



**Alternative methods for agribusiness
Analytical performances certified**

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

Certificate No : BRD 07/13 – 05/07

Validation date :	24.05.2007
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	26.01.2009
	05.02.2010
End of validity :	24.05.2011

The company **BIO-RAD**
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FRANCE

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

iQ-Check™ *Listeria* spp (Cat. # 357-8113)

Protocol reference: **808465 – Rev.C**

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". The signature is fluid and cursive, with a long horizontal stroke at the end.

**Deputy General Manager
Jacques BESLIN**

AFNOR Certification

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PRINCIPLE OF THE METHOD

The iQ-Check *Listeria* spp test is based on gene amplification and real-time PCR detection. It uses primers and a DNA probe specific to *Listeria*. Following the enrichment step in *Listeria* Special broth (LSB), 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 conventional 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[®]*Listeria* spp or RAPID[®]*L.mono* method from the enrichment step. The presence of characteristic colonies confirms the iQ-Check result.
- By implementing any other method certified AFNOR VALIDATION based on a principle different from the iQ-Check *Listeria* spp 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 following 2 protocols were used during the validation study in 2007:

- (1) Enrichment in LSB broth followed by the standard lysis protocol
- (2) Enrichment in LSB broth followed by the easy lysis protocol

2) Additional internal assays were conducted in 2007 and accepted by the AFNOR VALIDATION Technical Board, in order to compare two new thermal cyclers (iQ[™]5 and MiniOpticon[™]) to one of the thermal cyclers initially accepted (Chromo4[™]). These tests are not detailed in this certificate. The four thermal cyclers (iCycler iQ[™], Chromo4[™], iQ[™]5 and MiniOpticon[™]) 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 Monitor[™] 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 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

Performance comparison of the alternative and reference methods

In 2007, tests were performed on 363 product samples, including 182 naturally contaminated, 30 artificially contaminated and 151 non-contaminated, belonging to the following main food categories : meats, dairy products (including cheese from raw milk), seafood, fruits and vegetables, and environmental samples (except breeding samples).

All samples were analysed in single by both methods.

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 = 177 ⁽¹⁾	Positive deviation A+ / R- PD = 22 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 13 ⁽²⁾	Negative agreement A- / R- NA = 151 ⁽³⁾

(1) Confirmed positives

(2) Of which 2 sample presumed positive by the alternative method with the standard lysis protocol were negative after confirmation

(3) Of which 8 samples presumed positive by the alternative method with the standard lysis protocol were negative after confirmation, and of which 6 samples presumed positive by the alternative method with the easy lysis protocol were negative after confirmation

The final results are identical regardless of the lysis protocol used.

The percentages obtained, with respect to the reference method, are as follows:

- Relative accuracy: **AC = 90.4%**
- Relative specificity: **SP = 87.3%**
- Relative sensitivity: **SE = 93.2%**

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):

Alternative method:

$$(PA + PD) / (PA + PD + ND) = 93.9\%$$

Reference method:

$$(PA + ND) / (PA + PD + ND) = 89.6\%$$

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

PD = 22, ND = 13, Y = PD + ND = 35

According to Mc Nemar test: d minimum = 12; d = 22-13 = 9

Conclusion

The two methods are statistically equivalent.

The two methods use different enrichment broths and the percentage of naturally contaminated samples is high (86%), factors which strongly influence the number of discordant results.

Relative DETECTION LEVEL

Performance comparison of the alternative and reference methods

Tests were carried out in 2007, 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 (except breeding samples).

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

Results obtained, identical regardless of the 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
Rillettes	<i>Listeria welshimeri</i>	0.3 [0.2 - 0.5]	0.4 [0.2 - 0.6]
Raw milk	<i>Listeria monocytogenes</i> 1/2b	0.9 [0.6 - 1.5]	0.7 [0.4 - 1.3]
Mixed vegetables	<i>Listeria seeligeri</i>	0.7 [0.3 - 1.4]	0.6 [0.3 - 0.9]
Smoked salmon	<i>Listeria monocytogenes</i> 1/2a	0.6 [0.3 - 1.2]	0.6 [0.3 - 1.1]
Process water	<i>Listeria innocua</i> 1/2c	0.6 [0.3 - 1.1]	0.6 [0.4 - 1.1]

(3) LOD₅₀: estimation of contamination level required to achieve 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

The detection level of the alternative method, for both lysis protocols, is between 0.2 and 1.5 CFU/25g. The detection level of the reference method is between 0.2 and 1.3 CFU/25g.

INCLUSIVITY/EXCLUSIVITY

Implementation of alternative method only

- All 50 strains of *Listeria monocytogenes* and 34 strains of *Listeria* other than *monocytogenes* were detected.
- The study of 32 non-*Listeria* strains resulted in no cross-reactions.

PRACTICABILITY

Implementation of alternative method only

- **Time required for results:**
 - **Positive** results are obtained:
 - For *Listeria* genus: in 2 days using the alternative method (after confirmation), compared to 3 days using the reference method.
 - For *Listeria* species: in 2 to 9 days using the alternative method (depending on the confirmation method) compared to 3 to 11 days using the reference method.
 - **Negative** results are obtained in 1 day using the alternative method, compared to 5 to 11 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 RAPID'*Listeria* spp), or 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, 2 days of training are required.

INTER-LABORATORY STUDY

The inter-laboratory study was conducted in 2007, involving 13 participating laboratories. Analyses were performed on samples of pasteurized milk, artificially contaminated with a strain of *Listeria innocua* 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 48 analyses for each participating laboratory.

Results:

Contami- nation 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	104	104	80	80	80	0	0
1	104	104	80	1	1	79	79
2	104	104	80	0	0	80	80

* Two laboratories received samples beyond the deadline, at a temperature superior to the set criteria. One laboratory encountered difficulties implementing the alternative method. These 3 sets of results were not taken into account in the statistical analysis.

Calculations

- Relative accuracy is **99.2%**
- Specificity is **100%**
- Sensitivity is **98.8%**

Interpretation

Then values obtained for the collaborative study are higher than those obtained for the preliminary study. This can be explained by the fact that a single artificially contaminated matrix was analyzed.

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

$$\text{Alternative method:} \\ (PA + PD) / (PA + PD + ND) = 99.4\%$$

$$\text{Reference method} \\ (PA + ND) / (PA + PD + ND) = 99.4\%$$

Accordance, concordance and concordance odds ratio:

Accordance: % chance of obtaining the same result for two identical samples analysed by the same laboratory under repeatability conditions. The accordance is the average (mean) of the probabilities that two replicates should have the same result for each laboratory.

Concordance: % chance of obtaining the same result for two identical samples analysed by two different laboratories (reproducibility conditions). Concordance is the % of all replicate pairs having the same result.

Concordance odds ratio (COR): this ratio is 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** and for the **reference method**:

Contamination level	Accordance (%)	Concordance (%)	COR
L0	100	100	1.00
L1	98	97.5	1.15
L2	100	100	1.00

Conclusion

The 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.

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