



Alternative methods for agribusiness
Analytical performances certified

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

Certificate No.: RAY-32/02-06/08

Validation date: 30/06/2008
Extension date: 04/02/2010
Extension date: 30/06/2012

The company

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

RAYAL Salmonella OPTIMA

Protocol reference: RayAI Salmonella Optima – QCF31 – Issue 2

SCOPE

Food and feed products and environmental samples (excluding breeding samples).

RESTRICTIONS OF USE

None.

REFERENCE METHOD

EN ISO 6579 (2002) - Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp.

A handwritten signature in black ink, appearing to read "JBESLIN".

Deputy General Manager
Jacques BESLIN

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PRINCIPLE OF THE METHOD

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

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), starting from unheated RVS.
- Alternatively, it is possible to perform confirmation tests directly 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.

NOTE (History of validation)

1/ The first validation of *Salmonella* OPTIMA method was performed for the company Boline, certificate number BLN 26/02-03/04, dated March 2004.

The validation study performed in 2008, which current certificate is related to, was performed according to ISO 16140.

The results of the 2004 preliminary study about relative accuracy, relative specificity and relative sensitivity were kept and completed. The results of 2004 inclusivity/exclusivity study were kept. The collaborative study was done again in its entirety.

In the present study, several options were tested:

- The integration of one confirmation option with identification without prior purification when the colony are well isolated
- The possibility to store RVS broths for 48 hours at $5\pm 3^{\circ}\text{C}$ prior to the immuno-enzymatic test.
- The possibility to perform the last incubation step of the immuno-enzymatic test (prior to adding the stop solution), equally for 15 or 30 minutes at $20\text{-}25^{\circ}\text{C}$.

2/ In February 2010, the AFNOR VALIDATION technical committee accepted to extend the scope of analysis of the method RAYAL *Salmonella* OPTIMA to the detection of non motile *Salmonella*, taking into account results obtained by the AFNOR VALIDATION expert laboratory in charge of the first validation study done in 2008, and on the basis of internal results.

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

In 2004 and 2008 tests were carried out on 528 product samples, of which 59 were naturally contaminated, 139 artificially contaminated, and 330 non-contaminated, belonging to the following principal food product categories:

Dairy products, meat products, miscellaneous (egg based products, pastries, etc.), fish products, vegetables, feed 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 = 194 ⁽¹⁾	Positive deviation A+ / R- PD = 2 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 2 ⁽²⁾	Negative agreement A- / R- NA = 330 ⁽³⁾

(1) Confirmed positives

(2) and (3) all the ELISA positive results were confirmed positive

Percentages obtained compared to the reference method are as follows:

- Relative accuracy: **99.1%**
- Relative specificity: **99.1%**

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

- Relative sensitivity: **99.0%**

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

Alternative method:

$$(PA + PD) / (PA + PD + ND) = 99.0\%$$

Reference method:

$$(PA + ND) / (PA + PD + ND) = 99.0\%$$

Storage of the RVS broths at 2-8°C for 48 hours

For all the samples tested, the result obtained after the cold storage is the same to the result obtained directly after incubation.

Relative DETECTION LEVEL

Comparison of performances of the alternative method and the reference method

Tests were carried out in (2008), on 6 combinations of food products/strains.

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 ₅₀	
		Alternative method	Reference method
Minced meat (poultry)	Salmonella hadar	0.4 [0.2 – 0.6]	0.4 [0.2 – 0.6]
Raw milk	Salmonella typhimurium	0.5 [0.3 – 0.8]	0.5 [0.3 – 0.8]
Liquid egg	Salmonella enteritidis	0.4 [0.2 – 0.7]	0.4 [0.2 – 0.7]
Fish filet	Salmonella virchow	0.5 [0.3 – 0.9]	0.5 [0.3 – 0.9]
Dog food	Salmonella senftenberg	0.5 [0.3 – 1.0]	0.5 [0.3 – 1.0]
Process water	Salmonella newport	0.4 [0.3 – 0.7]	0.4 [0.3 – 0.7]

(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"

Conclusion

The detection level of both alternative and reference methods is between 0.2 and 1.0 CFU per 25 grams.

INCLUSIVITY / EXCLUSIVITY (2004)

Implementation of alternative method only

- 55 strains of *Salmonella* were detected out of 55 tested.
- The study of 30 strains not belonging to the genus *Salmonella* did not detect the presence of any cross-reaction (when the strains are grown in RVS).

PRACTICABILITY

Implementation of alternative method only

- **Response time :**
 - **Positive** results are obtained in 3 to 4 days using the alternative method (*including confirmation according to classical tests of the reference method, with purification step included*) against 5 to 7 days 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 3 to 4 days.

INTER-LABORATORY STUDY

The inter-laboratory study was conducted in 2008 with 15 participating laboratories. The analyses were carried out on samples of pasteurized milk, artificially contaminated with a *Salmonella typhimurium* strain at the following 3 levels of contamination:

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

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 CFU	120	112	112	112	0	0	
3 CFU	120	112	112	0	0	112	112
30 CFU	120	112	112	0	0	112	112

* One laboratory did not communicate their results.

Calculations

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

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):

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

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

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:

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

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

Contamination level	Accordance	Concordance	COR
L0	100%	100%	1.0
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

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