Alternative methods for agribusiness
Analytical performances certified

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

Certificate No.: BRD 07/11 – 12/05

Validation date :	**09.12.2005**
Extension date :	**03.07.2009**
	21.05.2010
Renewal date :	**24.09.2009**
End of validity :	**09.12.2013**

The company
(head office, production site and distribution)

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

RAPID'*Salmonella*

Protocol reference: RAPID'Salmonella/Agar (356-3961 / 356-4705) **V9**

SCOPE

All human and animal food products and environmental samples (excluding breeding samples)

RESTRICTIONS OF USE

ONPG confirmation test excludes confirmation of lactose+ *Salmonella*.

LATEX SALMONELLA test excludes confirmation of *Salmonella* others than those of group B to E and G.

REFERENCE METHOD

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

Deputy General Manager
Jacques BESLIN

PRINCIPLE OF THE METHOD

RAPID'*Salmonella* is a chromogenic agar medium, the principle of which relies on demonstration of two enzymatic activities. *Salmonella* spp present as typical magenta colonies (detection of C8-esterase activity). Counter selection is used to reveal other bacteria by a different colour. RAPID'*Salmonella* permits detection of motile and non-motile salmonella, as well as lactose-positive *Salmonella*, including *Salmonella typhi* and *Salmonella paratyphi.*

In the context of AFNOR Validation, all samples identified as positive by the RAPID'*Salmonella* method must be confirmed by one of the following means:

- According to classical tests described in methods standardized by CEN or ISO, using colonies isolated on chromogenic medium (including purification step).
- Using nucleic probes as described in ISO 7218 standard using isolated colonies (including or not purification step).
- Using 1 to 3 isolated suspect colonies, by evaluation of oxidase activity (oxidase test), followed by omnivalent Omni-O test. If reaction is positive to the Omni-O test, proceed with an ONPG biochemical test. *Salmonella* are negative to oxidase test, positive to Omni-O test and negative to ONPG test.
- Performing a SALMONELLA LATEX test using an isolated colony. *Salmonella* of groups B to E and G are positive to the latex test.
- Use of any other AFNOR VALIDATION certified method based on a different principle from that of RAPID'*Salmonella*. The validated protocol of the second method must be respected in its entirety.

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 1: Some strains of *Salmonella* (a few are part of Dublin serovar and *S. Bongori* specie) can show a weak magenta color (or no color) due to a low esterase activity.

NOTE 2: protocols validated in the context of AFNOR VALIDATION

Three protocols are validated in the context of AFNOR VALIDATION:

- Short protocol : enrichment in Buffered Peptone Water supplemented incubated 18h±2h at 41,5°C±1°C for the following categories: all human and animal food products and environmental samples (excluding breeding samples) **(1)**

- Double enrichment protocol : enrichment in Buffered Peptone Water incubated at 37°C±1°C for 18h ±2h, followed by second enrichment in selective RVS broth incubated as follows:
  - 8h±2h to 24h±2h at 41,5°C±1°C for the following categories: seafood products, vegetable products, dairy and egg products **(2)**
  - 24h±2h at 41.5°C±1°C for the following categories: meat products and animal foodstuffs **(3)**

NOTE 3: Validation history

1/ In July 2009, additional tests permitted to extend the validation to a new "short protocol" and to the SALMONELLA LATEX test confirmation. The results available in this certificate were completed as follows:

- By analysing 118 samples (representing all categories of human and animal food products) implementing the double enrichment protocol (RVS).
- By doing new study of relative accuracy/specificity/sensitivity, analysing 324 samples with the short protocol. Also inclusivity and exclusivity were completed.

Modification of the agar medium (opacification) was also validated. The results are not available in this certificate.

2/ In September 2009, the renewal of validation was pronounced without performing additional tests, since neither the RAPID'*Salmonella* method, or the reference method, nor the protocol validated has been modified.

3/ Extension study of May 2010 allowed to validate the analysis of environmental samples (excluding breeding samples) using the "short protocol". The following parameters were tested: relative accuracy/specificity/sensibility and relative detection level. Results were in accordance with those expected. Validation results for the short protocol which are available in this certificate were recalculated taking into account results obtained for environmental samples.

Relative ACCURACY, relative SPECIFICITY and relative SENSITIVITY

Comparison of performances of the alternative method and the reference method

Tests were carried in 2005 out on 408 product samples, of which 84 were naturally contaminated, 107 artificially contaminated, and 217 non-contaminated, belonging to the following principal food product categories: meat products, dairy products, seafood and vegetable products, egg products, animal foodstuffs.

All samples were analysed **in single** by the **two methods**. The two ways (second enrichment in RVS) of the "double enrichment protocol"were tested.

For "double enrichment protocol", with RVS incubation for 6 hours

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 = **78** (1)	Positive deviation A+ / R- PD = **6** (1)
Alternative method negative (A-)	Negative deviation A- / R+ ND = **11** (2)	Negative agreement A- / R- NA = **180** (3)

(1) Confirmed positives

(2) Of which 4 samples presumed positive by the alternative method, 3 negative after confirmation with oxydase test

(3) Of which 23 samples presumed positive by the alternative method were negative after confirmation, 14 of which immediately eliminated by oxydase test.

For "double enrichment protocol", with RVS incubation for 24±2 hours

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 = **166** (1)	Positive deviation A+ / R- PD = **13** (1)
Alternative method negative (A-)	Negative deviation A- / R+ ND = **12** (2)	Negative agreement A- / R- NA = **217** (3)

(1) Confirmed positives

(2) Of which 4 samples presumed positive by the alternative method, all negative after confirmation with oxydase test

(3) Of which 50 samples presumed positive by the alternative method were negative after confirmation, 30 of which immediately eliminated by oxydase test.

Additional tests were carried in 2009 and in 2010, out on 409 product samples, of which 66 were naturally contaminated, 137 artificially contaminated, and 206 non-contaminated, belonging to the following principal food product categories: meat products, dairy products, seafood and vegetable products, egg products, animal foodstuffs and environmental samples.

All samples were analysed **in single** by the **two methods**. The short protocol of the alternative method was used.

For "short protocol"

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 = **167** (1)	Positive deviation A+ / R- PD = **17** (1)
Alternative method negative (A-)	Negative deviation A- / R+ ND = **19** (2)	Negative agreement A- / R- NA = **206** (3)

(1) Confirmed positives

(2) Of which 1 sample presumed positive by the alternative method, negative after confirmation with latex test

(3) Of which 3 samples presumed positive by the alternative method, negatives after confirmation with latex test

Percentages obtained compared to the reference method are as follows:

	Double enrichment protocol		**Short protocol**
	Incubation RVS for 6h	Incubation RVS for 24h±2h	
Relative accuracy: **AC (%)**	**91.4**	**93.9**	**91.2**
Relative specificity*: **SP (%)**	**94.4**	**94.3**	**92.4**
Relative sensitivity: **SE (%)**	**87.6**	**93.3**	**89.8**

*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):

"Double enrichment protocol", with RVS incubation for 6 hours:

Alternative method : (PA + PD) / (PA + PD + ND) = **88.4%**

Reference method : (PA **+ ND)** / (PA **+ PD +** ND) = **93.7%**

"Double enrichment protocol", with RVS incubation for 24 ± 2 hours:

Alternative method : (PA + PD) / (PA + PD + ND) = **93.7%**

Reference method : (PA **+ ND)** / (PA **+ PD +** ND) = **93.2%**

"Short protocol":

Alternative method : (PA + PD) / (PA + PD + ND) = **90.6%**

Reference method : (PA + ND) / (PA + PD + ND) = **91.6%**

Analysis of discrepant results (according to Annex F of Standard EN ISO 16140)

"Double enrichment protocol", with RVS incubation for 6h

Y = ND + PD = 17 $6<y<22$ n = PD = 6 M = 5 $m<M$

"Double enrichment protocol", with RVS incubation for 24h±2h

Y = ND + PD = 25 $y>2$
Application of Mc Nemar test: $X^2 = d^2/y$ d = | PD-ND | = 1 $x^2 = 0{,}04$ $x^2 < 3.841$)

Short protocol

Y = ND + PD = 36 $y>22$
Application of Mc Nemar test: $X^2 = d^2/y$ d = | PD-ND | = 2 $x^2 = 0{,}105$ ($x^2 < 3.841$)

Conclusion

In both cases (for the two protocols tested) no statistical difference exists between the two methods.

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.

Products were analysed **6 times** by the **2 methods** at **4 levels** of contamination. The two ways of the "double enrichment protocol" were tested (second enrichment in RVS).

Results obtained are as follows:

"Double enrichment protocol", with RVS incubation for 6h

		Relative detection level LOD_{50}(3) With confidence interval (UFC/25g or 25 ml)	
Matrix	**Strain**	**Alternative method**	**Reference method**
Minced steak	*Salmonella* Infantis	0.8 [0.3 – 2.4]	0.8 [0.3 – 2.4]
Non-pasteurised milk	*Salmonella* Typhimurium	1.8 [0.6 – 5.6]	1.8 [0.6 – 5.6]
Fish fillet	*Salmonella* Saintpaul	0.4 [0.1 – 2.1]	0.4 [0.1 – 2.1]
Raw egg	*Salmonella* Enteritidis	0.8 [0.2 – 3.4]	0.5 [0.1 – 2.2]
Morsels in aspic	*Salmonella* Agona	0.5 [0.1 – 2.5]	0.5 [0.1 – 2.5]

(3) **LOD_{50}**: see table below.

"Double enrichment protocol", with RVS incubation for 24h ± 2h

		Relative detection level LOD_{50}(3) With confidence interval (UFC/25g or 25 ml)	
Matrix	**Strain**	**Alternative method**	**Reference method**
Minced steak	*Salmonella* Infantis	0.7 [0.3 – 2.1]	0.8 [0.3 – 2.4]
Non-pasteurised milk	*Salmonella* Typhimurium	0.6 [0.1 –2.6]	1.8 [0.6 – 5.6]
Fish fillet	*Salmonella* Saintpaul	0.4 [0.1 – 2.1]	0.4 [0.1 – 2.1]
Raw egg	*Salmonella* Enteritidis	0.8 [0.2 –3.4]	0.5 [0.1 – 2.2]
Morsels in aspic	*Salmonella* Agona	0.5 [0.1 – 2.5]	0.5 [0.1 – 2.5]

(3) **LOD_{50}**: see table below.

Additional tests were carried in 2009 and in 2010 out on 6 combinations of food product/strain described in the table below.

Products were analysed **6 times** by the **2 methods** at **4 levels** of contamination. The short protocol of the alternative method was used.

Results obtained are as follows:

"Short protocol"

Matrix	Strain	Relative detection level LOD_{50}(3) With confidence interval (UFC/25g or 25 ml)	
		Alternative method	**Reference method**
Minced steak	*Salmonella* Infantis	0.4 [0.1 – 1.4]	0.3 [0.1 – 1.1]
Raw milk	*Salmonella* Derby	0.4 [0.2 – 0.9]	0.5 [0.2 – 1.3]
Fish fillet (haddock)	*Salmonella* Saintpaul	0.4 [0.2 – 1.1]	0.6 [0.2 – 1.5]
Raw egg	*Salmonella* Enteritidis	0.4 [0.1 – 1.2]	0.3 [0.1 – 1.0]
Croquettes for dog	*Salmonella* Agona	0.6 [0.2 – 1.6]	0.3 [0.1 – 1.1]
Water process	*Salmonella* Typhimurium	0.5 [0.1 – 1.8]	0.6 [0.2 – 1.8]

(3) **LOD_{50}**: estimation of level of contamination enabling positive detection by alternative method in 50% of cases.
"Hitchins A. Proposed Used 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

Detection level of the reference method ranges between 0.1 and 5.6 UFC/25g.

Detection level of the alternative method ranges between:
- 0.1 et 5.6 CFU/25 g for "double enrichment protocol" with RVS incubation for 6h
- 0.1 et 3.4 CFU/25 g for "double enrichment protocol" with RVS incubation for 24h
- 0.1 et 1.8 CFU/25 g for the "short protocol"

INCLUSIVITY / EXCLUSIVITY
Implementation of alternative method only

2005 study:

- 51 strains of *Salmonella* were detected out of 52 tested. The non-identified strain is a strain of *paratyphi A*. Two other strains of *Salmonella paratyphi A* were tested and presented magenta colonies on RAPID'*Salmonella* agar. All target strains show an Omni-0 positive / ONPG negative profile, with the exception of *Salmonella arizonae* (lactose-positive phenotype) presenting a positive ONPG test.

- The study of 30 non-*Salmonella* strains revealed typical colonies on RAPID'*Salmonella* agar in the case of a single strain of *Enterobacter sakazakii*. However this latter presents a negative Omni-0 test, non-characteristic of salmonella.

  Certain strains of *Escherichia hermanii* isolated during the course of the study demonstrate magenta colonies. Consequently 12 strains of this species were tested: 8 present a positive reaction to the Omni-0 test, but present a positive ONPG test, non-characteristic of *Salmonella*.

2009 study (short protocol):

- 47 strains of *Salmonella* were detected out of 51 tested. Three strains of *Salmonella* (*Salmonella Paratyphi* A ATCC 9150, *Salmonella Paratyphi* B Ad 301 and *Salmonella Paratyphi* C ATCC 13428) showed difficulty to grow, as well as *Salmonella* gallinarum Ad 300. Five strains of *Salmonella* gave a negative latex test: *Salmonella arizonae* Ad 450, *Salmonella bongori* Ad 599, *Salmonella cerro* Ad 689, *Salmonella Houtenae* Ad 596 and *Salmonella Veneziana* Adria 233.

- 42 non-*Salmonella* strains, of which 12 strains of *Escherichia hermanii*, were studied. 11 of the *Escherichia hermanii strains* tested, 1 strain of *Citrobacter diversus* Adria 140 and 1 strain of *Serratia marescens* Ad 447 gave magenta colonies, with a more mat coloration than that observed with *Salmonella*. All these strains gave a negative latex test.

PRACTICABILITY

Implementation of alternative method only

- **Response time:**
  - **Positive** results are obtained in 2 days ("short protocol"), between 3 to 4 days ("double enrichment protocol" with 6h RVS incubation) and 4 to 5 days ("double enrichment protocol" with RVS 24h±2h incubation) using the alternative method against 5 days using the reference method.
  - **Negative** results are obtained in 2 days ("double enrichment protocol" with 6h RVS incubation and "short protocol") and 3 days ("double enrichment protocol" with 24h±2h RVS incubation) using the alternative method against 3 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 depending on the confirmation protocol adopted.

INTER-LABORATORY STUDY

The inter-laboratory study was conducted in 2005 with 15 participating laboratories. The analyses were carried out on samples of half-cream pasteurized milk artificially contaminated with a *Salmonella typhimurium* strain at the 4 following 3 levels of contamination:

- 0
- slightly superior to relative detection level
- 10 times superior to previous level

The laboratories tested, using **both methods, 8 replicate samples** for **each level** of contamination.

The two ways of the "double enrichment protocol" were tested (second enrichment in RVS).

The following results were obtained:

Results for "double enrichment protocol", with incubation of 8h±2h:

Contami-nation level	Total number of samples	Number of samples analysed	Results exploited*	Negative results		Positive results	
				REF	ALT	REF	ALT
0	120	120	88	88	88	0	0
1	120	120	88	0	0	88	88
2	120	120	88	0	0	88	88

**Findings of 4 laboratories were excluded due to abnormal results apparently resulting from inter-contamination and/or discordance in identification tests.*

Results for "double enrichment protocol", with incubation of 24h±2h:

Contami-nation level	Total number of samples	Number of samples analysed	Results exploited*	Negative results		Positive results	
				REF	ALT	REF	ALT
0	120	120	80	80	78	0	2**
1	120	120	80	0	0	80	80
2	120	120	80	0	0	80	80

** Findings of 5 laboratories were excluded due to abnormal results apparently resulting from inter-contamination and/or discordance in identification tests*

*** Supplementary positives confirmed by alternative method.*

Calculations

	Protocol with incubation 8h±2h	Protocol with incubation 24h±2h
Relative accuracy	100%	99,2 % **
Sensitivity	100%	100%
Specificity*	100%	97,5 % **

*N.B. **relative specificity** of less than 100% results from a number of supplementary confirmed positives and not false positives

** Supplementary positives confirmed by alternative method.

Interpretation

Inter-laboratory study results are comparable to those of the preliminary study.

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

	Protocol 8h±2h	Protocol 24h±2h
Alternative method	100%	100%
Reference method	100%	98.7%

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:**

Level of contamination	Accordance		Concordance		COR	
	8h±2h	24h±2h	8h±2h	24h±2h	8h±2h	24h±2h
L0	100	96.2	100	97.6	1,00	0.62
L1	100	100	100	100	1,00	1,00
L2	100	100	100	100	1,00	1,00

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

Level of contamination	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, 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