

PRINCIPLE OF THE METHOD

The iQ-Check *Salmonella* II test is based on gene amplification and real-time PCR detection. It uses primers and a DNA probe specific to *Salmonella* spp. Following the pre-enrichment step (and enrichment step if necessary), lysis of bacteria releases bacterial DNA. The amplification and detection steps are then performed in a thermal cycler. Following the reaction, fluorescence is emitted and measured directly by the thermal cycler. The instrument software analyses the results and displays them in the form of curves for interpretation.

In the context of AFNOR Validation, all samples identified as positive by the iQ-Check *Salmonella* II method must be confirmed by one of the following means:

- According to conventional tests described in methods standardized by CEN or ISO (including a purification step), starting from the buffered peptone water enrichment broth.
- By implementing any other method certified AFNOR VALIDATION based on a principle different from the iQ-Check *Salmonella* II method, and following specifications in the test instructions.

In the event of discordant results (positive with the alternative method, unconfirmed with one of the above-mentioned options), the laboratory must take all necessary steps to ensure the validity of the result obtained.

NOTE (History of validation)

1) The iQ-Check *Salmonella* II test includes a **general protocol (Standard I)**. In the event of any inhibition the user has two solutions: using a **1/10 dilution** or using an **alternative protocol** (not applicable to environmental samples) proposed by the manufacturer and described in the instruction manual. This alternative protocol, tested during the AFNOR validation preliminary study in 2004, is still applicable but only for use with the standard extraction protocol.

2) The May 2007 extension study is a result of the following modifications made to the iQ-Check *Salmonella* II method:

- Addition of a simplified (easy) extraction protocol no longer requiring the first centrifugation step
- A shortened enrichment time (18 hours +/- 2 hours) for the general protocol
- Amplification mix and lysis reagent modifications

A new preliminary study was conducted with the following 2 extraction protocols:

- **Standard protocol I**, 18 hours ± 2 hours
- **Easy protocol I** in microplates, 21 hours ± 1 hour

The inter-laboratory study was not repeated. It was conducted in 2004 with the Standard protocol.

3) Additional internal assays were realised in 2007 and accepted by the AFNOR VALIDATION Technical Board, in order to compare two new thermal cyclers (iQ™5 and MiniOpticon™) to one of the thermal cyclers initially accepted (Chromo4™). These tests are not detailed in this certificate.

The four thermal cyclers (iCycler iQ™, Chromo4™, iQ™5 and MiniOpticon™) can now be used in the context of AFNOR VALIDATION.

4) In September of 2008, the validation was extended to 2 new specific protocols for the following matrices:

- **Standard protocol II**, specific for raw meat, in 10 hours ± 2 hours. The preliminary study was performed in its entirety, except for exclusivity.
- **Easy protocol II**, specific for raw beef in 20 hours ± 1 hour. All the parameters of the preliminary study were performed, except for inclusivity/exclusivity.

In November of 2008, during the **renewal** of the method and the change of the kit to version II, a new inter-laboratory study was performed using the Easy protocol I, in accordance with EN ISO 16140 requirements.

5) In January 2009, a new study was conducted extending the validation to include the use of a new version of the **Opticon Monitor™ software**, which offers in addition to a manual analysis, the option of automated data analysis.

Tests were conducted internally and by a third party, and followed the Standard I extraction protocol after enrichment in buffered peptone water. All iQ-Check tests were done in the Chromo4, and data analysed both manually and with the automated option of the Opticon Monitor™ software.

These assays demonstrated that manual and automated data analysis of samples gave equivalent results. For clarity, results of this study are not detailed in this certificate.

6) In February of 2010, the following extensions were validated by the AFNOR VALIDATION technical committee:

- Modification of the extraction step, using a new "**Deepwell plate**" format (in addition to the "tube" format validated before). Internal assays showed that these modifications did not have any impact on rendered results.
- The **CFX Manager™ software** can be used for a complete automated analysis for the CFX96™ and the Mini Opticon™ real-time PCR instruments. Internal assays showed that results obtained with these new combinations of automated systems were equivalent to those obtained with instruments and software validated before.

Relative ACCURACY, relative SPECIFICITY, relative SENSITIVITY Performance comparison of the alternative and reference methods

In 2007 and 2008, tests were performed on 582 product samples, including 64 naturally contaminated, 219 artificially contaminated and 299 non-contaminated, belonging to the following main food categories :

- Meat products
- Dairy products
- Fish-based and vegetable products
- Egg products
- Animal feed
- Environmental samples

All samples were analysed **In single** by **both methods**.

The study was performed using the Standard protocol I, the Easy protocol I, the standard protocol II (specific for raw meat) and the Easy protocol II (specific for raw beef meat). The tables of results are presented below:

Standard protocol I (2007)

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 = 191 ⁽¹⁾	Positive deviation A+ / R- PD = 3 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 5 ⁽²⁾	Negative agreement A- / R- NA = 228 ⁽³⁾

(1) Confirmed positives

(2) Of which no sample presumed positive by the alternative method was negative after confirmation

(3) Of which 6 samples presumed positive by the alternative method were negative after confirmation

Easy Protocol I (2007)

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 = 188 ⁽¹⁾	Positive deviation A+ / R- PD = 3 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 8 ⁽²⁾	Negative agreement A- / R- NA = 228 ⁽³⁾

(1) Confirmed positives

(2) Of which no sample presumed positive by the alternative method was negative after confirmation

(3) Of which 20 samples presumed positive by the alternative method were negative after confirmation

Standard protocol II (specific for raw meat) (2008)

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 = 51 ⁽¹⁾	Positive deviation A+ / R- PD = 0 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 1 ⁽²⁾	Negative agreement A- / R- NA = 37 ⁽³⁾

(1) Confirmed positives

(2) Of which no sample presumed positive by the alternative method was negative after confirmation

(3) Of which 1 sample presumed positive by the alternative method was negative after confirmation

Easy protocol II (specific for raw beef) (2008)

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 = 31 ⁽¹⁾	Positive deviation A+ / R- PD = 0 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 1 ⁽²⁾	Negative agreement A- / R- NA = 34 ⁽³⁾

(1) Confirmed positives

(2) Of which no sample presumed positive by the alternative method was negative after confirmation

(3) Of which no sample presumed positive by the alternative method was negative after confirmation

In 2004, tests were performed on 362 food samples, belonging to the following main food categories (excluding environmental samples for which the alternative protocol is not applicable):

- Meat products
- Dairy products
- Fish-based and vegetable products
- Egg products
- Animal feed

All samples were analysed in single by both methods.

The study was carried out using the alternative protocol. The table of results is presented below.

Alternative protocol, excluding environmental samples (2004)
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 = 155 ⁽¹⁾	Positive deviation A+ / R- PD = 0 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 4 ⁽²⁾	Negative agreement A- / R- NA = 203 ⁽³⁾

(1) Confirmed positives

(2) Of which no sample presumed positive by the alternative method was negative after confirmation

(3) Of which 1 sample presumed positive by the alternative method were negative after confirmation

The percentages obtained, with respect to the reference method, are as follows:

Criteria / protocol	Standard protocol I	Easy protocol I	Alternative protocol	Standard protocol II (raw meat)	Easy protocol II (raw beef)
Relative accuracy: AC	98.1%	97.4%	98.9%	98.9%	98.5%
Relative specificity: SP	98.7%	98.7%	100%	100%	100%
Relative sensitivity: SE	97.4%	95.9%	97.5%	98.1%	96.9%

Note: **relative specificity** below 100% is due to a number of additional confirmed positive results and not from false positives

Sensitivity was also recalculated, taking into account all confirmed positives (including the additional positive results of the alternative method):

All products	Alternative method (PA + PD) / (PA + PD + ND) =	Reference method (PA + ND) / (PA + PD + ND) =
Standard protocol I	97.5%	98.5%
Easy protocol I	96.0%	98.5%
Alternative protocol	97.4%	100%
Standard protocol II (raw meat)	98.1%	100%
Easy protocol II (raw beef)	96.9 %	100%

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

Standard protocol I

PD = 3, ND = 5, therefore $Y = PD + ND = 8$; $6 \leq Y \leq 22$ $m = 3, M = 0$ hence $m > M$

Easy protocol I

PD = 3, ND = 8, $Y = PD + ND = 11$; $6 \leq Y \leq 22$ $m = 3, M = 0$ hence $m > M$

Conclusion

For all protocols, the tests conclude that the results of the two methods are equivalent.

Relative DETECTION LEVEL

Performance comparison of the alternative and reference methods

Tests were carried out in 2007 and 2008, in all on the 6 combinations of food products/strain described below. Products were analysed 6 times, by both methods (including all four protocols for the alternative method), at 4 different contamination levels.

The table of results is presented below:

Matrix	Strain	Relative detection level LOD ₅₀ (3) With confidence interval (CFU/25g or 25 ml)				Reference method
		Alternative method				
		Standard protocol I	Easy protocol I	Standard protocol II (raw meat)	Easy protocol II (raw beef)	
Minced beef	S. Infantis	0.6 [0.2 - 1.7]	0.6 [0.2 - 1.7]	–	–	0.6 [0.2 - 1.7]
		–	–	0.4 [0.1 - 1.4]	–	0.4 [0.1 - 1.4]
		–	–	–	0.2 [0.1 - 0.7]	0.2 [0.1 - 0.7]
Raw milk	S. Typhimurium	0.7 [0.3 - 2.0]	0.9 [0.3 - 2.8]	–	–	0.4 [0.1 - 1.7]
Fish filet	S. Saintpaul	0.5 [0.2 - 1.5]	0.8 [0.4 - 1.5]	–	–	0.5 [0.2 - 1.5]
Liquid egg	S. Enteritidis	0.5 [0.2 - 1.6]	0.5 [0.2 - 1.6]	–	–	0.5 [0.2 - 1.6]
Animal feed	S. Agona	0.3 [0.1 - 1.1]	0.2 [0.1 - 0.7]	–	–	0.3 [0.1 - 1.1]
Process water	S. Derby	0.4 [0.2 - 1.1]	0.4 [0.2 - 1.1]	–	–	0.4 [0.2 - 1.1]

(3) LOD₅₀: estimation of contamination level required to achieve positive detection with the 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"

Previous tests were carried out in 2004, on 5 combinations of food products/strain. Products were analysed 6 times, by both methods, at 4 different contamination levels.

The results for the alternative protocol are as follows:

Matrix	Strain	Relative detection level LOD ₅₀ (3) With confidence interval (CFU/25g or 25 ml)	
		Alternative method - Alternative protocol	Reference method
Minced chicken	S. Hadar	0.7 [0.4 - 1.4]	0.7 [0.4 - 1.4]
Raw milk	S. Typhimurium	0.5 [0.4 - 0.8]	0.4 [0.3 - 0.7]
Fish fillets	S. Virchow	0.4 [0.3 - 0.6]	0.4 [0.3 - 0.6]
Raw eggs	S. Enteritidis	1.0 [0.5 - 2.2]	1.0 [0.5 - 2.2]
Animal feed	S. Seftenberg	0.6 [0.4 - 1.0]	0.6 [0.4 - 1.0]

(3) LOD₅₀: estimation of contamination level required to achieve positive detection with the 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

Globally, the detection limit for each protocol of the alternative method is between:

- 0.1 et 2.0 UFC/25 g for Standard protocol I,
- 0.1 to 2.8 UFC/25 g for Easy protocol I,
- 0.3 to 2.2 UFC/25g for Alternative protocol,
- 0.1 to 0.7 UFC/25g for Standard protocol II (raw meat),
- 0.1 to 0.7 UFC/25 g for the Easy protocol II (raw beef).

The detection limit of the reference method is between 0.1 and 1.7 CFU/25g.

INCLUSIVITY/EXCLUSIVITY

Implementation of alternative method only

2007 and 2008 tests:

- 156 strains of *Salmonella* were detected out of the 156 tested, regardless of the lysis protocol used.
- The study of 30 non-*Salmonella* strains resulted in no cross-reactions, regardless of the lysis protocol used.

2004 tests, including the alternative protocol:

- 51 strains of *Salmonella* were detected out of the 51 tested, regardless of the protocol used.
- The study of 31 non-*Salmonella* strains resulted in no cross reactions, regardless of the protocol used.

PRACTICABILITY

Implementation of alternative method only

- **Time required for results:**
 - **Positive** results are obtained in 3 to 6 days using the alternative method (*after confirmation using conventional tests, including the purification step*) similarly to the reference method.
 - **Negative** results are obtained in 1 day using the alternative method, or in 12 hours for raw meat protocol, compared to 3 days using the reference method.
 - In the case of presumed positive results using the alternative method, but shown to be negative after confirmation, negative results are obtained in 4 to 6 days.
- **Staff training:** for technicians with no PCR training, an initial training of 4 to 5 days would seem necessary. For technicians with training in standard microbiology and molecular biology techniques, one day of training is required.

INTER-LABORATORY STUDY (2008)

The inter-laboratory study was conducted in 2008, involving **19 participating laboratories**. Analyses were performed on samples of **pasteurized milk**, artificially contaminated with a strain of *Salmonella* Typhimurium at the following three contamination levels:

- 0 UFC/25 ml
- 1 – 10 UFC/25 ml
- 5 – 50 UFC/25 ml

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

Results:

Contamination levels	Total number of samples	Number of samples analyzed*	Number of results processed**	Number of negative results		Number of positive results	
				REF	ALT	REF	ALT
0	152	144	80	76	76	4	4
1	152	144	80	0	0	80	80
2	152	144	80	0	0	80	80

* One laboratory received samples too late and was not able to perform the tests. The results were not taken in account.

** Results from 8 laboratories were not processed because of intercontamination (samples highly contaminated)

Calculations

- Relative accuracy (AC) = 100%
- Specificity (SP) = 95 %
- Sensitivity (SE) = 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 (including additional positive results with the alternative method):

Alternative method:

$$(PA + PD) / (PA + PD + ND) = 100\%$$

Reference method:

$$(PA + ND) / (PA + PD + ND) = 100\%$$

Accordance, concordance and concordance odds ratio:

Accordance: % chance of obtaining the same result for two identical samples analysed by the same laboratory under repeatability conditions. The accordance is the average (mean) of the probabilities that two replicates should have the same result for each laboratory.

Concordance: % chance of obtaining the same result for two identical samples analysed by two different laboratories (reproducibility conditions). Concordance is the % of all replicate pairs having the same result.

Concordance odds ratio (COR): this ratio is defined by the following formula:

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

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

Contamination level	Accordance (%)	Concordance (%)	COR
L0	92,5%	90,3%	1,00
L1	100%	100%	1,00
L2	100%	100%	1,00

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

Contamination level	Accordance (%)	Concordance (%)	COR
L0	92,5%	90,3%	1,00
L1	100%	100%	1,00
L2	100%	100%	1,00

Conclusion

The 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