



**Alternative methods for agribusiness
Analytical performances certified**

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

Certificate No : BRD 07/10 – 04/05

Validation date:	07.04.2005
Extension dates:	15.12.2006
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	05.02.2010
First renewal:	26.03.2009
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The company **BIO-RAD**
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is hereby authorized to refer to this **AFNOR Validation certificate** for the following alternative **qualitative** analysis method:

iQ-Check™ *Listeria monocytogenes* II (Cat. # 357-8124)

Protocol reference: **808464 – Rev.D**

SCOPE

All human foodstuffs and environmental samples.

RESTRICTIONS OF USE

None.

REFERENCE METHOD

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

A handwritten signature in black ink, appearing to read "Jacques Beslin", written over a horizontal line.

**Deputy General Manager
Jacques BESLIN**

AFNOR Certification

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PRINCIPLE OF THE METHOD

The iQ-Check *Listeria monocytogenes* II test is based on gene amplification and real-time PCR detection. It uses primers and a DNA probe specific to *Listeria monocytogenes*. Following the pre-enrichment step (and enrichment step if necessary), lysis of bacteria releases bacterial DNA. The amplification and detection steps are then performed in a thermal cycler. Following the reaction, fluorescence is emitted and measured directly by the thermal cycler. The instrument software analyses the results and displays them in the form of curves for interpretation.

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 or ISO (including a purification step), after isolation on selective agars such as *Listeria* agar according to Ottaviani and Agosti, or PALCAM or Oxford agars.
- By implementing the RAPID'*L.mono* method from the enrichment step in half Fraser broth, or by isolation on RAPID'*L.mono* agar from the enrichment step in LSB broth. The presence of characteristic colonies confirms the iQ-Check result.
- By implementing any other AFNOR validated method based on a principle different from the iQ-Check *Listeria monocytogenes* II method, and following specifications in the test instructions.

In the event of discordant results (positive with the alternative method, unconfirmed with one of the three above-mentioned options), the laboratory must take all necessary steps to ensure the validity of the result obtained.

NOTE (History of validation)

1/ The December 2006 extension study is a result of the following modifications made to the iQ-Check *Listeria monocytogenes* method:

- Use of a specific enrichment broth (LSB broth) for improved *Listeria* resuscitation.
- Addition of a simplified (easy) lysis protocol no longer requiring the first centrifugation step.
- Fluorophore and lysis reagent modification.

These new protocols are alternatives to the originally validated protocol, involving enrichment in half Fraser broth and using a standard lysis protocol.

For human foodstuffs and environmental samples, a new preliminary study was conducted with the following 2 protocols:

- Enrichment in LSB broth followed by the standard lysis protocol
- Enrichment in LSB broth followed by the easy lysis protocol

For environmental samples only, an additional alternative protocol was tested during the new preliminary study, in order to take into account any possible inhibition reactions caused by these products: enrichment in half Fraser broth, followed by TSB broth, then the easy lysis protocol.

Results obtained in 2004 with the half Fraser protocol followed by standard lysis were kept. The inter-laboratory study was not repeated. It was conducted in 2005.

2/ Additional internal assays were realised in 2007 and accepted by the AFNOR VALIDATION Technical Board, in order to compare two new thermal cyclers (iQTM5 and MiniOpticonTM) to one of the thermal cyclers initially accepted (Chromo4TM). These tests are not detailed in this certificate.

The four thermal cyclers (iCycler iQTM, Chromo4TM, iQTM5 and MiniOpticonTM) can now be used in the context of AFNOR VALIDATION.

3/ In January 2009, a new study was conducted extending the validation to include the use of a new version of the Opticon MonitorTM software, which offers in addition to a manual analysis, the option of automated data analysis.

Tests were conducted internally and by a third party, and followed the standard extraction protocol after enrichment in LSB. All iQ-Check tests were done in the Chromo4, and data analysed both manually and with the automated option of the Opticon Monitor™ software.

These assays demonstrated that manual and automated data analysis of samples gave equivalent results. For clarity, results of this study are not detailed in this certificate.

4/ In March 2009, the validation of iQ-Check *Listeria monocytogenes* II was renewed with no additional study as the test had not been modified since the last validation and the reference method has remained unchanged.

5/ In February of 2010, the following extensions were validated by the AFNOR VALIDATION technical committee:

- Modification of the extraction step, using a new "Deepwell plate" format (in addition to the "tube" format validated before). Internal assays showed that these modifications did not have any impact on rendered results.
- The CFX Manager™ software can be used for a complete automated analysis for the CFX96™ and the Mini Opticon™ real-time PCR instruments. Internal assays showed that results obtained with these new combinations of automated systems were equivalent to those obtained with instruments and software validated before.

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

In 2004, tests were performed on 343 product samples, including 123 naturally contaminated, 38 artificially contaminated and 182 non-contaminated, belonging to the following main food categories : meats, dairy products, seafood, fruits and vegetables, and environmental samples (except breeding samples).

The tests were performed using the following protocol: Half Fraser followed by standard lysis.

All samples were analysed in **single** by **both** methods.

Table of results obtained using half Fraser + standard lysis protocol, all categories

(Cf. Table 1 of the NF 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 = 2 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 4 ⁽²⁾	Negative agreement A- / R- NA = 182 ⁽³⁾

(1) Confirmed positives

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

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

In 2006, additional tests were performed on 328 product samples, including 141 naturally contaminated, 30 artificially contaminated and 157 non-contaminated, belonging to the same main food categories.

The tests were conducted using two new protocols: LSB broth followed by standard lysis, or by easy lysis protocol.

All samples were analysed in **single** by **both** methods.

Table of results obtained using LSB broth incubated for 22h + standard lysis protocol, all categories

(Cf. Table 1 of the NF EN ISO 16140 standard):

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 138 ⁽¹⁾	Positive deviation A+ / R- PD = 22 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 11 ⁽²⁾	Negative agreement A- / R- NA = 157 ⁽³⁾

(1) Confirmed positives

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

(3) Of which 7 samples presumed positive by the alternative method were negative after confirmation

Table of results obtained using LSB broth incubated for 24h + easy lysis protocol, all categories

(Cf. Table 1 of the NF EN ISO 16140 standard):

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA=135 ⁽¹⁾	Positive deviation A+ / R- PD = 21 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 14 ⁽²⁾	Negative agreement A- / R- NA = 158 ⁽³⁾

(1) Confirmed positives

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

(3) Of which 4 samples presumed positive by the alternative method were negative after confirmation

Table of results obtained with half Fraser broth + TSB broth + easy lysis protocol, for the environmental samples category

(Cf. Table 1 of the NF EN ISO 16140 standard):

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 30 ⁽¹⁾	Positive deviation A+ / R- PD = 0 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 0 ⁽²⁾	Negative agreement A- / R- NA = 31 ⁽³⁾

(1) Confirmed positives

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

(3) Of which 2 samples presumed positive by the alternative method were negative after confirmation

For 2004 and 2006 studies, the percentages obtained, with respect to the reference method, are as follows:

Criteria / protocol	half Fraser + standard lysis	LSB broth 22h + standard lysis	LSB broth 24h + easy lysis	half Fraser + TSB + easy lysis
	All products	All products	All products	Environmental samples
Relative accuracy (AC)	98.3%	89.9%	89.3%	100%
Relative specificity (SP)	98.9%	87.7%	88.3%	100%
Relative sensitivity (SE)	97.5%	92.6%	90.6%	100%

Note: relative specificity below 100% is due to a number of additional confirmed positive results and not from false positives.

Sensitivity was also recalculated, taking into account all confirmed positives (including the additional positive results of the alternative method):

All products	Alternative method (PA + PD) / (PA + PD + ND) =	Reference method (PA + ND) / (PA + PD + ND) =
half Fraser + standard lysis	97.5%	98.6%
LSB broth 22h + standard lysis	93.6%	87.1%
LSB broth 24h + easy lysis	91.8%	87.6%

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

Half Fraser + standard lysis protocol

PD = 2, ND = 4, Y = PD + ND = 6

$6 \leq Y \leq 22$, $m = 2$, $M = 0$ hence $m > M$

LSB broth 22h + standard lysis protocol

PD = 22, ND = 11, Y = PD + ND = 33

According to Mc Nemar test: d minimum = 12; $d = 22 - 11 = 11$

LSB broth 24h + easy lysis protocol

PD = 21, ND = 14, Y = PD + ND = 35

According to Mc Nemar test: d minimum = 12; $d = 21 - 14 = 7$

Conclusion

For all three protocols, the tests conclude that the results of the two methods are equivalent.

Relative DETECTION LEVEL

Comparison of performances of the alternative method and the reference method

Tests were carried out, in 2004 and 2006, on 5 combinations of food products/strain described in the following table.

These products represent the following food categories:

Meats, dairy products, seafood, fruits and vegetables, and environmental samples (excluding breeding samples).

Products were analysed 6 times, by both methods, at 4 different contamination levels.

Results obtained in 2004, using the half Fraser and standard lysis protocol, were as follows:

Matrix	Strain	Relative detection level LOD ₅₀ (3) With confidence interval (CFU/25g or 25 ml)	
		Alternative method	Reference method
Traditional rillettes	<i>L. monocytogenes</i> 1/2c	0.7 [0.4 - 1.2]	0.7 [0.4 - 1.2]
Raw milk	<i>L. monocytogenes</i> 1/2b	0.5 [0.3 - 0.8]	0.5 [0.3 - 0.8]
Smoked fish fillets	<i>L. monocytogenes</i> 1/2a	0.3 [0.2 - 0.5]	0.3 [0.2 - 0.5]
4th range raw vegetables	<i>L. monocytogenes</i> 4b	0.9 [0.5 - 1.6]	0.9 [0.5 - 1.6]
Process water	<i>L. monocytogenes</i> 1/2c	0.6 [0.3 - 0.8]	0.6 [0.3 - 0.8]

(3) LOD₅₀: see table below

Results obtained in 2006, for both lysis protocols using LSB broth, whichever lysis protocol used, are as follows:

Matrix	Strain	Relative detection level LOD ₅₀ (3) With confidence interval (CFU/25g or 25 ml)	
		Alternative method	Reference method
Rillettes	<i>L. monocytogenes</i> 1/2b	0.5 [0.3 - 0.9]	0.5 [0.3 - 0.9]
Raw milk	<i>L. monocytogenes</i> 1/2b	0.6 [0.4 - 0.9]	0.5 [0.3 - 0.8]
Smoked salmon	<i>L. monocytogenes</i> 4b	0.4 [0.2 - 0.8]	0.5 [0.3 - 0.8]
Mixed vegetables	<i>L. monocytogenes</i> 1/2a	0.6 [0.3 - 1.2]	0.6 [0.3 - 1.1]
Process water	<i>L. monocytogenes</i> 1/2c	0.6 [0.3 - 1.2]	0.6 [0,3 - 1.1]

(3) LOD₅₀: see table below

Results obtained in 2006, for the "environmental samples" protocol with half Fraser broth, followed by TSB broth and easy lysis protocol, are as follows:

Matrix	Strain	Relative detection level LOD ₅₀ (3) With confidence interval (CFU/25g or 25 ml)	
		Alternative method	Reference method
Process water	<i>L. monocytogenes</i> 1/2c	0.6 [0.3-1.1]	0.6[0.3-1.1]

(3) LOD₅₀: estimation of level of contamination enabling positive detection with 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

For protocol with half Fraser then standard lysis protocol:

The detection level of the alternative method is between 0.2 and 1.6 CFU/25g. It is identical to that of the reference method.

For both protocols using LSB broth, whichever lysis protocol is used:

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

The detection level of the reference method is of between 0.3 and 1.1 CFU/25g.

For the "environmental samples" protocol with half Fraser and easy lysis protocol

The detection level of the alternative method is identical to that of the reference method, i.e. between 0.3 and 1.1 CFU/25g.

INCLUSIVITY/EXCLUSIVITY

Implementation of alternative method only

2004 tests using the half Fraser + standard lysis protocol:

- 50 strains of *Listeria monocytogenes* were detected out of the 50 tested.
- The study of 33 non-*Listeria monocytogenes* strains showed one cross-reaction with one strain of *Enterococcus faecium* grown in nutrient broth, but transfer to selective medium failed to confirm this result.

2006 tests using the following two protocols:

- LSB broth + easy lysis protocol
- half Fraser broth + TSB + easy lysis protocol
- 50 strains of *Listeria monocytogenes* were detected out of the 50 tested.
- The study of 36 non-*Listeria* strains resulted in no cross reactions.

PRACTICABILITY

Implementation of alternative method only

- **Time to results:**
 - **Positive** results are obtained in 2 days using the alternative method (after confirmation on Rapid'*L.mono*), or in 9 days (after confirmation using classical tests) compared to 9 to 11 days using the reference method.
 - **Negative** results are obtained in one day using the alternative method, compared to five days using the reference method.
 - In the case of results presumed positive using the alternative method, but shown to be negative after confirmation, negative results are obtained in 2 days (by isolation on RAPID'*L.mono*), or in up to 11 days (confirmation by conventional tests).
- **Staff training:** For technicians with no PCR training, an initial training of 4 to 5 days would seem necessary. For technicians with training in standard microbiology and PCR techniques, two days of training are required.

INTER-LABORATORY STUDY

The inter-laboratory study was conducted in 2005, involving 15 participating laboratories. Analyses were carried out on samples of pasteurized milk, artificially contaminated with a strain of *Listeria monocytogenes* at the following three contamination levels:

- 0
- slightly higher than the relative detection level
- 10 times greater than the previous level

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

For the iQ-Check *Listeria monocytogenes* method; the protocol tested was the initially validated protocol, involving enrichment in half Fraser broth and standard lysis.

Results:

Contamination levels	Total number of samples	Number of samples analyzed*	Number of results processed	Number of negative results		Number of positive results	
				REF	ALT	REF	ALT
0	120	112	112	111	111	1	1
1	120	112	112	1	2	111	110
2	120	112	112	0	0	112	112

* One laboratory failed to perform the analysis due to an organization problem that occurred the day the samples were received.

Calculations

- Relative accuracy: **AC = 99.7%**
- Specificity: **SP = 99.1%**

The percentage specificity obtained is due to a sample found positive by one laboratory with both methods (reference method and alternative method), although it had not been contaminated by the organizing laboratory.

- Sensitivity: **SE = 99.1%**

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 (including additional positive results with the alternative method):

$$\begin{array}{ll} \text{Alternative method:} & \text{Reference method} \\ (PA + PD) / (PA + PD + ND) = 99.6\% & (PA + ND) / (PA + PD + ND) = 100\% \end{array}$$

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 gives the values for the **alternative method**:

Contamination level	Accordance (%)	Concordance (%)	COR
L0	98	98.2	1.00
L1	97	96.5	1.00
L2	100	100	1.00

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

Contamination level	Accordance (%)	Concordance (%)	COR
L0	98	98.2	1.00
L1	98	98.2	1.00
L2	100	100	1.00

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