

**AFNOR CERTIFICATION VALIDATION STUDY
IBISA METHOD FOR THE DETECTION
OF SALMONELLA SPP**

SUMMARY REPORT

IBISA METHOD - S.R.(V0)
OCTOBER 2011



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Assays performed at ISHA: 25, avenue de la République 91300 Massy

Manufacturer:

AES CHEMUNEX

Route de Dol – BP 54
35270 COMBOURG - FRANCE

Expert laboratory :

I. S. H. A.

25, avenue de la République
91300 MASSY - FRANCE

For the AFNOR Certification validation of the IBISA method with confirmation according to the NF EN ISO 16140 standard

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Annexes

Annex 1: selectivity strains list

Annex 2: accordance calculations

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1. Introduction

1.1. Validation referential

The aim of this validation study is to evaluate the performance of the alternative method against the reference method ISO 6579 (2002). It consists in a preliminary study and a collaborative study. The studies follow the ISO 16140 standards.

1.2. Alternative method

The IBISA method is used for *Salmonella* spp detection in food and feed products and production environment samples. The principle of the method is based on the use of chromogenic substrates (esterase activity) specifically cleaved by *Salmonella* and on the simultaneous research of β -glucosidase activity, allowing a differentiation of *Salmonella* from other Enterobacteriaceae.

After incubation, *Salmonella* show typical green colonies while other non inhibited micro-organisms show colourless or magenta colonies.

The IBISA medium allows the detection of mobile and immobile cells and lactose positive *Salmonella*, including serovars Typhi and Paratyphi. The specific formulation of the media gives it a characteristic yellow colour. This singularity is to accentuate a colour contrast promoting the readability, and also slowing the growth of other Enterobacteriaceae.

The protocol of the method is showed in figure 1.

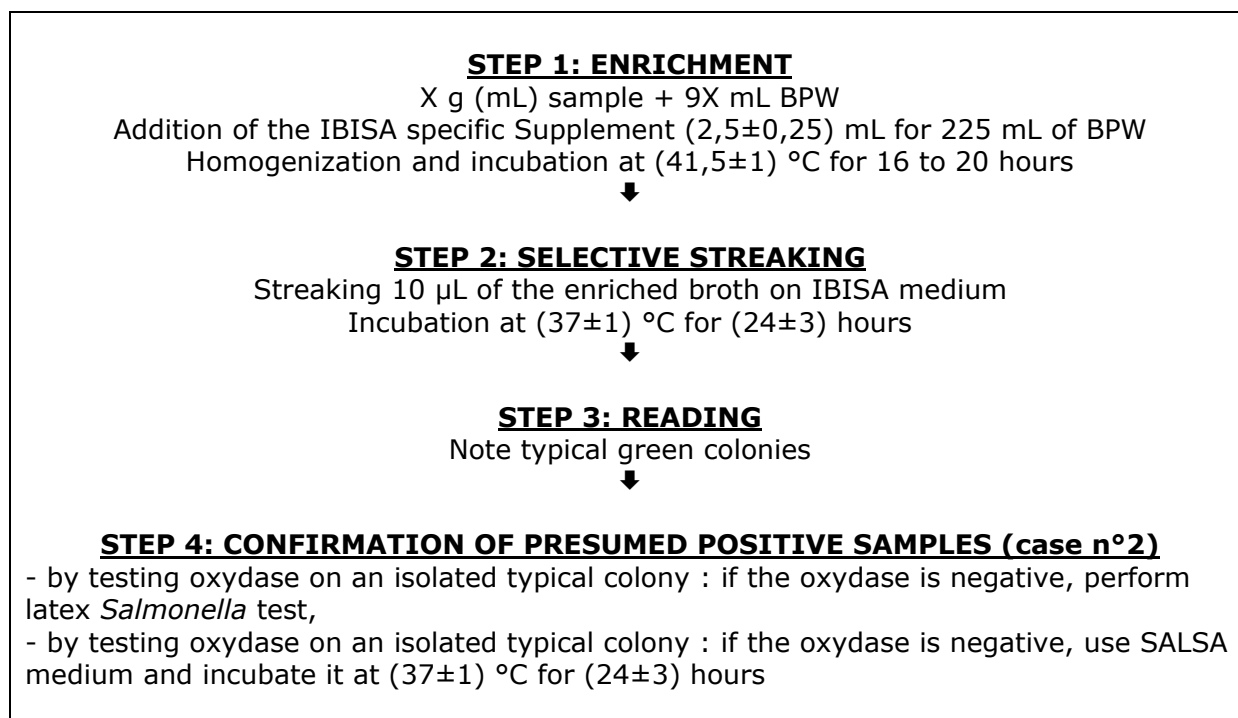


Figure 1: alternative method protocol

1.3. Scope of application

The alternative method was tested for all food and feed products and environmental samples.

1.4. Reference method(*)

The NF EN ISO 6579 (2002) standard: Horizontal method for the detection of *Salmonella* spp has been applied.

The protocol of this method is presented in figure 2.

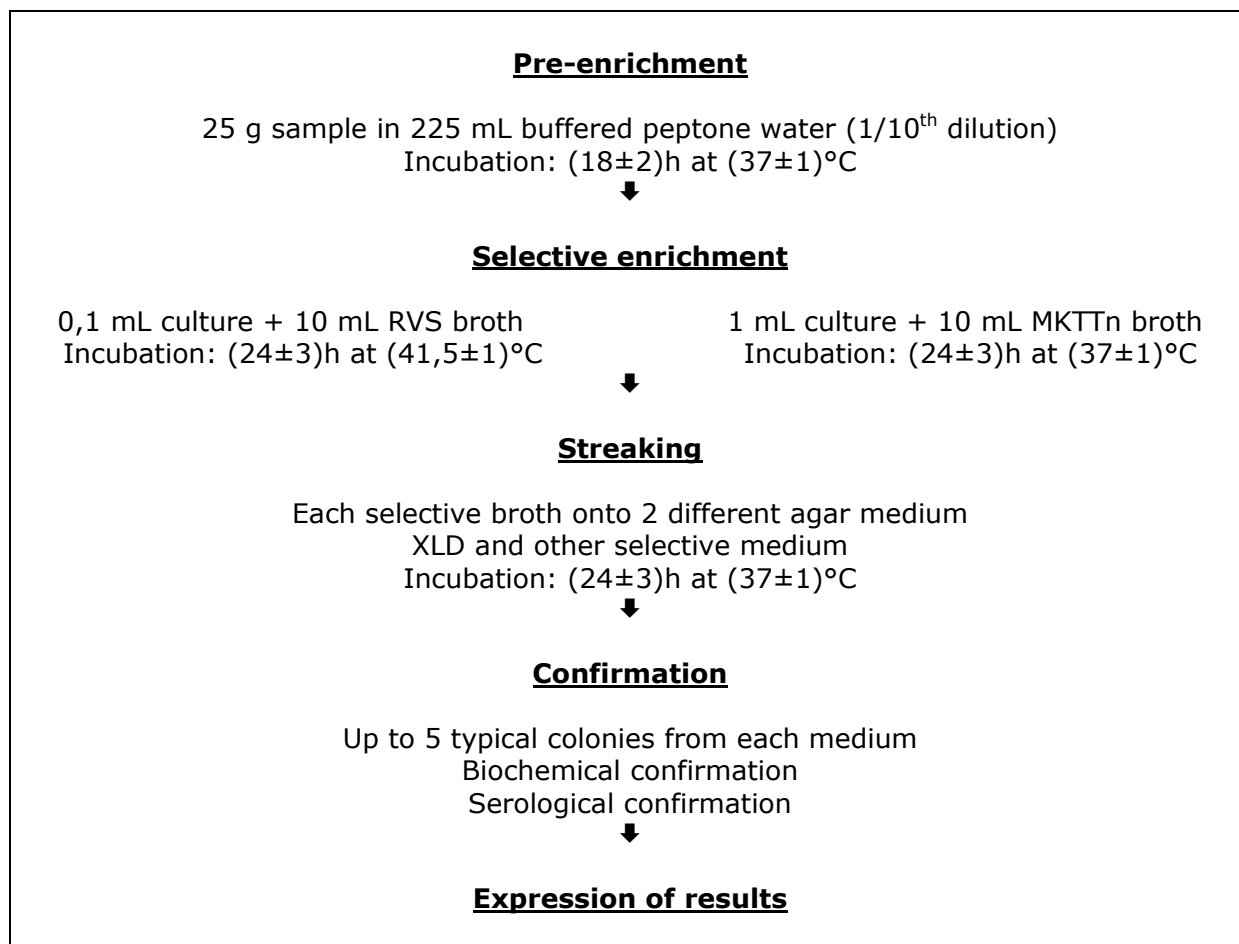


Figure 2: reference method protocol

2. Comparative study

The following characteristics are studied during the preliminary study:

- Relative accuracy (AC), relative specificity (SP) and relative sensitivity (SE)
- Relative detection level of the alternative method and the reference method
- Selectivity of the alternative method
- Practicability of the alternative method

2.1. Relative accuracy, relative specificity, relative sensitivity

The relative accuracy is the degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples.

The relative specificity is the ability of the alternative method to not detect the target microorganism when it is not detected by the reference method.

The relative sensitivity is the ability of the alternative method to detect the analyte when it is detected by the reference method.

The objective of this study is to evaluate the performance of both methods on contaminated and non-contaminated samples.

2.1.1. Number and nature of samples

The following categories are studied: meat products, dairy products, seafood and vegetable products, other products, feedstuffs products and environmental samples.

A number of 395 samples was analysed. Types of products are indicated in table 1.

Category	Type	Number of positive samples	Number of negative samples	Total
Meat products	Raw poultry meat	14	12	26
	Raw beef meat	9	8	17
	Others meats	2	3	5
	Delicatessen	8	9	17
	Total	33	32	65
Dairy products	Raw milk cheese	17	19	36
	Raw milk and other milks	6	5	11
	Pasteurized milk cheese	5	8	13
	Other dairy product	2	2	4
	Total	30	34	64
Seafood and vegetable products	Raw and cooked vegetables	13	15	28
	Raw and cooked fruit	4	5	9
	Spices and herbs	2	2	4
	Fish and shellfish	15	12	27
	Total	34	34	68
Other products	Eggs and egg products	10	11	21
	Pastries	10	10	20
	Others	10	10	20
	Total	30	31	61
Feedstuffs products	Pet food	26	27	53
	Bone meals	5	4	9
	Cattle feeding	10	5	15
	Total	41	36	77
Environmental samples	Process water	5	6	11
	Swabs	22	20	42
	Sponges	3	4	7
	Total	30	30	60
Total		198	197	395

Table 1: nature and number of analysed samples (*=positive results by either method)

2.1.2. Artificial contamination of samples

Naturally contaminated samples are seldom available. Therefore, artificial contaminations of food samples were mostly performed. For spiking, several strains were stressed using

different treatments and the stress intensity was evaluated (logarithmic difference between enumeration on non selective agar –TSA- and selective agar –XLD-).

49 naturally contaminated samples were analysed. 73,9 % of positive samples are the results of artificial spiking.

2.1.3. Confirmation protocol

The confirmation of presumed positive results obtained by the alternative method was realized:

- by testing oxydase on an isolated typical colony : if the oxydase is negative, perform latex *Salmonella* test,
- by testing oxydase on an isolated typical colony : if the oxydase is negative, use SALSA medium and incubate it at (37±1) °C for (24±3) hours

2.1.4. Results

Each sample was analysed once by the alternative method and once by the reference method.

Table 2 presents paired results of both methods.

Category	Response	Reference method^(*) positive (R+)	Reference method^(*) négative (R-)
Meat products	Alternative method positive (A+)	PA=17	PD=6
	Alternative method négative (A-)	ND=10 including 0 PPND	NA=32 including 2 PPNA
Dairy products	Alternative method positive (A+)	PA=22	PD=4
	Alternative method négative (A-)	ND=4 including 0 PPND	NA=34 including 0 PPNA
Sea food and vegetable products	Alternative method positive (A+)	PA=32	PD=1
	Alternative method négative (A-)	ND=1 including 0 PPND	NA=34 including 2 PPNA
Other products	Alternative method positive (A+)	PA=27	PD=1
	Alternative method négative (A-)	ND=2 including 0 PPND	NA=31 including 0 PPNA
Food stuff products	Alternative method positive (A+)	PA=38	PD=1
	Alternative method négative (A-)	ND=2 including 0 PPND	NA=36 including 0 PPNA
Environ-mental samples	Alternative method positive (A+)	PA=30	PD=0
	Alternative method négative (A-)	ND=0 including 0 PPND	NA=30 including 0 PPNA
All products	Alternative method positive (A+)	PA=166	PD=13
	Alternative method négative (A-)	ND=19 including 0 PPND	NA=197 including 4 PPNA

Table 2: results of relative accuracy for both methods (PA: positive agreement, NA: negative agreement, ND: negative deviation, PD: positive deviation, PP: presumed positive before confirmation, A+: confirmed positive, A-: negative immediately and negative after confirmation when presumed positive)

2.1.5. Calculation of relative accuracy (AC), relative specificity (SP) and relative sensitivity (SE)

For all products categories, these results permit to calculate the relative accuracy, relative specificity and relative sensitivity according to NF EN ISO standard. Results are indicated in table 3.

Category	PA	NA	ND	PD	N	Exactitude relative AC [(PA+NA)/N]	N+ PA+ND	Sensibilité relative SE [PA/N+]	N- NA+PD	Spécificité relative SP [NA/N-]
Meat products	17	32	10	6	65	75,4%	27	63,0%	38	84,2%
Dairy products	22	34	4	4	64	87,5%	26	84,6%	38	89,5%
Sea food and vegetable products	32	34	1	1	68	97,1%	33	97,0%	35	97,1%
Other products	27	31	2	1	61	95,1%	29	93,1%	32	96,9%
Food stuff products	38	36	2	1	77	96,1%	40	95,0%	37	97,3%
Environmental samples	30	30	0	0	60	100%	30	100%	30	100%
All products	166	197	19	13	395	91,9%	185	89,7%	210	93,8%

Table 3: relative accuracy, relative specificity and relative sensitivity of alternative method (PA: positive agreement, NA: negative agreement, ND: negative deviation, PD: positive deviation, AC = (PA+NA)/N x 100%, SE = PA/N+ x 100%, SP = NA/N- x 100%, N+ = PA+ND and N- = NA+PD)

Criteria values in percent are shown in table 4.

	Alternative method
Relative accuracy	91,9%
Relative sensitivity	89,7%
Relative specificity	93,8%

Table 4: AC, SE and SP in percent for alternative method

Sensitivity of both methods was recalculated considering all confirmed positive (including alternative method positive deviations). Results are shown in table 5.

	Alternative method (PA+PD)/(PA+PD+ND)	Reference method (PA+ND)/(PA+PD+ND)
Sensitivity	90,4%	93,4%

Table 5: sensitivity of both methods including all confirmed positive

2.1.6. Analysis of discordant results

Discordant results are examined according to annex F of NF EN ISO 16140 standard, with Y as the number of discordant results and m as the smallest of the two values of PD and ND.

In the present case, $Y = 13 + 19 = 32$, the McNemar's test is used with the chi square distribution for 1 degree of freedom to compare the two methods.

The following formula $X^2 = d^2/Y$, with $d = |PD - ND|$ permits to calculate $X^2 = 1.125$. The X^2 value is inferior to 3.841, both methods are comparable for $\alpha=0.05$.

- Negative deviations

-Sample numbers: 1 / 10 / 18 / 30 / 35 / 37 / 45 / 47 / 214 / 243 / 256 / 260 / 265 / 347 / 356 / 390 / 431 / 441:

A positive result is obtained by the reference method whereas a negative result is obtained by the alternative method. However analysis of the alternative method broth by the reference method didn't allow finding typical colonies or showed typical colonies not confirmed. Due to the difference of sampling between both methods, no cell of *Salmonella* may have been taken in the sampling for the alternative method.

Sample numbers: 31

A positive result is obtained by the reference method whereas a negative result is obtained by the alternative method. However the analysis of alternative method broth by reference method allowed finding typical colonies which were confirmed as *Salmonella*. This result is due probably at annexe flora of the sample and the low concentration of spiking.

- Positive deviations

-Sample numbers: 6 / 39 / 52 / 155 / 204 / 210 / 211 / 242 / 248 / 268 / 368 / 399 / 447

A positive result is obtained by the alternative method whereas a negative result is obtained by the reference method. Due to the difference of sampling between both methods, no cell of *Salmonella* may have been taken in the sampling for the reference method.

2.1.7. Broth stability study

A study of the stability of the enriched broths at $(5\pm 3)^\circ\text{C}$ for 72 hours was realized on the whole positive and discordant samples. The broths were analysed by the alternative method and the results were compared to those obtained in the relative accuracy study. No discordant result was observed.

A storage at $(5\pm 3)^\circ\text{C}$ for 72 hours of the IBISA Petri dishes showing typical colonies was done. These dishes were confirmed according to the two techniques described. The typical colonies that had first grown on the medium kept their typical. No discordant result was observed.

2.2. Relative detection level

The objective of this study is to determine the level of contamination for which less than 50% of the responses obtained are positive and that for which more than 50% of the responses obtained are positive.

2.2.1. Matrices

Six couples "matrix-strain" were studied in parallel with the reference method and the alternative method for each category. The total viable count of each matrix was enumerated. Characteristics of the strain and the matrix are shown in table 6.

Matrix	Strain	ISHA code	Origin
Minced meat	S. Typhimurium	SAL.1.133	Minced meat
Raw milk	S. Newport	SAL.1.98	Raw milk cheese
Fish	S. London	SAL.1.80	Sea product
Raw egg	S. Enteritidis	SAL.1.48	Egg product
Rodent food	S. Infantis	SAL.1.69	Horse food
Process water	S. Yoruba	SAL.1.162	Environmental sample

Table 6: "matrix-strain" couples of the relative detection level

2.2.2. Spiking protocol

Six levels of contamination were tested including the negative control.

Six replicates for each level of contamination were inoculated and analysed by the reference method and the alternative method.

As the two methods have no common step, 12 test portions of 25 g were prepared for each level of contamination and individually inoculated with a calibrated bacterial suspension. Bacterial suspension of about 10 cells per mL was prepared. From this initial suspension, volumes of 0.9 mL, 0.3 mL and 0.1 mL were used to spike 25 g of sample respectively for the 3 first levels. In parallel, the initial suspension was diluted ratio ½ and ¼ in order to inoculate the lower levels of contamination with 0.1 mL.

For all the levels of contamination, homogeneity of the inoculums was checked by enumeration on 30 TSA Petri dishes. Then, the confidence interval was determined according to Poisson law.

2.2.3. Results

Tables 7 and 8 present the relative detection level for each method.

Strain	Matrix	Relative detection level according to Spearman-Kärber method (cells in 25 g)	
		Reference method (*)	Alternative method
S. Typhimurium	Minced meat	0,702 [0,502 ; 0,983]	0,787 [0,562 ; 1,102]
S. Newport	Raw milk	0,553 [0,377 ; 0,812]	0,569 [0,387 ; 0,835]
S. Virchow	Fish	0,572 [0,306 ; 1,068]	0,539 [0,333 ; 0,871]
S. Enteritidis	Raw egg	1,100 [0,714 ; 1,695]	1,000 [0,649 ; 1,540]
S. Infantis	Rodent food	0,863 [0,534 ; 1,395]	0,760 [0,470 ; 1,228]
S. Yoruba	Process water	1,233 [0,839 ; 1,810]	1,173 [0,628 ; 2,190]

Table 7: relative detection level (3 significant numbers)

Strain	Matrix	Relative detection level according to Spearman-Kärber method (cells in 25 g)	
		Reference method (*)	Alternative method
S. Typhimurium	Minced meat	0,7 [0,5 ; 1,0]	0,8 [0,6 ; 1,1]
S. Newport	Raw milk	0,6 [0,4 ; 0,8]	0,6 [0,4 ; 0,8]
S. Virchow	Fish	0,6 [0,3 ; 1,1]	0,5 [0,3 ; 0,9]
S. Enteritidis	Raw egg	1,1 [0,7 ; 1,7]	1,0 [0,7 ; 1,5]
S. Infantis	Rodent food	0,9 [0,5 ; 1,4]	0,8 [0,5 ; 1,3]
S. Yoruba	Process water	1,2 [0,8 ; 1,8]	1,2 [0,6 ; 2,2]

Table 8: relative detection level (1 significant number)

The detection limit obtained with the alternative method is comprised between 0.3 and 2.2 CFU in 25 g and the detection limit obtained with the reference method is comprised between 0.3 and 1.8 CFU in 25 g

2.3. Inclusivity / exclusivity (selectivity)

The objective of this study is to test:

- the inclusivity: the detection of the target microorganism from a wide range of strains,
- the exclusivity: the lack of interference from a relevant range of non-target microorganisms.

According to the requirements of NF EN ISO 16140, 53 strains of *Salmonella* spp and 30 non-target strains were tested. A list of the strains figures in annex 1.

2.3.1. Test protocols

- **Inclusivity**

Each *Salmonella* strain was cultivated twice before inoculation in BPW (about 1 to 100 CFU/225 mL). The complete protocol of alternative method was applied with the minimum time of incubation.

- **Exclusivity**

Each non-target strain was cultivated twice before inoculation in growth medium (Trypticase Soy Broth) with a level of contamination expected to occur in the food matrices (about 10^5 CFU/mL). After 24 hours of incubation, the ADIAFOOD test was performed.

In cases where the target strains or non-target strains results were unexpected to interpret by the alternative method, the analysis was conducted once again in parallel with the alternative method and the reference method (complete protocol).

2.3.2. Results

The 53 *Salmonella* strains tested were detected by the alternative method.

No non target strain was detected by the alternative method except for two strains of *Pseudomonas* (little green colonies) but with positive oxydase test.

2.3.3. Conclusion

The selectivity of the method is satisfactory.

3. Collaborative study

The main object of the collaborative study is to determine the variability of the results obtained by different laboratories analysing identical samples and to compare these results within the framework of the comparative study of the methods.

3.1. Collaborative study implementation

3.1.1. Participating laboratories

The collaborative study was realized by the expert laboratory and fourteen participating laboratories.

3.1.2. *Salmonella* spp absence in the matrix

Before spiking, the absence of *Salmonella* spp was verified in the batch of minced meat used according to the reference method.

3.1.3. Strain stability in the matrix

The strain stability in minced meat matrix was evaluated for 4 days at (4±2)°C. The strain used was *Salmonella* Typhimurium (code ISHA : SAL.1.133) isolated from beef minced meat.

The two methods were performed. Inoculation of 10 cells in 25 mL minced meat. The samples were analysed at D0, D+1, D+2 and D+3 by the reference method and by the alternative method. The results are summarized in table 9.

Day	Alternative method	Reference method
D0	Presence in 25 g	Presence in 25 g
D+1	Presence in 25 g	Presence in 25 g
D+2	Presence in 25 g	Presence in 25 g
D+3	Presence in 25 g	Presence in 25 g

Table 9: results of the stability study of the strain SAL.1.133 in minced meat

The results show that the *Salmonella* strain used is stable for 3 days at (4±2)°C in minced meat.

3.1.4. Samples preparation and spiking

The matrix was inoculated with the target strain suspension to obtain 3 contamination levels:

- L0: 0 cell in 25 g
- L1: 3 cells in 25 g
- L2: 30 cells in 25 g

The matrix was distributed at 25 g in sterile bags. Every bag was individually spiked and homogenized. Eight samples per level, per laboratory and per method were prepared. Each laboratory received 48 samples to analyse, 1 sample to quantify the endogenous microflora and 1 water sample containing a temperature probe.

The results of the enumerations of the TVC, the target levels and the real levels of contamination are presented in table 10.

Matrix	Total viable count (CFU/g)	Target level (cells / 25 g)	Real level (cells / 25 g)	Confidence interval
Minced meat	1,3.10 ⁶	0	0	0
		3	4,4	[1 ; 9]
		30	36	[25 ; 48]

Table 10: target level, real level and TVC of the matrix

3.1.5. Samples labeling

The labelling of the bags was realized as follows: a code to identify the laboratory: from A to N (cf. table 11) and a code to identify each sample, only known by the expert

laboratory. The samples and the temperature control vials (water sample with a temperature probe) were stored at 4°C before shipping.

Contamination level	Sample code
L0	3/8/15/18/21/22/23/24
L1	2/4/9/10/11/12/14/16
L2	1/5/6/7/13/17/19/20

Table 11: sample code by contamination level

3.1.6. Samples shipping

The samples were shipped in a coolbox the 23rd of May 2011.

3.1.7. Samples reception and analysis

The coolboxes were received the 24th of June 2011 by all the participating laboratories. The control temperature was recorded upon receipt of the package and the temperature probe sent to the expert laboratory. The samples were analysed the same day. The expert laboratory concurrently analysed a set of samples under the same conditions with both methods

3.2. Results

3.2.1. Temperature and state of the samples

The temperature readings upon reception and the state of the samples are shown in table 12.

Laboratory	Temperature (°C)	State of the samples
A	3,4	Correct
B	4,5	Correct
C	3,3	Correct
D	4,0	Correct
E	3,3	Correct
F	5,3	Correct
G	4,9	Correct
H	5,7	Correct
I	6,7	Correct
J	5,1	Correct
K	3,5	Correct
L	5,8	Correct
M	7,3	
N	6,1	Correct

Table 12: temperature and state of the samples upon reception

The temperature measurements are inferior to 8.4°C for all the laboratories.. The analysis of thermal profiles is shown in table 13.

Laboratory	A	B	C	D	E	F	G	H	I	J	K	L	M	N	
T (°C)	Mean	4,4	3,7	4,3	4,4	3,0	6,4	6,7	4,6	4,8	3,0	nd	6,4	nd	nd
	SD	1,5	1,4	0,8	0,8	1,4	0,9	1,2	1,3	1,3	0,5	nd	1,6	nd	nd

Table 13: data of the temperature probes for the transportation time of samples

For three laboratories (K, M and N) temperature probes were failure but the final temperature was checked. The thermal profiles analysis indicates for all laboratories mean temperatures comprises between 3 and 6.7°C.

3.2.2. Total viable counts

For the whole laboratories, the total viable counts at 30°C vary between 5.0×10^5 and 6.4×10^5 CFU/g.

3.2.3. Expert laboratory results

The results obtained by the expert laboratory are summarized in table 14.

Contamination level	Alternative method	Reference method (*)
L0	0/8	0/8
L1	8/8	8/8
L2	8/8	8/8

Table 14: positive results obtained by expert laboratory by both methods

The results are consistent with those expected.

3.2.4. Participating laboratories results

The results are summarized in tables 15 and 16.

- Alternative method results

Laboratory	Contamination level		
	L0	L1	L2
A	0/8	8/8	8/8
B	0/8	8/8	8/8
C	0/8	8/8	8/8
D	0/8	8/8	8/8
E	0/8	8/8	8/8
F	0/8	8/8	8/8
G	0/8	8/8	8/8
H	0/8	8/8	8/8
I	0/8	8/8	8/8
J	0/8	8/8	8/8
K	0/8	8/8	8/8
L	0/8	8/8	8/8
M	0/8	8/8	8/8
N	0/8	7/8	8/8

Table 15: alternative method positive results for all laboratories

- Reference method results

Laboratory	Contamination level		
	L0	L1	L2
A	1/8	8/8	8/8
B	0/8	8/8	8/8
C	0/8	8/8	8/8
D	0/8	8/8	8/8
E	0/8	8/8	8/8
F	0/8	8/8	8/8
G	0/8	8/8	8/8
H	0/8	8/8	8/8
I	0/8	8/8	8/8
J	0/8	8/8	8/8
K	0/8	8/8	8/8
L	0/8	8/8	8/8
M	0/8	8/8	8/8
N	0/8	8/8	8/8

Table 16: reference method positive results for all laboratories

- Results analysis

The laboratory N presented a negative result by the alternative method for a low level contaminated sample. Due to the low level of contamination of this sample (3 CFU/25 mL), no *Salmonella* cell may have been inoculated in the matrix.

The laboratory A presented a positive result by the reference method for a no contaminated sample. All investigations showed that it was a cross-contamination.

Final analysis was consequently conducted using data supplied by thirteen laboratories.

3.2.5. Specificity (SP) and sensitivity (SE) calculations

The specificity and sensitivity calculations of both methods are presented in table 17, with the low critical value (LCL). Formulas used are:

For level L0, $SP = [1 - (FP/N-)] \times 100\%$, N-: total number of L0 tests
FP: number of false positive

For levels L1 and L2, $SE = (TP/N+) \times 100\%$, N+: total numbers of L1 or L2 tests
TP: number of true positive

Specificity / sensitivity	Alternative method	LCL	Reference method	LCL
SP (level L0)	100%	98%	100%	98%
SE (level L1)	99,1%	98%	100%	98%
SE (level L2)	100%	98%	100%	98%
SE (level L1+L2)	99,6%	98%	100%	98%

Table 17: specificity (SP), sensitivity (SE) and LCL of alternative and reference method

3.2.6. Relative accuracy calculations

Pairs of results of the different levels of contamination are presented in table 18.

Level	Alternative method	Reference method		
		RM+	RM-	Total
L0	AM+	PA=0	PD=0	0
	AM-	ND=0	NA=104	104
	Total	0	104	104
L1	AM+	PA=103	PD=0	103
	AM-	ND=1	NA=0	1
	Total	104	0	104
L2	AM+	PA=104	PD=0	104
	AM-	ND=0	NA=0	0
	Total	104	0	104
L0+L1+L2	AM+	PA=207	PD=0	207
	AM-	ND=1	NA=104	105
	Total	208	104	312

Table 18: tests results for both methods (PA: positive agreement, NA: negative agreement, ND: negative deviation, PD: positive deviation)

Relative accuracy values of the different contamination levels are presented in table 19 with their LCL. Formula used is the following:

$AC = (PA+NA)/N \times 100\%$, PA: number of positive agreements
NA: number of negative agreements

Level	Relative accuracy (AC)	LCL (Low Critical Value)
L0	100%	98,0%
L1	99,0%	98,0%
L2	100%	98,0%
L1+L2	99,5%	98,0%
Total	99,7%	98,0%

Table 19: relative accuracy values (AC) and LCL of alternative method

3.2.7. Discordant results analysis

Discordant results are analysed according to the annex F of ISO 16140 standard. The total number of discordant results is given by the following formula: $Y = PD + ND$.

In the present case, $Y = 0 + 1 = 1$, with $Y < 6$ so, the two methods are considered as equivalent.

3.3. Interpretation

3.3.1. Accordance

The accordance is the 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).

To derive the accordance from the results of an interlaboratory study, the probability that two samples give the same result is calculated for each participating laboratory in turn, and this probability is then averaged over all laboratories. Values of accordance are shown in table 20. Calculations of accordance by level and method are presented in annex 2.

Level	Alternative method	Reference method
L0	100%	100%
L1	98,3%	100%
L2	100%	100%

Table 20: accordance by level and method

3.3.2. Concordance

The concordance is the percentage chance of finding the same result for two identical samples analysed in two different laboratories.

To calculate the concordance from the results of an interlaboratory study, take in turn each replicate in each participating laboratory, pair it with identical results of all the other laboratories. The concordance is the percentage of all pairings giving the same results on all the possible pairings of data. Values of concordance are shown in table 21. Calculations of concordance by level and method are presented in annex 3.

Level	Alternative method	Reference method
L0	100%	100%
L1	98,1%	100%
L2	100%	100%

Table 21: concordance by level and method

3.3.3. Concordance odds ratio

If the concordance is smaller than the accordance, it indicates that two identical samples are more likely to give the same result if they are analysed by the same laboratory than if they are analysed by different ones, suggesting that there can be variability in

performance between laboratories. Unfortunately, the magnitude of the concordance and accordance is strongly dependent on the level of accuracy, making it difficult to assess easily the degree of between-laboratory variation.

It is therefore helpful to calculate the concordance odds ratio (COR) defined as follows:

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

Values of COR for both methods are shown in table 22.

A value for the odds ratio of 1.00 would be expected if accordance and concordance were equal, and the larger the odds ratio is, the more inter-laboratory variation is predominant. Nevertheless, values above 1.00 can occur by chance variation, and so a statistical significance test should be used to confirm whether the evidence for extra variation between laboratories is convincing. The "exact test" is the best recommended test for this). The philosophy behind such tests is that the probabilities of occurrence are calculated for all sets of replicate results that could have produced the overall numbers of positives and negatives.

Level	Alternative method			Reference method		
	Accordance	Concordance		Accordance	Concordance	
L0	100	100	1,0	100	100	1,0
L1	98,3	98,1	1,1	100	100	1,0
L2	100	100	1,0	100	100	1,0

Table 22: COR values for each method by contamination level

3.3.4. AC, SP, SE comparison

Table 23 summarizes the values obtained for AC, SP and SE parameters for the preliminary study and the interlaboratory study.

Parameter	Preliminary study	Interlaboratory study
AC	91,6%	99,7%
SP	89,1%	100%
SE	97,9%	99,5%

Table 23: AC, SP and SE comparison between preliminary and interlaboratory study

The values obtained during the collaborative study are better than those obtained during the preliminary study, probably because of the greater variety of samples and strains tested during the preliminary study.

The sensitivity of both methods is recalculated in table 24 by including all confirmed positive results.

Alternative method (PA+PD)/(PA+PD+ND)	Reference method (PA+ND)/(PA+PD+ND)
99,5%	100%

Table 24: sensitivity recalculated by both methods

4. Practicability

The practicability was evaluated according to the 13 criteria defined by AFNOR Technical Committee.

1- Mode of packaging of test components

2- Volume of reagents

3- Storage conditions of components and shelf-life of unopened products (expiration of not opened products)

4- Modalities after first use

The media is manufactured in Petri dishes (to be conserved between 2 and 8 °C)

AEB520059: case of 120 dishes. 90 mm

AEB520060: case of 20 dishes. 90 mm

IBISA® specific supplement (to be conserved between 2 and 8 °C)

After incubation, it is possible to keep the enrichment broth for 72 hours at 2-8 °C. After incubation, IBISA dishes can be stored cold (72 h maximum) before reading and confirmation tests.

5- Equipment and specific local requirements

Equipment

- Two single channel pipettes
- *Stomacher* (homogenizer)
- Incubators
- Dilutor
- Bunsen burner
- Serological pipette pump
- Stomacher bag holder
- Refrigerator 4°C (2 to 8°C)
- Colour printer
- Pipettes supports
- Tubes racks

Consumables

- Bags filter
- Enrichment media

6- Reagents ready to use or for reconstitution

None

7- Training period for operator with no experience with the method

Half day is required for technicians with microbiology knowledge.

8- Handling time and flexibility of the method in relation to the number of samples

Steps- Manipulation time – Negative samples	Time (minutes)			
	Alternative method		Reference method	
	1 analysis	20 analyses	1 analysis	20 analyses
Dilution - Suspension	3	30	3	30
2 nd enrichment	/	/	1	16
Streaking on IBISA media	1	12	/	/
Isolation on XLD and another selective medium	/	/	2	20
Reading of the Petri dishes	0,5	6	1	12
Total	4,5	48	7	78

Steps- Manipulation time – Positive samples	Time (minutes)			
	Alternative method		Reference method	
	1 analyse	20 analyses	1 analyse	20 analyses
Dilution - Suspension	3	30	3	30
2 nd enrichment	/	/	1	16
Streaking on IBISA media	1	12	/	/
Isolation on XLD and another selective medium	/	/	2	20
Reading of the Petri dishes	0,5	6	1	12
IBISA test confirmation	2	30	/	/
Biochemical test confirmation	/	/	3	40
Total	6,5	78	10	118

9- Time required for results

Steps –Time for negative results	Alternative method	Reference method
Dilution - Suspension	D0	D0
2 nd enrichment	/	D1
Isolation on IBISA media	D1	/
Isolation on XLD and another selective medium	/	D2
Reading of the Petri dishes	D2	D3

Steps –Time for positive results	Alternative method	Reference method
Dilution - Suspension	D0	D0
2 nd enrichment	/	D1
Isolation on IBISA media	D1	/
Isolation on XLD and another selective medium	/	D2
Reading of the Petri dishes	D2	D3
IBISA test confirmation	D2 ou D3	/
Biochemical test confirmation	/	D5

10- Operator qualification

Identical as necessary for the reference method

11- Steps common with the reference method

None.

12- Traceability of analysis results

None.

13- Maintenance by laboratory

None.

5. Conclusion

Concerning the preliminary study, the performances of the IBISA test for the detection of *Salmonella* spp are comparable to those of the standard NF EN ISO 6579 (2002).

This study concerned 395 samples of five categories of products (meat, dairy, seafood, vegetable, food stuffs and environmental products).

Values obtained for the 3 criteria are the following:

- relative accuracy: 91.9%
- relative sensitivity: 89.7%
- relative specificity: 93.8 %

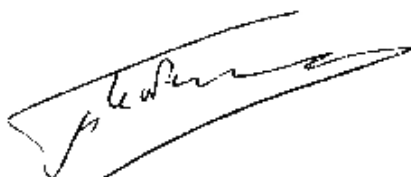
Several discordant results were observed (13 PD and 19 ND). Most of these results are due to the difference of sampling between both methods, no cell of *Salmonella* may have been taken in the sampling for the alternative method.

The relative level of detection of the alternative method and the reference method was evaluated for all categories. The detection limit obtained with the alternative method is comprised between 0.3 and 2.2 CFU in 25 g and the detection limit obtained with the reference method is comprised between 0.3 and 1.8 CFU in 25 g

The specificity of the method is satisfactory.

Concerning the interlaboratory study, the results obtained for the 12 selected laboratories showed that the values of relative accuracy, relative sensitivity and relative specificity are comparable to those obtained during the preliminary study. The variability of the alternative method, demonstrated by the calculations of accordance, concordance and concordance odds ratio, is similar to that of the reference method.

The study of the practicability of the alternative method shows a simple and easy-to-use method and a significant time savings compared to the reference method.



Massy, the 4th of October 2011
François Le Nestour
Manager of Biology Innovation Unit

Annex 1 : selectivity

Inclusivity list

Code	Microorganism	Origin (French designation)
SAL.1.5	<i>Salmonella</i> Anatum	Sésame décortiqué
SAL.1.7	<i>Salmonella</i> Arizonae (48 : z4, z23 :-)	Canard
SAL.1.8	<i>Salmonella</i> Arizonae (18 : z4, z23 :-)	Canard
SAL.1.10	<i>Salmonella</i> Braenderup	Env. atelier (alim. humaine)
SAL.1.17	<i>Salmonella</i> Brandenburg	Canard
SAL.1.21	<i>Salmonella</i> Bredeney	Blanc de poulet cru
SAL.1.23	<i>Salmonella</i> Cerro	Farine de lapin
SAL.1.29	<i>Salmonella</i> Derby	Echine de porc
SAL.1.38	<i>Salmonella</i> Derby	Chiffonnette salaisonerie
SAL.1.40	<i>Salmonella</i> Diarizonae	Semoule de blé
SAL.1.42	<i>Salmonella</i> Diarizonae	Boue station épuration
SAL.1.43	<i>Salmonella</i> Dublin	Lait
SAL.1.44	<i>Salmonella</i> Dublin	Quality management UK
SAL.1.47	<i>Salmonella</i> Enteritidis	Poulet
SAL.1.52	<i>Salmonella</i> Enteritidis	Chiffonnette pâtisserie
SAL.1.170	<i>Salmonella</i> Gallinarum	Env. élevage de pintade
SAL.1.171	<i>Salmonella</i> Gallinarum	Elevage de poules
SAL.1.57	<i>Salmonella</i> Hadar	Escalope de volaille
SAL.1.60	<i>Salmonella</i> Havana	Env. atelier (alim. humaine)
SAL.1.61	<i>Salmonella</i> Heidelberg	Viande de volaille
SAL.1.64	<i>Salmonella</i> Indiana	Filet de bœuf
SAL.1.69	<i>Salmonella</i> Infantis	Farine de viande
SAL.1.163	<i>Salmonella</i> Infantis	Lait (alim. humaine)
SAL.1.169	<i>Salmonella</i> Kedougou	Couenne de porc
SAL.1.76	<i>Salmonella</i> Kottbus	Sauté de dinde cru
SAL.1.78	<i>Salmonella</i> Livingstone	Environnement atelier de production
SAL.1.83	<i>Salmonella</i> London	Abattoir de volaille (alim. humaine)
SAL.1.84	<i>Salmonella</i> Manhattan	Bovin
SAL.1.85	<i>Salmonella</i> Mbandaka	Pintadeau
SAL.1.91	<i>Salmonella</i> Montevideo	Tartare pur bœuf
SAL.1.97	<i>Salmonella</i> Napoli	Canard
SAL.1.98	<i>Salmonella</i> Newport	Fromage au lait cru
SAL.1.101	<i>Salmonella</i> Orion	Canard
SAL.1.102	<i>Salmonella</i> Paratyphi A	CIP 55 39
SAL.1.104	<i>Salmonella</i> Paratyphi A	CIP A 220
SAL.1.110	<i>Salmonella</i> Paratyphi B	Filet de poulet cru
SAL.1.111	<i>Salmonella</i> Paratyphi B	Paupiette de lapin cuite
SAL.1.112	<i>Salmonella</i> Paratyphi C	CIP 55.108
SAL.1.114	<i>Salmonella</i> Poona	Environnement atelier (alim. animale)
SAL.1.115	<i>Salmonella</i> Regent	Manchon de canard
SAL.1.116	<i>Salmonella</i> Rissen	Environnement atelier de production
SAL.1.120	<i>Salmonella</i> Saint-Paul	Viande surgelée
SAL.1.121	<i>Salmonella salamae</i>	Lait cru
SAL.1.122	<i>Salmonella</i> Schwarzengrund	Sauté de porc cru
SAL.1.126	<i>Salmonella</i> Senftenberg	Tourteau de soja (alim. animale)
SAL.1.129	<i>Salmonella</i> Typhi	CIP 54 136
SAL.1.147	<i>Salmonella</i> Typhimurium	Cordon bleu surgelé
SAL.1.158	<i>Salmonella</i> Virchow	11337 (intox)
SAL.1.181	<i>Salmonella bongori</i>	Environnement industriel
SAL.1.182	<i>Salmonella</i> Typhimurium variant immobile (S.I 1,4,[5],12:-:-)	Tiramisu
SAL.1.183	<i>Salmonella</i> Typhimurium variant monophasique (S.I 1,4,[5],12:i:-)	porc à la tahitienne
SAL.1.184	<i>Salmonella</i> Typhimurium variant monophasique (S.I 1,4,[5],12:-:1,2)	Environnement élevage poule
SAL.1.185	<i>Salmonella</i> Blockley	Environnement élevage poule

Exclusivity list

Code	Microorganism	Origin (French designation)
CIT.1.1	<i>Citrobacter freundii</i>	CIP 53.62
CIT.1.2	<i>Citrobacter freundii</i>	ATCC 8090
CIT.2.4	<i>Citrobacter koseri</i>	Effluent secondaire
CIT.2.1	<i>Citrobacter koseri</i>	CIP 72.11
CIT.2.2	<i>Citrobacter diversus</i>	CIP 82.87 T
CIT.2.3	<i>Citrobacter diversus</i>	CIP 82.94
ENTB.1.1	<i>Enterobacter aerogenes</i>	Industrie laitière
ENTB.2.1	<i>Enterobacter cloacae</i>	Eaux usagées
ENTB.3.1	<i>Enterobacter sakazakii</i>	Poudre de lait
ENTB.3.2	<i>Enterobacter sakazakii</i>	CIP 57.33
ESC.1.6	<i>Escherichia coli</i>	Ravioli poulet
ESC.1.3	<i>Escherichia coli</i>	Industrie laitière
ESC.2.1	<i>Escherichia hermanii</i>	CIP 103176
ESC.3.1	<i>Escherichia vulneris</i>	CIP 103177T
HAF.1.1	<i>Hafnia alvei</i>	Taboulé
HAF.1.2	<i>Hafnia alvei</i>	CNRZ 713
KLE.1.1	<i>Klebsiella oxytoca</i>	Salade soja
KLE.2.1	<i>Klebsiella pneumoniae</i>	Pâtisserie
PAN.1.1	<i>Pantoea agglomerans</i>	A181
PRO.1.1	<i>Proteus mirabilis</i>	CIP 103181
PRO.2.1	<i>Proteus vulgaris</i>	Environnement industrie
PSE.1.2	<i>Pseudomonas aeruginosa</i>	Omelette gruyère
PSE.2.2	<i>Pseudomonas fluorescens</i>	CIP102127
SER.1.1	<i>Serratia ficaria</i>	CIP 79.23
SER.2.1	<i>Serratia fonticola</i>	CIP 103580
SER.3.1	<i>Serratia marcescens</i>	Environnement industrie
SHI.1.1	<i>Shigella flexneri</i>	CIP 82.48T
SHI.2.1	<i>Shigella sonnei</i>	ATCC 9290
PROV.1.1	<i>Providencia stuartii</i>	HPA RM
YER 1.1	<i>Yersinia enterocolitica</i>	CIP 80.27

Annex 2 - Accordance calculation

Alternative method

Number of replicates:

8

Level L0

Laboratory	Number of positive	Probability of positive	Probability of paired positive	Probability of negative	Probability of paired negative	Probability of paired identical results
B	0	0,000	0,000	1,000	1,000	1,000
C	0	0,000	0,000	1,000	1,000	1,000
D	0	0,000	0,000	1,000	1,000	1,000
E	0	0,000	0,000	1,000	1,000	1,000
F	0	0,000	0,000	1,000	1,000	1,000
G	0	0,000	0,000	1,000	1,000	1,000
H	0	0,000	0,000	1,000	1,000	1,000
I	0	0,000	0,000	1,000	1,000	1,000
J	0	0,000	0,000	1,000	1,000	1,000
K	0	0,000	0,000	1,000	1,000	1,000
L	0	0,000	0,000	1,000	1,000	1,000
M	0	0,000	0,000	1,000	1,000	1,000
N	0	0,000	0,000	1,000	1,000	1,000
Mean						100,0%

Level L1

Laboratory	Number of positive	Probability of positive	Probability of paired positive	Probability of negative	Probability of paired negative	Probability of paired identical results
B	8	1,000	1,000	0,000	0,000	1,000
C	8	1,000	1,000	0,000	0,000	1,000
D	8	1,000	1,000	0,000	0,000	1,000
E	8	1,000	1,000	0,000	0,000	1,000
F	8	1,000	1,000	0,000	0,000	1,000
G	8	1,000	1,000	0,000	0,000	1,000
H	8	1,000	1,000	0,000	0,000	1,000
I	8	1,000	1,000	0,000	0,000	1,000
J	8	1,000	1,000	0,000	0,000	1,000
K	8	1,000	1,000	0,000	0,000	1,000
L	8	1,000	1,000	0,000	0,000	1,000
M	8	1,000	1,000	0,000	0,000	1,000
N	7	0,875	0,766	0,125	0,016	0,781
Mean						98,3%

Level L2

Laboratory	Number of positive	Probability of positive	Probability of paired positive	Probability of negative	Probability of paired negative	Probability of paired identical results
B	8	1,000	1,000	0,000	0,000	1,000
C	8	1,000	1,000	0,000	0,000	1,000
D	8	1,000	1,000	0,000	0,000	1,000
E	8	1,000	1,000	0,000	0,000	1,000
F	8	1,000	1,000	0,000	0,000	1,000
G	8	1,000	1,000	0,000	0,000	1,000
H	8	1,000	1,000	0,000	0,000	1,000
I	8	1,000	1,000	0,000	0,000	1,000
J	8	1,000	1,000	0,000	0,000	1,000
K	8	1,000	1,000	0,000	0,000	1,000
L	8	1,000	1,000	0,000	0,000	1,000
M	8	1,000	1,000	0,000	0,000	1,000
N	8	1,000	1,000	0,000	0,000	1,000
Mean						100,0%

Reference method

Number of replicates:

8

Level L0

Laboratory	Number of positive	Probability of positive	Probability of paired positive	Probability of negative	Probability of paired negative	Probability of paired identical results
B	0	0,000	0,000	1,000	1,000	1,000
C	0	0,000	0,000	1,000	1,000	1,000
D	0	0,000	0,000	1,000	1,000	1,000
E	0	0,000	0,000	1,000	1,000	1,000
F	0	0,000	0,000	1,000	1,000	1,000
G	0	0,000	0,000	1,000	1,000	1,000
H	0	0,000	0,000	1,000	1,000	1,000
I	0	0,000	0,000	1,000	1,000	1,000
J	0	0,000	0,000	1,000	1,000	1,000
K	0	0,000	0,000	1,000	1,000	1,000
L	0	0,000	0,000	1,000	1,000	1,000
M	0	0,000	0,000	1,000	1,000	1,000
N						
Mean						100,0%

Level L1

Laboratory	Number of positive	Probability of positive	Probability of paired positive	Probability of negative	Probability of paired negative	Probability of paired identical results
B	8	1,000	1,000	0,000	0,000	1,000
C	8	1,000	1,000	0,000	0,000	1,000
D	8	1,000	1,000	0,000	0,000	1,000
E	8	1,000	1,000	0,000	0,000	1,000
F	8	1,000	1,000	0,000	0,000	1,000
G	8	1,000	1,000	0,000	0,000	1,000
H	8	1,000	1,000	0,000	0,000	1,000
I	8	1,000	1,000	0,000	0,000	1,000
J	8	1,000	1,000	0,000	0,000	1,000
K	8	1,000	1,000	0,000	0,000	1,000
L	8	1,000	1,000	0,000	0,000	1,000
M	8	1,000	1,000	0,000	0,000	1,000
N	8	1,000	1,000	0,000	0,000	1,000
Mean						100,0%

Level L2

Laboratory	Number of positive	Probability of positive	Probability of paired positive	Probability of negative	Probability of paired negative	Probability of paired identical results
B	8	1,000	1,000	0,000	0,000	1,000
C	8	1,000	1,000	0,000	0,000	1,000
D	8	1,000	1,000	0,000	0,000	1,000
E	8	1,000	1,000	0,000	0,000	1,000
F	8	1,000	1,000	0,000	0,000	1,000
G	8	1,000	1,000	0,000	0,000	1,000
H	8	1,000	1,000	0,000	0,000	1,000
I	8	1,000	1,000	0,000	0,000	1,000
J	8	1,000	1,000	0,000	0,000	1,000
K	8	1,000	1,000	0,000	0,000	1,000
L	8	1,000	1,000	0,000	0,000	1,000
M	8	1,000	1,000	0,000	0,000	1,000
N	8	1,000	1,000	0,000	0,000	1,000
Mean						100,0%

Annex 3 - Concordance calculation

Alternative method

Level L0	Laboratory	Number of negative	Interlaboratory pairs with the same result	Total number of interlaboratory pairs
	B	8	768	768
	C	8	768	768
	D	8	768	768
	E	8	768	768
	F	8	768	768
	G	8	768	768
	H	8	768	768
	I	8	768	768
	J	8	768	768
	K	8	768	768
	L	8	768	768
	M	8	768	768
	N	8	768	768
	Total		9984	9984
	Concordance			100,0%

Level L1	Laboratory	Number of positive	Interlaboratory pairs with the same result	Total number of interlaboratory pairs
	B	8	760	768
	C	8	760	768
	D	8	760	768
	E	8	760	768
	F	8	760	768
	G	8	760	768
	H	8	760	768
	I	8	760	768
	J	8	760	768
	K	8	760	768
	L	8	760	768
	M	8	760	768
	N	7	672	768
	Total		9792	9984
	Concordance			98,1%

Level L2	Laboratory	Number of positive	Interlaboratory pairs with the same result	Total number of interlaboratory pairs
	B	8	768	768
	C	8	768	768
	D	8	768	768
	E	8	768	768
	F	8	768	768
	G	8	768	768
	H	8	768	768
	I	8	768	768
	J	8	768	768
	K	8	768	768
	L	8	768	768
	M	8	768	768
	N		768	768
	Total		9984	9984
	Concordance			100,0%

Reference method

Level L0	Laboratory	Number of negative	Interlaboratory pairs with the same result	Total number of interlaboratory pairs
	B	8	768	768
	C	8	768	768
	D	8	768	768
	E	8	768	768
	F	8	768	768
	G	8	768	768
	H	8	768	768
	I	8	768	768
	J	8	768	768
	K	8	768	768
	L	8	768	768
	M	8	768	768
	N	8	768	768
	Total		9216	9216
	Concordance		100,0%	

Level L1	Laboratory	Number of positive	Interlaboratory pairs with the same result	Total number of interlaboratory pairs
	B	8	768	768
	C	8	768	768
	D	8	768	768
	E	8	768	768
	F	8	768	768
	G	8	768	768
	H	8	768	768
	I	8	768	768
	J	8	768	768
	K	8	768	768
	L	8	768	768
	M	8	768	768
	N	8	768	768
	Total		9216	9216
	Concordance		100,0%	

Level L2	Laboratory	Number of positive	Interlaboratory pairs with the same result	Total number of interlaboratory pairs
	B	8	768	768
	C	8	768	768
	D	8	768	768
	E	8	768	768
	F	8	768	768
	G	8	768	768
	H	8	768	768
	I	8	768	768
	J	8	768	768
	K	8	768	768
	L	8	768	768
	M	8	768	768
	N	8	768	768
	Total		9216	9216
	Concordance		100,0%	