



**Alternative methods for agribusiness  
Analytical performances certified**

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

**Certificate No.:** EUR 15/2 – 11/00

**Validation date :** 29.11.2000  
**Extension date :** 07.03.2002  
**Renewal dates \*:** 08.04.2005  
 18.05.2009  
**End of validity :** 29.11.2012

*\* EN ISO 16140 protocol was used in 2005 for the preliminary study and in 2008 for the interlaboratory study*

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

**LUMIPROBE 24 *Salmonella* species**

Protocol reference : FTLST-V-05/09 (tube) and FTLSP-V-05/09 (microplaque)

**SCOPE**

All human and animal food products.

**RESTRICTIONS OF USE**

None.

**REFERENCE METHOD**

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

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## PRINCIPLE OF THE METHOD

The *LUMIPROBE 24* Salmonella species method is a test which associates an enrichment in specific broths with a hybridisation of nuclear probes in solid phase, allowing the rapid and specific detection of the Salmonella. The rRNA of the targeted bacteria, released by lysis, is captured by an oligonucleotide coated on a support. It is then combined by hybridisation with a second oligonucleotide, labelled by a tracer; hybrids are revealed by a chemiluminescent reaction.

In the context of AFNOR Validation, all samples identified as positive by the alternative method must be confirmed, starting from the selective broth used for LUMIPROBE 24 test (RV), by classical tests described in methods standardized by CEN or ISO (including a purification step).

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/ In 2005, the renewal of the validation has taken in account the following changes, compared to the first validation in 2000 (egg products), extended to all products in 2002 :

- New reference method EN ISO 6579 (2002) replacing EN 12824
- New validation protocol according to EN ISO 16140 standard

Some tests run in 2000 and 2002 have been kept interlaboratory study realized according to AFNOR Validation rules for study-Rev 7), and the comparison study has been completed on the following points : accuracy, relative sensitivity and specificity, relative detection level, inclusivity.

2/ In 2009, the validation of the kit was renewed. Since the last renewal, the method as well as the reference method were not modified. To comply with the requirements of the EN ISO 16140 standard, the inter-laboratory study was completely repeated. Also, complementary assays were performed for the dairy products food category (relative accuracy/specificity/sensitivity, relative detection level and inclusivity). All results are detailed in this certificate.

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

In 2005 tests were carried out, completed in 2008, on 334 product samples, of which 51 were naturally contaminated, 110 artificially contaminated, and 173 non-contaminated, belonging to the following principal food product categories : meat products, sea food products, vegetables, dairy products, egg products and pet food products.

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 = 151 <sup>(1)</sup>	Positive agreement A+ / R- PD = 3 <sup>(1)</sup>
Alternative method negative (A-)	Negative deviation A- / R+ ND = 7 <sup>(2)</sup>	Negative agreement A- / R- NA = 173 <sup>(3)</sup>

(1) Confirmed positives

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

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

Percentages obtained compared to the reference method are as follows :

- Relative accuracy : **97,0%**
- Relative specificity : **98,3%**

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

- Relative sensitivity : **95,6%**

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

Alternative method :

$$(PA + PD) / (PA + PD + ND) = 95,6\%$$

Reference method :

$$(PA + ND) / (PA + PD + ND) = 98,1\%$$

### Conclusion

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

$$PD = 3, ND = 7; \quad Y = PD + ND = 10; \quad 6 \leq Y \leq 22; \quad m = 3, M = 1; \quad \text{so } m > M$$

### Conclusion

The two methods are not statistically different.

## Relative DETECTION LEVEL

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

Tests were carried out in 2005, on 5 combinations of food products/strains, described in the table below.

These products represent the following food products categories : meat products, sea food products, vegetables, dairy products, egg products and pet food products.

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
Whole eggs	S. Enteritidis S38	0.6 [0.4 - 1.0]	0.8 [0.5 - 1.2]
Raw minced meat	S. Typhimurium S15	1.6 [1.0 - 2.4]	1.3 [1.0 - 1.8]
Raw milk	S. Dublin S59	8.2 [5.6 - 11.9]	1.9 [1.1 - 3.4]
Smoked salmon	S. Enteritidis S63	1.0 [0.6 - 1.7]	1.0 [0.6 - 1.7]
Granules for rodents	S. spp S65	0.6 [0.4 - 1.1]	1.0 [0.6 - 1.7]

(3) **LOD<sub>50</sub>** : 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 10<sup>th</sup> December, 2003"

In 2008, news assays were conducted on dairy products on the combination of food product/strain, described below.

Results obtained are as follows :

		Relative detection level (CFU/25g or 25 ml) With confidence interval (3) LOD <sub>50</sub>	
Matrix	Strain	Alternative method	Reference method
Raw milk	S. Dublin	0.8 [0.6 – 1.2]	0.9 [0.7 – 1.3]
Raw milk	S. Newport	0.6 [0.5 – 0.8]	0.6 [0.4 – 0.8]

(3) LOD<sub>50</sub> : 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 10<sup>th</sup> December, 2003"

### Conclusion

The detection level of the alternative method is between 0.4 and 11.9 CFU/25g.

The detection level of the reference method is between 0.4 and 3.4 CFU/25g.

## INCLUSIVITY / EXCLUSIVITY

### Implementation of alternative method only

#### 2000 and 2002 studies

- 50 strains of *Salmonella* were detected out of 50 tested.
- The study of 30 strains not belonging to the genus *Salmonella* showed cross reactions with 2 strains of *Citrobacter diversus* (*C.diversus* 140 and *C.diversus* CIP8294) and 1 strain of *Enterobacter sakazakii* 95. Those cross reactions were not found on liquid egg ; the tests of 7 others strains of *E. sakazakii* gave negative results.
- Two strains of *Citrobacter freundii* (23 and 175), and one strain of *Enterobacter agglomerans* (II) showed a cross reaction when testing BHI, but this was not the case using the protocol of LUMIPROBE 24 method (RM+RV).

#### 2005 complementary study

- The 6 tested strains have been detected (1Typhi, 2 Paratyphi A, 2 Paratyphi B, 1 Paratyphi C).

#### 2008 complementary study

- 21 other strains of *Salmonella* were detected on 24 tested.  
3 strains of *Salmonella* Gallinarum were not detected initially. For a contamination level between 50 to 70 CFU/225 ml, the detection threshold of LUMIPROBE 24 *Salmonella* species kit was not reached for this serovar growing slower than other serovars of *Salmonella*.

## PRACTICABILITY

### Implementation of alternative method only

- **Response time :**
  - **Positive** results are obtained in 4 to 6 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 1 day using the alternative method against 5 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 5 days.
- **Personnel training** : One day training for an operator trained in classical tests of microbiology.

## INTER-LABORATORY STUDY

The inter-laboratory study was conducted in 2008 with 12 participating laboratories. The analyses were carried out on samples of pasteurized milk artificially contaminated with a strain of *Salmonella* Enteritidis at the following 3 levels of contamination :

- level 0 : 0 CFU/ml
- level 1 : 3 CFU/ml
- level 2 : 30 CFU/ml

The laboratories tested, using **both methods, 8 replicate samples for each level** of contamination, giving 24 analyses for each participating laboratory, and a total of 288 analysis for all.

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	96	96	88	87	88	1	0
1	96	96	88	6	12	82	76
2	96	96	88	1	1	87	87

\* The results of one laboratory were not taken into account because the protocol of the alternative method was not respected.

### Calculations

- Relative accuracy = **92%**
- % specificity = **100%**
- % sensitivity = **92,6%**

**Sensitivity** was also recalculated taking into account all confirmed positive results (this includes supplementary positives with alternative method) :

$$\begin{aligned} & \text{Alternative method :} \\ & (PA + PD) / (PA + PD + ND) = \mathbf{96\%} \end{aligned}$$

$$\begin{aligned} & \text{Reference method :} \\ & (PA + ND) / (PA + PD + ND) = \mathbf{92\%} \end{aligned}$$

### 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** :

Contamination level	Accordance	Concordance	COR
L0	100	100	1.00
L1	78	78	1.00
L2	98	98	1.00

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

Contamination level	Accordance	Concordance	COR
L0	98	98	1.00
L1	90	87	1.30
L2	98	98	1.00

### **Conclusion**

Variability of the alternative method (accordance, concordance, concordance odds ratio) is comparable 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)