Alternative methods for agribusiness
Analytical performances certified

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

Certificate No.: BIO 12/14 – 04/05

Validation date:	**07.04.2005***
Extension dates:	**14.09.2006**
	14.12.2006*
	17.01.2008
	27.03.2008
Renewal dates:	**26.03.2009**
	29.03.2013
End of validity:	**07.04.2017**

**** EN ISO 16140 protocol was used in 2005 for the preliminary study and in 2006 for the interlaboratory study***

The company (Head office, distributor and production site)
bioMérieux
Chemin de l'Orme
69280 MARCY L'ETOILE - FRANCE

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

ChromID™ Ottaviani Agosti Agar (OAA)

Validated for the detection of *Listeria monocytogenes* and other *Listeria* spp

Protocol reference: Ref. 43 641 and 43 649 - 12695 **version H**

SCOPE

All human food product and environmental samples.

RESTRICTIONS

None.

REFERENCE METHOD

EN ISO 11290-1 (February 1997) and its **amendment A1** (February 2005): Microbiology of food and animal feeding stuffs. Horizontal method for the detection and enumeration of *Listeria monocytogenes* – Part 1: detection method.

Managing Director
Florence MÉAUX

PRINCIPLE OF THE METHOD

ChromID™ Ottaviani Agosti Agar (OAA) contains a nutritive base combining different peptones and two substrates including a chromogenic one. It permits growth of all *Listeria* species and enables the corresponding enzyme activity to be revealed. The differentiation of *Listeria monocytogenes* is based on the appearance of an opaque halo around the colony (phospholipase C activity).

After enrichment in half Fraser broth, incubated for 22 to 26 hours at 30°C, isolate on chromID™ Ottaviani Agosti agar and incubate for 22 to 26 h at 37°C.

In the context of NF VALIDATION, all samples identified as presumptive positives of ***Listeria monocytogenes*** by the OAA method must be confirmed by one of the following means:

- According to classical tests described in standardized methods by CEN or ISO (including a purification step), from isolated colonies on chromogenic medium.
- By identification tests defined in standardized methods, with preparation of the *Listeria* API test strip without prior purification if the colonies are sufficiently well isolated (by first checking the purity of the strain submitted for confirmation by isolation on blood agar)
- By *L. monocytogenes* Accuprobe reagent ref. 39500 from colonies that have been isolated or not, directly collected on OAA agar.
- From a colony isolated on chromID™ Ottaviani Agosti agar, by performing a RAPIDEC® Lmono strip.
- By implementing the VIDAS® LMO2 test directly from a suspension of characteristic colonies that have been isolated or not on OAA agar.

In the context of NF VALIDATION, all samples identified as presumptive positives of ***Listeria* non-*monocytogenes*** by the OAA method must be confirmed by one of the following means:

- According to classical tests described (gram catalase and mobility if necessary) described in the standardized methods by CEN or ISO (including a purification step) from isolated colonies on chromogenic medium.
- By performing a gram coloration and the use of the ID Color Catalase reagent directly on characteristic colonies isolated on OAA agar.
- From colonies isolated on OAA agar, by plating on PALCAM agar, (until 15 bites by agar)

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

NOTE 1: Certification concerns the use of the OAA method for the detection with the short protocol.

NOTE 2 (History of validation)

1/ In September 2006, an extension study was to provide an additional confirmation (by isolation ALOA CONFIRMATION® agar, on RAPID'*L.mono* agar, or CHROMagar® *Listeria* Identification).

Assays were performed on pure strains growing on chromID™ OAA and liable to present characteristic colonies:

- 150 *Listeria monocytogenes* strains of different serotypes and origins were tested ;
- 100 non-target strains (50 *Listeria* species other than *monocytogenes* and 50 non-*Listeria* strains) were tested.

The three proposed methods of confirmation were systematically tested. The results obtained complied with the expected results.

2/ In December 2006, the inter-laboratory study was completely repeated in accordance with the requirements of standard EN ISO 16140, subject of an extension of validation.

3/ In January 2008, the study allows the integration of a new case of confirmation, the RAPIDEC® Lmono test. Assays were performed on pure strains growing on ChromID™ OAA and liable to present characteristic colonies:

- 150 *Listeria monocytogenes* strains of different serotypes and origins were tested.
- 100 non-target strains (50 *Listeria* species other than *monocytogenes* and 50 non-*Listeria* strains) were tested.

The obtained results are conformed to those expected, except for one *Listeria monocytogenes* strain, on 150 tested. Furthermore the results obtained after storage till 72 hours after inoculation of RAPIDEC® Lmono strips at room temperature, are identical to those obtained after incubation.

4/ In March 2008, a new extension study allows to extend the validation to all *Listeria spp.* research.

The short protocol of the method was not modified, only the reading has changed: the presence of blue colonies without halo presumptive *Listeria monocytogenes* other than *L. ivanovii* and *L. monocytogenes.*

Complementary assays were performed for relative accuracy/specificity/sensitivity, and relative detection level. Results are detailed in this certificate.

5/ In March 2009, the right to use the NF VALIDATION mark was renewed, without neither modification of the validation protocol nor the reference method since the latest validation.

A new option of confirmation corresponding to the VIDAS LMO2 test has been validated for *Listeria monocytogenes* detection protocol, on the basis of the following results from previous validation studies:

- Results of relative accuracy/specificity/sensitivity and relative detection level obtained in 2005 during the initial validation of OAA method, which included the possibility to confirm with VIDAS LMO2 test.
- Specificity results obtained in 2002 during the initial validation of VIDAS LMO2 method (BIO 12/09 - 07/02) :
  - 50 strains of *Listeria monocytogenes* were detected out 50 tested.
  - The study of 43 strains non- *Listeria monocytogenes*, of which 28 strains not belonging to the *Listeria* genus, did not detect the presence of cross-reactions.

The results, in accordance with those expected, do not modify the following content of this certificate.

6/ In March 2013, the validation of ChromID™ OAA was renewed without conducting an additional validation study, because nor the alternative method, nor the reference method, nor the validation protocol have changed since the previous validation study.

Relative ACCURACY, relative SPECIFICITY and relative SENSITIVITY

Comparison of performances of the alternative method and the reference method

In 2004 assays were performed on ***Listeria monocytogenes*** on 335 product samples, of which 121 were naturally contaminated, 31 artificially contaminated, and 183 non-contaminated, belonging to the following principal food product categories : meat products, dairy products, seafoods products, vegetables and environmental samples.

In 2008, assays were performed on ***Listeria* spp.** on 373 product samples, of which 87 naturally contaminated, 76 artificially contaminated and 210 non-contaminated, belonging to the following main food categories : meat products, dairy products, (of which 20 raw milk cheese), seafood products (of which 20 smoked fish), vegetables, environmental samples.

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

For *Listeria monocytogenes* - Table of results (Cf. Table 1 of the EN ISO 16140 standard):

Answer	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = **145** (1)	Positive deviation A+ / R- PD = **3** (1)
Alternative method negative (A-)	Negative deviation A- / R+ ND = **4** (2)	Negative agreement A- / R- NA = **183** (3)

(1) Confirmed positives

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

(3) Of which 1 sample presumed positive by the alternative method were negative after confirmation

For *Listeria* spp - Table of results (Cf. Table 1 of the EN ISO 16140 standard):

Answer	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = **156** (1)	Positive deviation A+ / R- PD = **2** (1)
Alternative method negative (A-)	Negative deviation A- / R+ ND = **5** (2)	Negative agreement A- / R- NA = **210** (3)

(1) Confirmed positives

(2) Of which 1 sample presumed positive by the alternative method was negative after confirmation

(3) Of which 9 samples presumed positive by the alternative method (and 8 by the reference method) were negative after confirmation

Percentages obtained compared to the reference method are as follows:

	Relative Accuracy **AC**	Relative Specificity **SP**	Relative Sensitivity **SE**
L. monocytogenes research	**97.9 %**	**98.4 %**	**97.3 %**
L. spp research	**97.9 %**	**98.9 %**	**96.9 %**

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

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

	Alternative method : (PA + PD) / (PA + PD + ND) =	Reference method (PA + ND) / (PA + PD + ND) =
L. monocytogenes research	**97.4 %**	**98.0 %**
L. spp research	**96.9 %**	**98.8 %**

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

Listeria monocytogenes:
PD = 3, ND = 4, Y = PD + ND = 7; $6 \leq Y \leq 22$, m = 3, M = 0 So m > M

Listeria spp:
PD = 2, ND = 5, Y = PD + ND = 7; $6 \leq Y \leq 22$, m = 2, M = 0 So m > M

Conclusion

The two methods are not statistically different, whether for *Listeria spp* or *Listeria monocytogenes* research.

Tests of storage of OAA agar plates during 48 hours at 2-8°C

During the accuracy study, OAA agar plates were stored at 2 – 8°C for 48 hours before making a second reading, in order to postpone reading of the plates if needed. The cold storage of OAA agar plates didn't have impact on the results.

Relative DETECTION LEVEL

Comparison of performances of the alternative method and the reference method

Tests were performed in 2004, on 5 combinations of "food product/strain" described in the table below.

These products represent the following categories of food: dairy products, meat products, vegetable products, seafood products and environmental samples.

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

Results obtained are as follows:

		Relative detection level (CFU/25g or 25 ml) With confidence interval (3) LOD_{50}	
Matrix	**Strain**	**Alternative method**	**Reference method**
Traditional "rillettes"	*L.monocytogenes* 1/2c	0.8 [0.4 – 1.5]	0.7 [0.4 – 1.3]
Raw milk	*L.monocytogenes* 1/2b	0.4 [0.3 – 0.7]	0.4 [0.3 – 0.7]
Smoked salmon	*L.monocytogenes* 1/2a	0.4 [0.2 – 0.8]	0.4 [0.2 – 0.8]
Red cabbage	*L.monocytogenes* 4b	0.7 [0.5 – 1.1]	0.7 [0.5 – 1.1]
Process Water	*L.monocytogenes* 1/2c	0.5 [0.3 – 1.0]	0.5 [0.3 – 1.0]

(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.*

In 2008, the detection level has been determined for two *Listeria* strains other than *monocytogenes*, and with combinations of products in the following table:

		Relative detection level (CFU/25g or 25 ml) With confidence interval (3) LOD_{50}	
Matrix	**Strain**	**Alternative method**	**Reference method**
Traditional "rillettes"	*Listeria innocua*	0.6 [0.4 – 1.0]	0.6 [0.4 – 1.0]
Raw milk	*Listeria ivanovii*	0.7 [0.4 – 1.4]	0.7 [0.4 – 1.4]

(3) **LOD_{50}**: See above

Conclusion

For *Listeria monocytogenes* research the limit detection of the alternative method ranges between 0.2 and 1.5 cells per 25 grams. The limit detection of the reference method ranges between 0.2 and 1.3 cells per 25 grams.
For *Listeria* other than *monocytogenes*, the detection limits of the two methods are identical and range between 0.4 and 1.4 cells per 25 grams.

INCLUSIVITY / EXCLUSIVITY
Implementation of alternative method only

Study 2005 (*L. monocytogenes*):

- 50 strains of *Listeria monocytogenes* were detected out of 50 tested.
- 16 non-*Listeria monocytogenes* strains all developed by giving blue colonies without a halo, except for 3 strains of *Listeria ivanovii* (out of 3 tested) which gave characteristic colonies with a halo.

  These 3 strains also gave characteristic colonies on the media of the reference method.
- The study of 18 strains not belonging to the *Listeria* genus did not detect the presence of cross-reactions.

Studies 2006 and 2007 (*L.* spp):

Results of *Listeria* strains other than *monocytogenes* including in the 2005 exclusivity study have been taken again for the inclusivity study. Also a significant number of strains other than *Listeria* were tested in exclusivity, during the first validation and during the 2006 extension.

- In total 62 non-*Listeria monocytogenes* strains of which 29 *Listeria ivanovii* and 153 *Listeria monocytogenes* strains were tested and all give expected results.
- Among 63 strains other genus, some *Bacillus* strains and *Enterococcus* gave blue colonies on chromID^{TM} OAA agar, but were considered as negative after the confirmation step. These strains gave presumptive positives with the reference method.

PRACTICABILITY
Implementation of alternative method only

- **Positive** results are obtained in 2 to 8 days using the alternative method (according to the confirmation level used) 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 positive presumptive results using the alternative method, but found negative following confirmation, these negative results are obtained in 2 to 4 days depending on the method of confirmation used.

INTERLABORATORY STUDY

The interlaboratory study was conducted in 2006 with 16 participating laboratories. The analysis were carried out on samples of pasteurized milk, artificially contaminated with a *Listeria monocytogenes* strain at 3 following levels of contamination:

- Level 0: 0 CFU / 25ml
- Level 1: 3 CFU / 25ml
- Level 2: 30 CFU / 25ml

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

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	128	112	104	104	104	0	0
1	128	108	104	0	0	104	104
2	128	112	104	0	0	104	104

** Two laboratories received the samples late and did not perform the analyses and one laboratory did not conduct the analyses on four samples because of leaks.*
*** In conclusion, these three laboratories were excluded.*

Calculations

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

Interpretation

Results of the collaborative study are similar 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):

Alternative method:	Reference method:
(PA + PD) / (PA + PD + ND) = 100%	(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 samples 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 (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:
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	100 %	100 %	1.0
L2	100 %	100 %	1.0

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

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

Conclusion

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