried out on 483 product samples, of which 202 were naturally contaminated, 47 artificially contaminated, and 234 non-contaminated, belonging to the following main food product categories :

Dairy products, meat products, vegetables, fishery products, environmental samples.

All samples were analysed **once** by the **two methods**.

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

Responses	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 239 ⁽¹⁾	Positive deviation A+ / R- PD = 4 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 5 ⁽²⁾	Negative agreement A- / R- NA = 235 ⁽³⁾

(1) Confirmed positives

(2) and (3) of which no sample presumed positive by the alternative method was negative after confirmation

Percentages obtained compared to the reference method are as follows :

- Relative accuracy : **AC = 98.1%**

- Relative specificity : **SP = 98.3%%**

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

- Relative sensitivity : **SE = 98.0%**

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

Alternative method :

$$(PA + PD) / (PA + PD + ND) = 98.0 \%$$

Reference method :

$$(PA + ND) / (PA + PD + ND) = 98.4 \%$$

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

PD = 4, ND = 5, thus Y = PD + ND = 9; $6 \leq Y \leq 22$ m = 4, M = 1 thus m > M

Conclusion

The two methods are no different in statistical terms.

Response of *Listeria other than monocytogenes* (after 48 hours of incubation)

In 2006 tests were carried out on 420 product samples, of which 99 were naturally contaminated, 87 artificially contaminated, and 234 non-contaminated, belonging to the following main food product categories :

Dairy products, meat products, vegetables, fishery products, environmental samples.

All samples were analysed **once** by the **two methods**.

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

Responses	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 174 ⁽¹⁾	Positive deviation A+ / R- PD = 5 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 7 ⁽²⁾	Negative agreement A- / R- NA = 234 ⁽³⁾

- (1) Confirmed positives
- (2) Of which no sample presumed positive by the alternative method was negative after confirmation
- (3) Of which 7 samples presumed positive by the alternative method after 48 hours incubation were negative after confirmation

Percentages obtained compared to the reference method are as follows :

- Relative accuracy : **AC = 97.1%**
- Relative specificity : **SP = 97.9%**

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

- Relative sensitivity : **SE = 96.1%**

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

Alternative method :

$$(PA + PD) / (PA + PD + ND) = 96.2 \%$$

Reference method :

$$(PA + ND) / (PA + PD + ND) = 97.3\%$$

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

PD = 5, ND = 7, thus Y = PD + ND = 12; $6 \leq Y \leq 22$ m = 5, M = 2 thus m > M

Conclusion

The two methods are no different in statistical terms.

Relative DETECTION LEVEL

Comparison of performances of the alternative method and the reference method

Tests were carried out in 2006, on 7 combinations of food products/strains described in the table below.

These products represent the following food product categories : Dairy products, meat products, vegetables, fishery products, environmental samples.

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

Results obtained are as follows:

Matrix	Strain(s)	Relative detection level (CFU/25g or 25 ml) With confidence interval (3) LOD ₅₀	
		Alternative method	Reference method
Rillettes	<i>L. monocytogenes</i> 1/2c	0.7 [0.4 - 1.2]	0.7 [0.4 - 1.2]
	<i>L. welshimeri</i>	0.7 [0.4 - 1.0]	0.7 [0.4 - 1.0]
Untreated milk	<i>L. monocytogenes</i> 1/2b	0.4 [0.2 - 0.8]	0.4 [0.2 - 0.8]
Smoked salmon	<i>L. monocytogenes</i> 1/2a	0.7 [0.4 - 1.3]	0.7 [0.4 - 1.3]
Red cabbage	<i>L. monocytogenes</i> 4b	0.9 [0.5 - 1.6]	0.6 [0.3 - 1.2]
Process water	<i>L. monocytogenes</i> 1/2c	0.3 [0.2 - 0.4]	0.3 [0.2 - 0.4]
	<i>L. innocua</i>	0.6 [0.3 - 1.1]	0.6 [0.3 - 1.1]

(3) **LOD₅₀**: estimation of level of contamination enabling positive detection by the 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 detection level of the alternative method is between 0.2 and 1.6 CFU/25 g.

The detection level of the reference method is between 0.2 and 1.2 CFU/25 g.

SELECTIVITY (INCLUSIVITY / EXCLUSIVITY) 1998, 1999, 2006 et 2008

Use of alternative method only

Inclusivity study (carried out in 2006 and 2008)

- 200 strains of *Listeria monocytogenes* were detected out of 200 tested.
- 82 strains of *Listeria* other than *monocytogenes* were detected out of 82 tested.

Exclusivity study (carried out in 1998 and 1999 and 2008)

- The study of 100 strains not belonging to the genus *Listeria monocytogenes* did not detect the presence of any cross-reaction (blue colonies with no yellow halo) even with strains listed in the bibliography as having PIPLC activity: *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus aureus*.
- During the accuracy studies carried out in 2006, several strains were identified because they produced colonies that looked similar to *Listeria* other than *monocytogenes*: these were *Bacillus*, *Enterococcus faecium*, *Oeiskovia xanthineolytica*, *Gardnerella vaginalis* and *Lactobacillus*. However, these few strains have a different appearance from *Listeria* in the GRAM test.

PRACTICABILITY

Implementation of alternative method only

Response time for *Listeria monocytogenes*:

- **Positive** results are obtained in 3 to 10 days using the alternative method (depending on the type of confirmation used) against 9 to 12 days using the reference method.
- **Negative** results are obtained in 2 days using the alternative method against 5 days using the reference method.

Response time for *Listeria* other than *monocytogenes*:

- **Positive** results are obtained in 2 to 3 days using the alternative method against 3 to 6 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 3 to 10 days (depending on the type of confirmation used).

INTER-LABORATORY STUDY

The inter-laboratory study was conducted in 2006 with 15 participating laboratories. The analyses were carried out on samples of pasteurized milk artificially contaminated with a *Listeria monocytogenes* strain at the following 3 levels of contamination:

- 0 level
- 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.

The following results were obtained:

Contamin- ation 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	120	120	112	112	112	0	0
1	120	120	112	9	9	103	103
2	120	120	112	0	0	112	112

* One laboratory did not carry out manipulations correctly and the results were not used.

Calculations

- Relative accuracy = **100%**
- specificity = **100%**
- sensitivity = **96%**

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

Interpretation

Results of the collaborative 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 the alternative method) :

Alternative method :

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

Reference method :

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

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. 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 (reproducibility conditions). 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	87%	85%	1.03
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	87%	85%	1.03
L2	100%	100%	1.00

The reduced percentages of accordancy and concordance for level 1 are due to the fact that some contaminated samples were shown to be not contaminated (these results were concordant between the alternative method and the reference method).

Conclusion

Variability of the alternative method (accordancy, 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.

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