



**Alternative methods for agribusiness
Analytical performances certified**

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

Certificate No.: QUA-18/05-07/08

**Validation date : 01/07/2008
Extension date: 26/01/2009
End of validity : 01/07/2012**

The company OXOID Thermo Fisher Scientific
6, route de Paisy – BP13
69571 DARDILLY cedex
FRANCE

Production Site DuPont Qualicon
ESL Building 400
PO Box 80400
Route 141 & Henry Clay Road
Wilmington DE 19880-0400 USA

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

**BAX[®] System PCR Assay *Listeria monocytogenes* 24E
(QB8125C)**

Protocol reference:

User guide : **2CQ-049.4-0307/FR0908-2** (BAX Q7)
Protocol summary : **2C-058-1207 FR0109** (BAX Q7)
Technical notice : **Rev 27C-007-1207 FR0409**
Software version : **2** (BAX Q7)

SCOPE

All human food products and environmental samples.

RESTRICTIONS OF USE

None.

REFERENCE METHOD

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

**Deputy General Manager
Jacques BESLIN**

A handwritten signature in black ink, appearing to read "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 BAX[®] system for detection of *Listeria monocytogenes* is a detection kit using PCR (Polymerase Chain Reaction) technology. There is a three step protocol: 1) preparation of DNA, 2) amplification and 3) detection. After a lysis step, the BAX[®] cyclor/detector performs both amplification and automated detection.

In the context of AFNOR Validation, all samples identified as positive by the alternative method must be confirmed, within 24 hours of the end of incubation from the final enrichment broth which must be stored at 4°C, by one of the following means:

- According to classical tests described in methods standardized by CEN or ISO (including a purification step);
- By inoculating 10 µl of 24 LEB (enrichment broth) onto *Brilliance*[™] Listeria Agar incubated at 37°C for 24-48 hours.

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

Note 1: Scope of validation

The following protocols of the BAX[®] System PCR Assay Genus Listeria 24E method are in the scope of AFNOR Validation:

- 1) General protocol (human food products and environmental samples, except smoked fish and raw "charcuteries"): enrichment in BAX 24 LEB for 26 hours ±2hours at 37°C ±1°C.
- 2) Specific protocol (smoked fish and raw "charcuteries"), also suitable for cooked "charcuteries": enrichment for 26 hours ±2 hours at 37°C ±1°C in BAX 24 LEB supplemented with a non-selective buffer supplement, and then mixing of the incubated enrichment broth before the lysis step.

Note 2: Validation history

In 2009, complementary tests were performed to validate a new protocol specific to the analysis of smoked fish and raw "charcuteries", which can also be used for cooked "charcuteries" (see above the description of protocols validated). Relative accuracy, relative specificity, relative sensitivity and relative detection level were retested. The results are presented in this certificate.

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

In 2008 tests were carried out, by implementing the general protocol, on 368 product samples, of which 88 were naturally contaminated, 75 artificially contaminated, and 205 non-contaminated, belonging to the following principal food product categories: Dairy products, meat products (except raw "charcuteries"), vegetables, seafood (except smoked fish) and environmental samples.

All samples were analysed in single by the two methods.

Table of results (Cf. Table 1 of the EN ISO 16140 standard) for the **general protocol**:

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 140 ⁽¹⁾	Positive deviation A+ / R- PD = 10 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 13 ⁽²⁾	Negative agreement A- / R- NA = 205 ⁽³⁾

(1) Confirmed positives

(2) Of which no samples, presumed positive by the alternative method, were found to be negative after confirmation

(3) Of which 5 samples, presumed positive by the alternative method, were found to be negative after confirmation

Supplementary tests were carried out in 2009, by implementing the specific protocol, on 78 product samples, of which 41 were naturally contaminated, none artificially contaminated, and 37 non-contaminated, belonging to the following principal food product categories: Smoked fish and raw/cooked "charcuteries".

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

Table of results (Cf. Table 1 of the EN ISO 16140 standard) for the **specific protocol**:

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 26 ⁽¹⁾	Positive deviation A+ / R- PD = 12 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 3 ⁽²⁾	Negative agreement A- / R- NA = 37 ⁽³⁾

(1) Confirmed positives

(2) Of which no samples, presumed positive by the alternative method, were found to be negative after confirmation

(3) Of which 4 samples, presumed positive by the alternative method, were found to be negative after confirmation.

Percentages obtained compared to the reference method are as follows:

	General protocol	Specific protocol
Relative accuracy : AC	93.4 %	80.8 %
Relative specificity : SP	94.9 %	75.5 %
Relative sensitivity : SE	91.5 %	89.7 %

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):

	Alternative method : (PA + PD) / (PA + PD + ND) =	Reference method : (PA + ND) / (PA + PD + ND) =
General protocol	92.0%	93.9%
Specific protocol	92.7 %	70.7%

Analysis of discrepancies (following annex F of standard EN ISO 16140):

		Conclusion
General protocol	PD = 10, ND = 13 ; Y = PD + ND = 23 ; donc Y > 22 McNemar test: D minimal = 10 ; d = PD - ND = 3 ; donc d ≤ D minimal	Equivalent
Specific protocol	PD = 12, ND = 3 ; Y = PD + ND = 15 ; donc Y < 22 ; M=3 ; m=3	No equivalent*

*The results of the statistical test are the consequence of a high number of supplementary results confirmed by the alternative method.

Relative DETECTION LEVEL

Comparison of performances of the alternative method and the reference method

Tests were carried out in 2008 (during the first validation and extension of validation), on 7 combinations of food products/strains.

These products belong to the following principal food product categories:

- For the general protocol: Dairy products, meat products (except raw "charcuteries"), vegetables, seafood (except smoked fish) and environmental samples ;
- For the specific protocol: smoked fish and raw/cooked "charcuteries".

Results obtained are as follows for the general protocol:

Matrix	Strain	Relative detection level (CFU/25g or 25 ml) With confidence interval (3) LOD ₅₀	
		Alternative method	Reference method
Minced meat	<i>L.monocytogenes 1/2b</i>	0.3 [0.2–0.4]	0.4 [0.3–0.6]
Raw milk	<i>L.monocytogenes 1/2b</i>	0.5 [0.3–0.7]	0.6 [0.4–0.9]
Raw fish	<i>L.monocytogenes 1/2a</i>	0.6 [0.3–1.3]	0.4 [0.2–0.9]
Mixture of raw vegetables	<i>L.monocytogenes 4b</i>	0.7 [0.4–1.2]	0.8 [0.6–1.2]
Process water	<i>L.monocytogenes 1/2c</i>	0.4 [0.2–0.6]	0.3 [0.2–0.4]

(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"

Results obtained are as follows for the specific protocol:

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>	0.6 [0.4 – 1.0]	0.6 [0.4 – 0.9]
Smoked salmon	<i>L.monocytogenes 1/2a</i>	0.4 [0.3 – 0.6]	0.5 [0.3 – 0.8]

(3) LOD₅₀: see table above

Conclusion

For the general protocol (all human food products -except smoked fish and raw "charcuteries"- and environmental samples):

The detection limit of the alternative method is between 0.2 and 1.3 CFU/25 g.

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

For the specific protocol (smoked fish and raw/cooked "charcuteries"):

The detection limit of the alternative method is between 0.4 and 1.0 CFU/25 g.

The detection limit of the reference method is between 0.3 and 0.9 CFU/25 g.

INCLUSIVITY / EXCLUSIVITY

Implementation of alternative method only

- 60 strains of *Listeria monocytogenes* were detected out of 60 tested.
- The study of 30 strains not belonging to the genus *Listeria* and the study of 32 strains of *Listeria non-monocytogenes* did not detect the presence of any cross-reaction.

PRACTICABILITY

Implementation of alternative method only

- **Response time :**
 - **Positive** results are obtained in 2 to 3 days using the alternative method (with confirmation onto *Brilliance*TM *Listeria* Agar) against 4 to 7 days using the reference method.
 - **Negative** results are obtained in 1 day 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 3 days.

INTER-LABORATORY STUDY

The inter-laboratory study was conducted in 2008 with 13 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 / 25ml
- 3 CFU / 25ml
- 30 CFU / 25ml

The laboratories tested, using **the reference method and the alternative method (general protocol)**, **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	104	80	80	80	80	0	0
1	104	80	80	0	1	80	79
2	104	80	80	0	0	80	80

* Three laboratories received the samples after the deadline.

Calculations

- Relative accuracy = **99.6%**
- % specificity = **100%**
- % sensitivity = **99.4%**

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 alternative method):

Alternative method :
 $(PA + PD) / (PA + PD + ND) = 99.6\%$

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

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

Contamination level	Accordance	Concordance	COR
L0	100%	100%	1,00
L1	98%	97,2%	1,3
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