



Alternative methods for agribusiness  
Analytical performances certified

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

Certificate No.: RAY 32/03 – 07/10

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

## RAYAL *Listeria*

Protocol reference: RayAl *Listeria* – QCF36 – Issue 1

### SCOPE

All human food products and environmental production samples

### RESTRICTIONS FOR 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 "JBESLIN".

Deputy General Manager  
Jacques BESLIN

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#### AFNOR Certification

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## PRINCIPLE OF THE METHOD

RayAI *Listeria* is an immuno-enzymatic test steps using a microtiter plate coated with specific antibodies directed against *Listeria* antigens and ready-to-use reagents. The test allows the detection of *Listeria* strains after enrichment steps and a heat shock releasing *Listeria* antigens that can eventually be present in the sample.

In the context of AFNOR VALIDATION, all samples identified as positive by the RayAI *Listeria* method must be confirmed by one of the following means:

- Isolate (from unheated RELM stored at 30°C or 2-8°C) on *Listeria* selective agar plate, then use classical tests described in methods standardized by CEN or ISO from colonies (including a purification step)
- Isolate (from unheated RELM stored at 30°C or 2-8°C) on Ottavioni Agosti agar or Rapid Lmono, then perform genus confirmation tests (Gram and catalase). It is possible to perform confirmation tests directly (without prior purification) if the colonies are well isolated.

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.

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

In 2010 tests were carried out on 316 product samples, of which 116 were naturally contaminated, 43 artificially contaminated and 157 non-contaminated, belonging to the following principal food product categories:

Meat products, dairy products, vegetables, seafood products 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):

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 152 <sup>(1)</sup>	Positive deviation A+ / R- PD = 3 <sup>(1)</sup>
Alternative method negative (A-)	Negative deviation A- / R+ ND = 4 <sup>(2)</sup>	Negative agreement A- / R- NA = 157 <sup>(3)</sup>

(1) Confirmed positives

(2) (3) Of which none sample presumed positive by the alternative method was negative after confirmation

Percentages obtained compared to the reference method are as follows:

- Relative accuracy **(AC): 97.8%**
- Relative specificity **(SP): 98.1%**
- Relative sensitivity **(SE): 97.4%**

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 :	Reference method :
$(PA + PD) / (PA + PD + ND) = 97.5\%$	$(PA + ND) / (PA + PD + ND) = 98.1\%$

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

PD = 3, ND = 4, Y = PD + ND = 7 ;  $6 \leq Y \leq 22$  ; m = 3 and M = 0 so m > M

### Conclusion

The two methods are not statistically different.

### Storage of broths during 72 hours at 2-8°C

Results obtained just after storage of RELM broths at 2-8°C during 72 hours were compared to those obtained immediately after incubation.

The cold storage of incubated broths did not modify results obtained just after incubation.

## Relative DETECTION LEVEL

### Comparison of performances of the alternative method and the reference method

Tests were carried out in 2010, on 5 combinations of food products/strains.

Products samples were belonging to the following food categories:

Meat products, dairy products, vegetables, seafood products and environmental samples

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

Results obtained are as follows:

Matrix	Strain	Relative detection level (CFU/25g or 25 ml) With confidence interval (3) LOD <sub>50</sub>	
		Alternative method	Reference method
Potted meat	<i>Listeria welshimeri</i>	0.4 [0.2 – 0.7]	0.4 [0.2 – 0.7]
Raw milk	<i>Listeria ivanovii</i>	0.5 [0.3 – 1.0]	0.5 [0.3 – 1.0]
Grated red cabbage	<i>Listeria monocytogenes</i>	0.4 [0.2 – 0.9]	0.4 [0.2 – 0.9]
Smoked Salmon	<i>Listeria monocytogenes</i>	0.3 [0.2 – 0.4]	0.3 [0.2 – 0.4]
Water process	<i>Listeria innocua</i>	0.3 [0.2 – 0.5]	0.3 [0.2 – 0.5]

(3) LOD<sub>50</sub>: 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.

### Conclusion

The relative detection level of the alternative method is identical to the relative detection level of the reference method. It is between 0.2 and 1.0 CFU/25g.

## INCLUSIVITY / EXCLUSIVITY

### Implementation of alternative method only

- 51 strains of *Listeria* were detected out of 51 tested.
- The study of 30 strains not belonging to the genus *Listeria* did not detect the presence of any cross-reaction.

## PRACTICABILITY

### Implementation of alternative method only

- **Time of results:**
  - **Positive** results are obtained in 5 to 7 days using the alternative method as for using the reference method.
  - **Negative** results are obtained in 2 days using the alternative method against 3 to 7 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 5 to 7 days.
- **The major interest of the RayAI Listeria method** rests on sorting negative samples from suspicious samples, facilitating the step of confirmation by reducing the number of samples, as well as saving important labour for samples series analysis.

## INTERLABORATORY STUDY

The interlaboratory study was conducted in 2010 with 14 participating laboratories. The analyses were carried out on samples of pasteurized milk, artificially contaminated with a *Listeria monocytogenes* 1/2b strain at the following levels of contamination:

- 0 CFU/25 ml
- 3 CFU/25ml
- 30 CFU/25ml

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 processed**	Number of negative results		Number of positive results	
				REF	ALT	REF	ALT
0	112	96	80	80	80	0	0
1	112	96	80	0	0	80	80
2	112	96	80	0	0	80	80

\* Two laboratories did not perform the assays.

\*\* Results of two laboratories were excluded because delivery temperature was not in accordance with that expected.

### Calculations

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

## Interpretation

Results of the interlaboratory 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):

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

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

### 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:  
COR= accordance x (100 - concordance) / concordance x (100 – accordance)

The following table indicates values for the **alternative method** and 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 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](http://www.afnor-validation.com)