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NF VALIDATION
Validation study according to the EN ISO 16140 standard

Summary report

EN ISO 16140 validation study of the NEOGEN ANSR™ for *Salmonella* for the detection of *Salmonella* spp in meat products, dairy products, and seafood and vegetables

Qualitative methods

This report includes 42 pages, with 6 appendixes.
Only copies including the totality of this report are authorised.
Competences of the laboratory are certified by COFRAC accreditation for the analyses marked with symbol◆.

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Before comment

Quality assurance documents related to this study can be consulted upon request by NEOGEN.

The technical protocol and the result interpretation were realised according to the EN ISO 16140 and the AFNOR technical rules.

✓ **Company**:	NEOGEN Cunningham Building Auchincruive, Ayr, KA6 5HW Scotland, U.K.
✓ **Expert Laboratory**:	ADRIA Développement ZA Creac'h Gwen 29196 QUIMPER Cedex
✓ **Studied method**:	**Validation of the NEOGEN ANSR™ *Salmonella***
✓ **Validation standard**:	EN ISO 16140 (October 2003) : Food microbiology – Protocol for the validation of alternative methods
✓ **Reference method**♦:	NF EN ISO 6579 (2002): Horizontal method for the detection of *Salmonella* spp.
✓ **Scope**:	**Meat products** **Dairy products** **Seafood and vegetables**
✓ **Certification organism**:	AFNOR Certification

♦ Analyses performed according to the COFRAC accreditation

1 AIM OF THE STUDY

The validation study of the **NEOGEN ANSR™ for *Salmonella* in meat products, dairy products and seafood and vegetables** was performed according to the EN ISO 16140 protocol and the AFNOR technical rules.

The following criteria were evaluated during the validation study:

- the method comparison study:
  - the practicability,
  - the inclusivity and the exclusivity,
  - the relative detection limit,
  - the relative accuracy, the relative sensitivity and the relative specificity.

- the interlaboratory study.

2 METHODS PROTOCOLS

2.1 Reference method protocol ♦

The reference method corresponds to the ISO 6579 standard: Horizontal method for the detection of *Salmonella* spp (See ***Appendix 1***).

2.2 Alternative method protocol

❒ *Principle*

ANSR™ for *Salmonella* is an isothermal, amplified nucleic acid assay. The ANSR method is based on nicking enzyme amplification reaction (NEAR™) technology. Target nucleic acid is amplified through a mechanism of polymerization from the ends of nicks created in double-stranded DNA by the action of a specific endonuclease. Amplified target sequences are detected in real time using fluorescent molecular beacon probes.

♦ Analysis performed according to the COFRAC accreditation

❒ ***Protocol*** (See ***Appendix 2***)

- enrichment step:
    * **Protocol 1**: unprocessed raw food, even frozen, with high background microflora, as well as pet foods: in BPW supplemented with selective reagents for 22 h ± 2 h at 41.5°C ± 1°C,
    * **Protocol 2**: processed food with low background microflora: in BPW for 22 h ± 2 h at 37°C ± 1°C
- lysis step:
    * 50 µl enrichment broth + 450 µl lysis buffer
    * incubation at 37°C for 10 min in a heater block
    * heat treatment at 80°C for 20 min
- ANSR test on 50 µl DNA extract. The result is obtained within 10 min.
- confirmation by performing an enrichment step in RVS (0.1 ml in 10 ml RVS) for 24 h ± 3 h at 41.5°C, and streaking onto a selective agar plate (followed by a latex test performed directly on isolated colonies). During the validation study, biochemical confirmatory tests were applied.

Enrichment storage for 72 h at 2-8°C was also evaluated on positive samples in order to offer sufficient practicability to the users. This was done for the positive samples in the relative accuracy, specificity and selectivity study.

3 METHODS COMPARISON STUDY

3.1 Relative accuracy, relative specificity and relative sensitivity

The relative accuracy is the closeness of agreement between a test result and the accepted reference value.

The relative specificity is defined as the degree to which a method is affected (or not) by the other components present in a multi-component sample; that is, it is the ability of the method to measure exactly a given analyte, or its amount, within the sample without interference from non-target components such as matrix effect or background noise.

The r*elative sensitivity is defined as the ability of the alternative method to detect two different amounts of analyte measured by the reference method within a given matrix over the whole measurement range; that is, it is the minimal quantity variation (increase of the analyte concentration x) which gives a significant variation of the measured signal (response y).*

3.1.1 Number and nature of samples

A total of **202 samples** was analysed: 126 samples with the Protocol 1 and 76 samples with the Protocol 2.

The distribution per tested category, type and pre-enrichment protocol is given in Table 1.

Table 1 - Distribution per tested category and type

Category	Type	Positive samples		Negative samples		Total		TOTAL
		Protocol 1	Protocol 2	Protocol 1	Protocol 2	Protocol 1	Protocol 2	
Meat products (processed and unprocessed)	Fresh meat products (unprocessed)	16	/	18	/	34	/	34
	Cooked meat products (ready-to-eat food or ready-to-reheat food)	/	11	/	10	/	21	21
	Fermented and cured meat products (sausages, salami, bacon…)	8	/	10	/	18	/	18
	Total	**24**	**11**	**28**	**10**	**52**	**21**	**73**
Dairy products	Thermisation / pasteurised products (milk powders, pasteurized milks)	3	7	7	4	10	11	21
	Fermented / acidified products (cheese, fermented milks)	11	/	11	/	22	/	22
	Raw milk based products (cheeses, milks …)	12	/	10	/	22	/	22
	Total	**26**	**7**	**28**	**4**	**54**	**11**	**65**
Seafood & vegetables	Fresh, raw, frozen products	10	/	10	/	20	/	20
	Heat treated products	/	11	/	10	/	21	21
	Composite foods (deli-salads, sandwiches, …)	/	13	/	10	/	23	23
	Total	**10**	**24**	**10**	**20**	**20**	**44**	**64**
TOTAL		**60**	**42**	**66**	**34**	**126**	**76**	**202**

3.1.2 Artificial contamination of samples

Artificial contaminations were done by spiking. The strains were stressed using various injury protocols. The injury efficiency was evaluated by comparing enumeration results onto selective and non selective agars (respectively XLD and TSYE). 88 samples were artificially contaminated, using 22 different strains. 85 gave a positive result. Most of the inoculation levels, after injury protocols on the inoculum, were lower or equal to 5 CFU/sample (Cf. figures 1 and 2). No more than 6 positive results were obtained with a same strain.

Figure 1 – Inoculation levels used for spiking

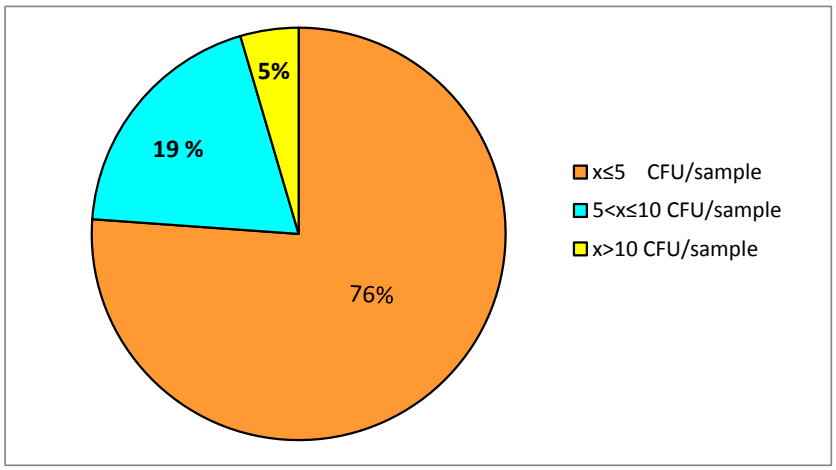
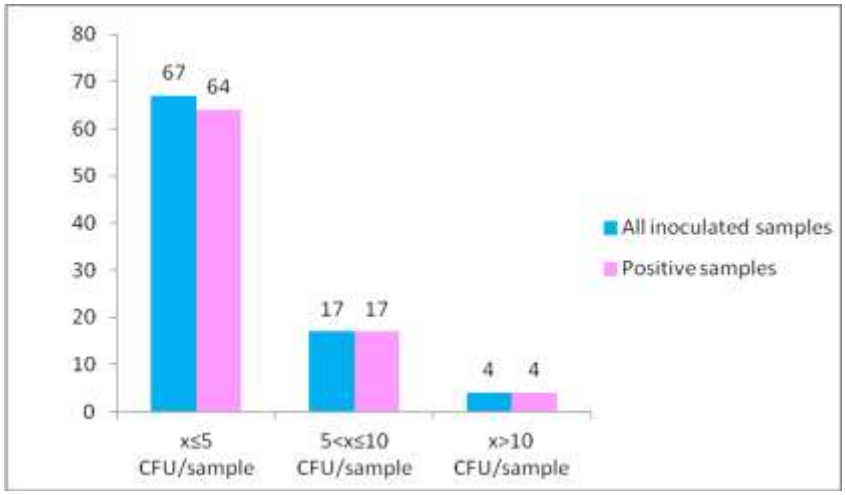


Figure 2 – Inoculated and positive samples according to the level of contamination



17 positive samples were naturally contaminated.

16.7% of the samples were naturally contaminated.

3.1.3 Confirmation protocols

The positive results of the ANSR™ *Salmonella* method were confirmed by a subculture in RVS broth, followed by streaking onto XLD and ASAP. The isolated colonies were confirmed by:

- latex tests directly on isolated colonies
- the tests described in the ISO 6579 method.

3.1.4 Test results

The paired results per category are given in tables 2, 3 and 4 and for all products in table 5; the paired results per protocol are given tables 6 and 7.

Results per category and protocol

PD = positive deviation (R-/A+) ND = negative deviation (A-/R+)
PA = positive agreement (R+/A+) NA = negative agreement (A-/R-)
PP = positive presumptive and non confirmed result

Table 2 – Meat products (processed and unprocessed)

Response	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement (A+/R+) **PA = 27**	Positive deviation (R-/A+) **PD = 6**
Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 2 (PPND = 0)**	Negative agreement (A-/R-) **NA = 38 (PPNA = 0)**

Table 3 – Dairy products

Response	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement (A+/R+) **PA = 27**	Positive deviation (R-/A+) **PD = 4**
Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 2 (PPND = 0)**	Negative agreement (A-/R-) **NA = 32 (PPNA = 0)**

Table 4 – Seafood and vegetables

Response	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement (A+/R+) **PA = 32**	Positive deviation (R-/A+) **PD = 0**
Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 2 (PPND = 0)**	Negative agreement (A-/R-) **NA = 30 (PPNA =0)**

Table 5 – All products

Response	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement (A+/R+) **PA = 86**	Positive deviation (R-/A+) **PD = 10**
Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 6 (PPND = 0)**	Negative agreement (A-/R-) **NA = 100 (PPNA = 0)**

Table 6 – Protocol 1

Response	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement (A+/R+) **PA = 47**	Positive deviation (R-/A+) **PD = 10**
Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 3 (PPND = 0)**	Negative agreement (A-/R-) **NA = 66 (PPNA = 0)**

Table 7 – Protocol 2

Response	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement (A+/R+) **PA = 39**	Positive deviation (R-/A+) **PD = 0**
Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 3 (PPND = 0)**	Negative agreement (A-/R-) **NA = 34 (PPNA = 0)**

Table 8 – Calculation of relative accuracy (AC), relative sensitivity (SE) and relative specificity (SP)

Category	PA	NA	ND	PD	N	Relative accuracy AC (%) [100x(PA+NA])/N]	N+ PA + ND	Relative sensitivity SE (%) [100xPA]/N+]	N- NA + PD	Relative specificity SP (%) [100xNA]/N-]
Meat products	27	38	2	6	73	89.0	29	93.1	44	86.4
Dairy products	27	32	2	4	65	90.8	29	93.1	36	88.9
Seafood and vegetables	32	30	2	0	64	96.9	34	94.1	30	100.0
All products*	**86**	**100**	**6**	**10**	**202**	**92.1**	**92**	**93.5**	**110**	**90.9**

3.1.5 Calculation of relative accuracy (AC), relative sensitivity (SE) and relative specificity (SP) (See Appendix 3)

The alternative method percentage values are:

	Alternative method
Relative accuracy : ***AC***	92.1
Relative specificity : ***SP***	90.9
Relative sensitivity : ***SE***	93.5

Sensitivity of both methods, when the positive deviations of the alternative method are considered, is presented below:

	Alternative method
Alternative method	94.1
Reference method	90.2

3.1.6 Analysis of discordants

The discordant results observed for meat products, dairy products, and seafood and vegetables are given below.

- ***Negative deviations: 6***

Sample n°	Product	Protocol	ANSR™ *Salmonella* Result	Confirmatory tests	Contamination (contamination level CFU/g)
5604	Cured pork meat	1	-	+	Natural *Salmonella* Typhimurium
5605	Pork meat	1	-	-	Natural *Salmonella* Derby
6281	Pasteurized milk cheese	1	-	-	Artificial *Salmonella* Indiana Ad 174 (1.6)
6471	Deli salad (vegetable mix, cheese)	2	-/-/-	+	Artificial *Salmonella* Mbandaka Ad 1723 (5.8)
1350	Deli salad (vegetables)	2	-/-/+ (negative curve)	+	Artificial *Salmonella* Infantis Ad 1646 (8.2)
1352	Milk powder	2	-/-/-	+	Artificial *Salmonella* Infantis F401B (3.4)

- **Protocol 1**:
  - for two samples (n° 5605 and 6281), the ANSR™ *Salmonella* and confirmatory tests gave negative results. Note that the enrichment broths were different for these samples. The deviations are thus probably due to the sampling heterogeneity.
  - for one sample (n° 5604), the presence of *Salmonella* spp. was confirmed in the enrichment broth, while the ANSR *Salmonella* test gave a negative result. The detection level was probably not reached.

- **Protocol 2**:
  - for three samples (n° 6471, 1350 and 1352), the presence of *Salmonella* spp. was confirmed in the enrichment broth. For these samples, the detection level of the ANSR *Salmonella* method was probably not reached.

- ***Positive deviations: 10***

Sample n°	Product	Protocol	ANSR™ *Salmonella* Result	Confirmatory tests	Contamination (contamination level CFU/g)
5545	Pork fat	1	+	+	Natural
5547	Sausage	1	+	+	Natural
5553	Meat skewers with pepper	1	+	+	Natural
5556	Pork meat	1	+	+	Natural
5603	Ground poultry meat	1	+	+ (MKTTn)	Natural
5606	Pork meat	1	+	+	Natural
6427	Fermented milk	1	+	+	Artificial *Salmonella* Mbandaka Ad 1722 (8.4)
1298	Fermented milk	1	+	+ (XLD)	Artificial *Salmonella* Dublin Ad 531 (4.0)
1302	Fermented milk	1	+	+	Artificial *Salmonella* Ohio Ad 1482 (0.2)
1303	Fermented milk	1	+	+	Artificial *Salmonella* Ohio Ad 1482 (0.2)

For these three categories, the analysis of discordant is the following:

Y = ND + PD = 6 + 10 = 16
6 < Y < 22
M = 3
m = 6
m > M

The two methods are not different at α 0.05.

3.1.7 Confirmations

The confirmations were performed by using:

- the tests described in the reference method;

- a sub-culture in RVS broth, followed by streaking onto one selective agar and performing a latex test directly on isolated characteristic colonies. Note that XLD and ASAP were used as selective agar during the validation study. All the alternative method positive results were confirmed by using this protocol, except for sample n° 5603 which needed a subculture in MKTTn to confirm the presence of *Salmonella*.

For samples n° 1044, 1298 to 1301, 1341 to 1346, 1370 and 1371, inoculated with *Salmonella* Dublin, characteristic colonies were observed only on XLD plates, atypical colonies (white) were observed on ASAP plates. For sample n° 5613, 2 typical colonies were observed only on XLD.

3.1.8 Enrichment broth storage at 2 - 8°C for 72 h

The positive sample enrichment broths of the alternative method were stored at 2 – 8°C for 72 h and analysed a second time.

The following changes were observed:

Sample n°	Product	Before BPW storage	After BPW storage
5602	Ground poultry meat	PA	ND
1350	Deli salad	ND	PA

The analysis of discordant results remains the same for BPW storage.

3.2 Relative detection level

The relative detection level is the smallest number of culturable micro-organisms that can be detected in the sample in 50% of occasions by the alternative and reference methods.

3.2.1 Matrices

The objective of this study is (i) to determine the target species minimal quantity that can be detected in food matrices, (ii) to compare both method results.

Detection limits were defined by analysing the different matrix/strain pairs. Four levels were tested. Six replicates of each combination were prepared.

The following matrices were tested:

- ground beef inoculated by *Salmonella* Infantis 128 (Protocol 1),
- raw milk, inoculated by *Salmonella* Montevideo 510 (Protocol 1),
- produces inoculated by *Salmonella* Virchow F276 (Protocol 1),
- Ready-to-reheat food inoculated by *Salmonella* Typhimurium 4874 (Protocol 2).

3.2.2 Contamination protocol

Contaminations and enumerations were realised according to the AFNOR technical rules (protocol for low level inoculations). The contamination levels are presented below:

The inoculation's levels were the following:

- 0 CFU/ g or ml,
- level required to get 0 to 50 % positive samples,
- level required to get 50 to 75 % positive samples,
- level required to get 75 to 100 % positive samples.

The samples were analysed by both methods, and the background microflora was enumerated.

3.2.3 Results

Raw data are given in ***Appendix 4***. Detection levels are presented in the table 9.

Table 9 – Relative detection level results

Strain / matrix pairs	Relative detection level (CFU / 25 g) according to Spearman-Kärber test[1]	
	Reference method	Alternative method
Ground beef / *Salmonella* Infantis 128	0.664 [0.402; 1.096]	0.995 [0.634; 1.564]
Raw milk / *Salmonella* Montevideo 510	0.554 [0.285; 1.074]	1.141 [0.561; 2.321]
Produces /*Salmonella* Virchow F276	0.502 [0.259; 0.974]	0.502 [0.259; 0.974]
Ready-to-reheat food / *Salmonella* Typhimurium 4874	0.602 [0.341; 1.062]	0.602 [0.341; 1.062]

Strain / matrix pairs	Relative detection level (CFU / 25 g) according to Spearman-Kärber test[1]	
	Reference method	Alternative method
Ground beef / *Salmonella* Infantis 128	0.7 [0.4; 1.1]	1.0 [0.6; 1.6]
Raw milk / *Salmonella* Montevideo 510	0.6 [0.3; 1.1]	1.1 [0.6; 2.3]
Produces /*Salmonella* Virchow F276	0.5 [0.3; 1.0]	0.5 [0.3; 1.0]
Ready-to-reheat food / Salmonella Typhimurium 4874	0.6 [0.3; 1.1]	0.6 [0.3; 1.1]

3.2.4 Conclusion

The relative detection level varies from 0.3 to 1.1 CFU/25 g for the reference method and from 0.3 to 2.3 CFU/25 g for the alternative method. The alternative and the standard methods show similar detection levels.

[1] *"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".*

3.3 Inclusivity / exclusivity

Inclusivity is the ability of the alternative method to detect the target analyte from a wide range of strains.

Exclusivity is the lack of interference from a relevant range of non-target strains of the alternative method.

3.3.1 Test protocols

❒ **Inclusivity**

Salmonella strains cultures were performed in BHI medium at 37°C. Dilutions were done in order to inoculate 10 cells/225 ml in supplemented BPW (Protocol 1). The broths were incubated for 20 h at 41.5°C ± 1°C. The alternative method was then performed.

❒ **Exclusivity**

Negative strains cultures were performed in BHI at 37°C. Dilutions were performed in order to inoculate 10^5 cells/ml BPW. The broths were incubated for 24 h at 37°C ± 1°C. The alternative method was then performed.

3.3.2 Results

Raw data are given in ***Appendix 5***.

❒ **Inclusivity**

51 *Salmonella* strains gave a positive ANSR™ *Salmonella* result. For three of them (*Salmonella arizonae* SIIIa 50;z4;z23 CIP 5526, *Salmonella* Havana Ad 930 and *Salmonella* Urbana Ad 501), a positive ANSR™ *Salmonella* result was observed only when BPW supplemented with milk was used for enrichment.

❒ **Exclusivity**

No cross reaction was observed with the 30 non target strains.

4 PRACTICABILITY

The alternative method practicability was evaluated according to the AFNOR criteria relative to method comparison study.

Packaging and reagents	Material provided into NEOGEN ANSR kit: - 12 strips of 8 clusters tubes 1.2 mL - 12 strips of 8 reaction tubes (200 µl) containing lyophilised ANSR *Salmonella* reagents - 12 strips of 8 permanent caps for reaction tubes - 1 kit insert - lysis buffer - lysis reagent
Storage conditions and shelf-life	The storage temperature is 2 – 8°C. The shelf-life is given on the package. The lysis buffer after reconstitution must be stored for 14 days at 2 – 8°C.
Specific equipment	Provided by NEOGEN: - ANSR incubator/reader - Computer and software - Heater block 80°C and 37°C - Micropipettes 50 µl - 8 channel micropipettes Not provided by NEOGEN: - Micropipettes (100 – 1 000 µl)
Reagents	*Salmonella* lysis buffer and reagent must be reconstituted with 18ml of *Salmonella* lysis buffer
Training	One day is required for technicians with microbiology knowledge.

Workflow (in minutes) for 24 samples	Steps	**Reference Method**	**Alternative method**
	Negative samples		
	Sampling	60	60
	Stomach	36	36
	Lysis and ANSR test	/	40
	RVS subculture and MKTTn	45	/
	Streaking onto selective plates	75	/
	Selective plate reading	30	/
	Total for negative samples analyses	**246**	**136**
	Total/negative sample	**10.3**	**5.7**
	Presumptive samples or positive samples		
	RVS subculture	/	25
	Streaking onto selective plates	/	20
	Selective plates reading	/	15
	Latex test	/	15
	Confirmatory tests	120	/
	Total for positive samples	**366**	**211**
	Total/positive sample	**15.3**	**8.8**

Time to result	Steps	**Reference Method**	**Alternative method**
	Negative samples		
	Sampling Pre-enrichment	Day 0	Day 0
	ANSR test	/	Day 1
	Subculture in RVS / MKTTn	Day 1	/
	Streaking onto selective plates	Day 2	/
	Reading plates	Day 3	/
	Presumptive positive or positive results		
	Subculture in RVS	/	Day 1
	Streaking onto selective plates	/	Day 2
	Selective plates reading	/	Day 3
	Latex test	/	Day 3
	Confirmatory test	Day 4 to Day 6	/
Technician background	Technician qualified in molecular microbiology or in food microbiology		
Common step with the reference method	Pre-enrichment step and confirmatory tests when Protocol 2 is used. No common step with Protocol 1.		
Traceability of the results	/		
Maintenance	Clean and decontaminate the instrument if any spills occur.		

Negative results are available within one day with the alternative method. The positive results are available within three days.

The workflow of the alternative method shortens the handling time and the time to results in comparison to the reference method.

5 INTERLABORATORY STUDY ORGANISATION AND RESULTS

5.1 Study organisation

- *Collaborators number*

Samples were sent to 16 laboratories.

- *Matrix and strain used*

The study was done with ground beef samples contaminated with *Salmonella* Typhimurimum A00C060.

- *Samples*

Samples were inoculated and sent on Monday 8th April 2013, as described below:

- 24 codified samples (25 g) for *Salmonella* research by the NEOGEN ANSR™ *Salmonella* method (red label)

- 24 codified samples (25 g) for *Salmonella* research by the ISO 6579 (2002) reference method (blue label),

- 1 ground beef sample (labelled “Sample for Total Count enumeration”) for aerobic mesophilic flora enumeration by ISO 4833 method,

- 1 water flask labelled “Temperature Control” with a temperature probe.

The analyses were started on Wednesday 10th April 2013.

- *Inoculation*

The targeted inoculation levels were:

- 0 CFU/25 g,
- 1 – 10 CFU/25 g,
- 5 – 50 CFU/25 g.

The samples were inoculated individually. 8 replicates were provided by tested contamination level.

- *Labelling and shipping*

Blinded samples were placed in isothermal boxes, which contained cooling blocks, and express-shipped to the different laboratories.

A temperature control flask containing a sensor was added to the package in order to register the temperature profile during the transport, the package delivery and storage until analyses.

Samples were shipped in 24 h to 72 h to the involved laboratories. The temperature conditions had to stay lower or equal to 8.4°C during transport, and between 0°C – 8.4°C in the labs.

- *Analyses*

Collaborators and ADRIA Développement carried out the analyses with the alternative and reference methods at Day 2.

5.2 Experimental parameters control

5.2.1 Contamination level before inoculation, levels obtained after the artificial contaminations of the samples

- *Before inoculation*

In order to detect *Salmonella*, the ISO 6579 method was performed on five ground beef test portions (25 g) before the inoculation. All the results were negative.

- *Sample stability*

Sample stability was checked by inoculating the matrix at 500 CFU/g and 5 CFU/g. Enumerations were performed for the high contamination level and detection analyses were performed for the low contamination level. *Triplicata* were analysed, and the results were the following:

Table 10

Day	Reference method (research)			CFU/g (XLD)			Aerobic mesophilic flora (CFU/g)
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3	
Day 0	+	+	+	570	480	550	$1.4\ 10^3$
Day 1	+	+	+	600	510	460	$1.4\ 10^3$
Day 2	+	+	+	460	520	470	$1.2\ 10^3$

No evolution was observed during storage at 4°C.

- *Contamination levels*

The contamination levels and the confidence intervals were:

Table 11

Level	Samples	Theoretical target level (b/25 g)	True level (b/25 g sample)	Low limit / 25 g sample	High limit / 25 g sample
Level 0	3 – 9 – 11 – 12 – 15 – 19 – 22 – 24	0	/	/	/
Low level	4 – 7 – 10 – 13 – 14 – 20 – 21 – 23	5	4.8	4.0	5.5
High level	1 – 2 – 5 – 6 – 8 – 16 – 17 – 18	25	30	26.1	34.6

5.2.2 Logistic conditions

Temperature conditions are given below:

Table 12 - Sample temperatures at receipt

Laboratories	Temperature measured by the sensor (°C)	Temperature measured at receipt (°C)	Receipt date and time	
A	1.0	7.0	09/04/2013	*11h30*
B	1.0	4.8	09/04/2013	*11h25*
C	0.5	**10.0**	09/04/2013	*08h00*
D	1.0	2.6	09/04/2013	*10h00*
E	1.0	5.7	09/04/2013	*09h30*
F	1.0	2.5	09/04/2013	*10h15*
G	1.5	4.7	09/04/2013	*09h00*
H	0.5	6.0	09/04/2013	*11h30*
I	*The Lab didn't realize the analyses*			
J	1.5	7.5	09/04/2013	*08h15*
K	2.5	*7.2*	09/04/2013	*12h15*
L	3.5	3.4	10/04/2013	*11h00*
M	1.5	7.0	09/04/2013	*13h30*
N	3.0	3.1	09/04/2013	*14h00*
O	1.0	2.2	09/04/2013	*09h00*
P	/	5.5	09/04/2013	*11h45*

5.2.3 Conclusion

No problem was encountered during the transport or at receipt for 16 labs, but Lab I decided finally to not participate to the ring trial.

5.3 Results analysis

5.3.1 Aerobic mesophilic flora enumeration

Depending on the Lab results, the enumeration levels varied from 3.8 10^2 to 2.1 10^3 CFU/g.

5.3.2 Expert lab results

The results obtained by the expert lab. are given below.

Table 13 – Results obtained by the expert Lab.

Level	Reference method	Alternative method
L0	0/8	0/8
L1	8/8	8/8
L2	8/8	8/8

5.3.3 Collaborator lab results

15 Labs participated to the study.

Labs C, D, H and J started the analyses on Tuesday 9th April 2013 (Day 1) instead of Wednesday 10th April 2013 (Day 2).

One Lab (E) encountered some problems with the incubator at 41.5°C (alternative enrichment incubation). The temperature felt at 37.3°C and it took 14h30 to reach 41.5°C.

In agreement with the AFNOR Technical Committee, Labs C, D, H and J were kept for interpretation, only Lab E was excluded.

Table 14 – Results obtained by the collaborator Labs.

Reference method

	Laboratory	L0	L1	L2
	A	0/8	8/8	8/8
	B	0/8	8/8	8/8
Analyses at Day 1	C	0/8	8/8	8/8
Analyses at Day 1	D	1/8	8/8	8/8
Problem with incubator	E	0/8	8/8	8/8
	F	0/8	7/8	8/8
	G	0/8	8/8	8/8
Analyses at Day 1	H	0/8	8/8	8/8
	I	This lab didn't realise the analyses		
Analyses at Day 1	J	0/8	8/8	8/8
	K	0/8	8/8	8/8
	L	1/8	8/8	8/8
	M	0/8	6/8	4/8
	N	0/8	8/8	8/8
	O	1/8	8/8	8/8
	P	0/8	8/8	8/8

At level 0, the observed positive data are probably due to cross contamination.

Alternative method

	Alternative before confirmation			
	Laboratory	L0	L1	L2
	A	1/8	8/8	8/8
	B	0/8	8/8	8/8
Analyses at Day 1	C	0/8	8/8	8/8
Analyses at Day 1	D	0/8	8/8	8/8
Problem with incubator	E	0/8	8/8	8/8
	F	0/8	8/8	8/8
	G	0/8	8/8	8/8
Analyses at Day 1	H	0/8	8/8	8/8
	I	This lab didn't realize the analyses		
Analyses at Day 1	J	2/8	8/8	8/8
	K	0/8	8/8	8/8
	L	0/8	8/8	8/8
	M	1/8	8/8	8/8
	N	0/8	8/8	8/8
	O	1/8	8/8	8/8
	P	0/8	8/8	8/8

Alternative after confirmation			
Laboratory	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	This lab didn't realize the analyses		
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	5/8
N	0/8	8/8	8/8
O	0/8	8/8	8/8
P	0/8	8/8	8/8

At level 0, the observed ANSR positive results which are not confirmed, are probably due do cross contamination in the molecular assays.

Salmonella isolates were recovered from blank samples in many cases. This may be due to cross contaminations, either in the reference method or in the alternative method. The isolates from the blank samples were analysed for Labs L and O by running molecular fingerprinting (PFGE) in order to confirm or infirm the hypothesis of cross-contaminations; the results are presented below (See figure 1).

Fingerprints were done according to the protocol described by PulseNet Network using Pulsed Field Gel Electrophoresis and *Xba*I as restriction enzyme:

Restriction enzyme	*Xba*I / 20U
Time and temperature	6 h at 37°C
Initial pulse (s)	2
Final pulse (s)	64
Run time (h)	20
Cooling module temperature (°C)	
Voltage (V)	200
Voltage (V/cm	6
Included angle (°)	120

Quality controls include the characterization of two strains.

The patterns were compared using the Dice band based coefficient. The observed and unique cluster was generated with the UPGMA (Unweighed Pair Group Method with Arithmetic Average) algorithm.

Figure 3 - Fingerprints

The fingerprint of the isolates from the blank samples O15 and L24 clearly matches with the fingerprint of the inoculated strain A00C060, confirming the hypothesis of cross contamination.

5.4 Results interpretation

5.4.1 Specificity and sensitivity for each method

For the L0 level and for each method, specificity percentages are calculated according to:

$$SP = \left[1 - \left(\frac{FP}{N-}\right) x\ 100\%\right]$$

with: N- = total number of all L0 assays
FP = number of false positive results

For each contamination level and each method, the sensitivity percentages are calculated according to:

$$SE = \frac{TP}{N+} x\ 100\%$$

with: N+ = total number of all L1 or L2 assays
TP = number of true positive results

Results are reported in Table 15.

Table 15 – Interpretation

Level	Reference method		Alternative method	
	SP/SE %	LCL%	SP/SE %	LCL%
Lo(SP)	97.3	94.3	100.0	98.0
L1(SE)	97.3	94.3	100.0	98.0
L2(SE)	96.4	92.2	97.3	94.3
L1+L2(SE)	96.9	93.6	98.7	96.5

LCL: confidence interval

5.4.2 *Relative accuracy (AC)*

Results for all levels (See ***Appendix 6***) are given below:

Table 16 - Paired results of the alternative and reference methods

Alternative method	Reference method		Total
	+	-	
+	PA = 215	PD = 6	221
-	ND = 5 (PPND = 2)	NA = 110 (PPNA = 6)	115
Total	N+ = 220	N- = 116	N = 336

Relative accuracy (AC) (in %) is calculated according to: $AC = \frac{(PA + NA)}{N} x\, 100\%$

with : N = number of samples analysed
PA = number of positive agreement
NA = number of negative agreement

The alternative method accuracy values with regard to the reference method are:

Table 17 – Interpretation

Level	AC %	LCL %
L0	97.3	94.3
L1	97.3	94.3
L2	95.5	91.6
L1 + L2	96.4	93.9
Total	**96.7**	**94.8**

5.4.3 *Discordant results*

6 positive deviations and 5 negative deviations were observed:

Y = ND + PD = 5 + 6 = 11
m = 5
M = 1

m > M, **the two methods are not different.**

5.5 Interpretation

5.5.1 Comparison of the relative accuracy, specificity and sensitivity values

The values obtained for the two parts of the validation study (comparative and inter-laboratory studies) are reported in Table 18.

Table 18 - Alternative method values calculated during the comparative and inter-laboratory studies

	Interlaboratory study	Methods comparative study
Relative accuracy (AC)	96.7 %	92.1 %
Sensitivity (SE)	98.7 %	93.5 %
Specificity (SP)	100.0 %	90.9 %

5.5.2 Accordance (DA)

Accordance values for both methods are:

Table 19 – Interpretation

Level	Reference method (DA)	Alternative method (DA)
L0	95.3 %	100.0 %
L1	95.8 %	100.0 %
L2	96.4 %	96.7 %

5.5.3 Concordance

Both methods concordance values are:

Table 20 – Interpretation

Level	Reference method	Alternative method
L0	94.7 %	100.0 %
L1	94.7 %	100.0 %
L2	92.9 %	94.6 %

5.5.4 Odds Ratio (COR)

The odds ratio value is determined according to:

$$COR = \frac{Accordance\,x\,(100 - condorcane)}{Concordane\,x\,(100 - accordance)}$$

Both method odds ratio values are:

Table 21 – Interpretation

Level	Reference method (COR)	Alternative method (COR)
L0	1.13	1.00
L1	1.26	1.00
L2	2.08	1.63

6 CONCLUSION

The **methods comparative study conclusions** are:

- ❒ The NEOGEN ANSR™ *Salmonella* method shows satisfying relative accuracy, specificity and sensitivity.

- ❒ The relative detection limits of the alternative method and the ISO standard are similar.

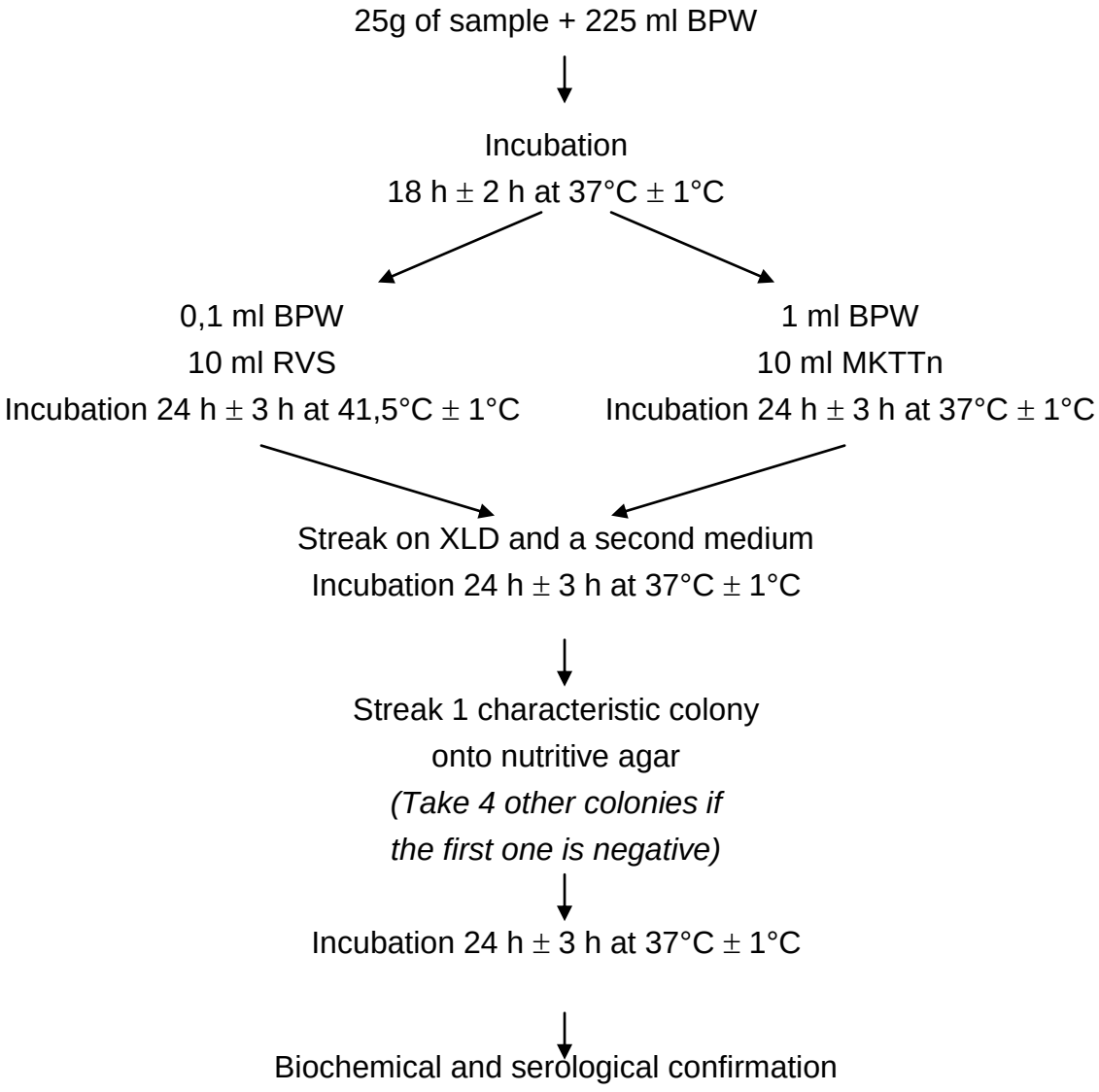
- ❒ The NEOGEN ANSR™ *Salmonella* method shows clearly satisfying inclusivity and exclusivity results.

- ❒ Negative results are available within one day using the NEOGEN ANSR™ *Salmonella* method.

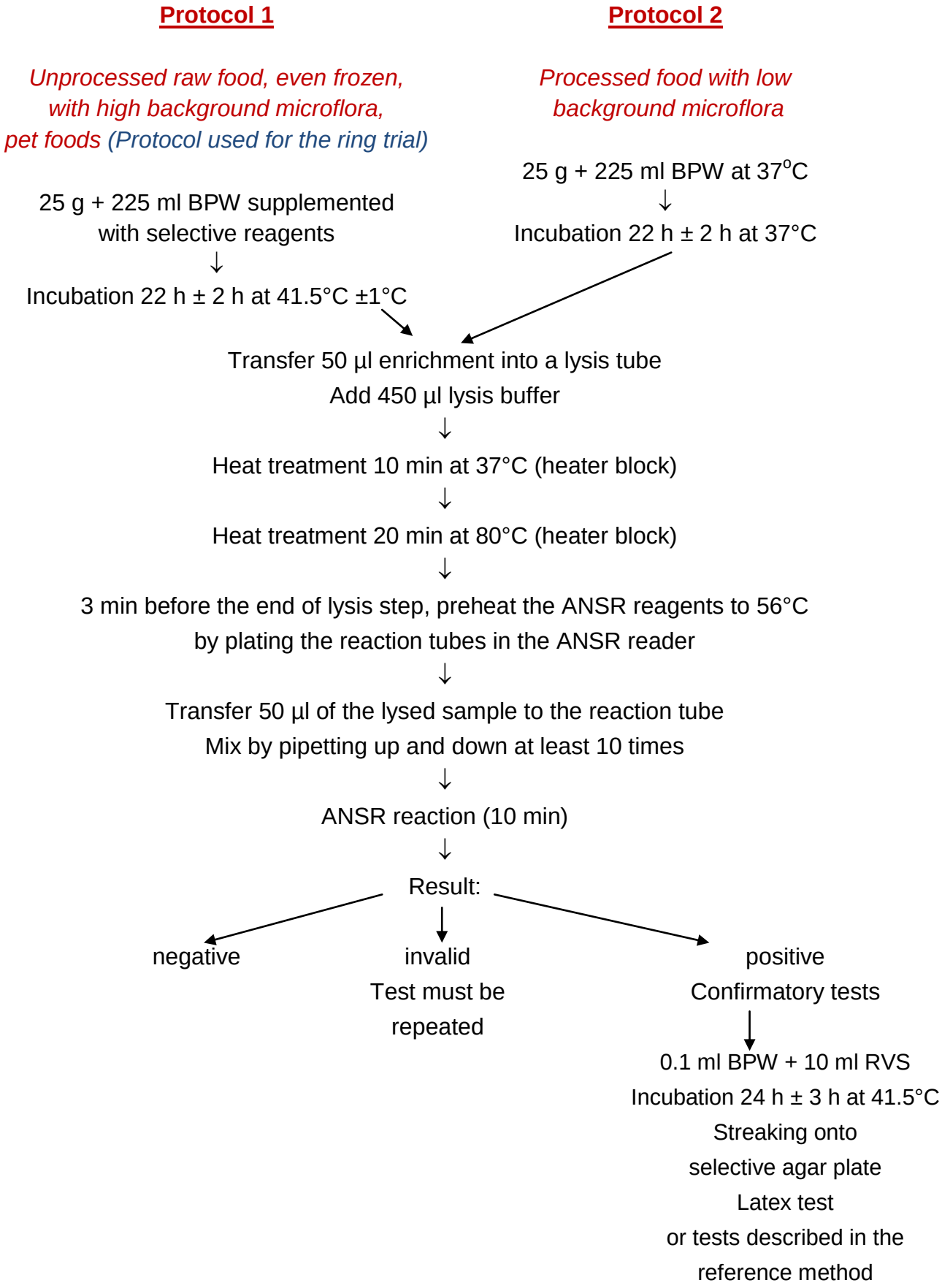
The **interlaboratory study conclusions** are:

- ☒ The observed data and results confirmed that the alternative method and reference method show equivalent performances (accordance, concordance, odds ratio).

Appendix 1 - NF EN ISO 6579: 2002:
Microbiology of food and animal feeding stuffs –
Horizontal method for the detection of Salmonella spp.



Appendix 2 – Flow diagram of the alternative method



Appendix 3 – Calculation of relative accuracy (AC), relative sensitivity (SE) and relative specificity (SP) integrating LCL (lower confidence limit)

Matrices	Types	PA	NA	ND	PD	N	Relative accuracy AC (%) [100x(PA+NA])/N]	LCL	N+ PA + ND	Relative sensitivity SE (%) [100xPA]/N+]	LCL	N- NA + PD	Relative specificity SP (%) [100xNA]/N-]	LCL
Meat products	Poultry	8	18	2	6	34			10			24		
	Pork	11	10	0	0	21			11			10		
	Beef and others	8	10	0	0	18			8			10		
	Total	27	38	2	6	73	89.0	81.7	29	93.1	83.7	44	86.4	76,0
Dairy products	Raw and fermented milks	8	11	2	0	21			10			11		
	Cheeses	7	11	0	4	22			7			15		
	Dessert, milk powder, ice creams	12	10	0	0	22			12			10		
	Total	27	32	2	4	65	90.8	83.6	29	93.1	83.7	36	88.9	78,4
Seafood and vegetables	Fresh, raw, frozen	10	10	0	0	20			10			10		
	Heated products	11	10	0	0	21			11			10		
	Composite foods	11	10	2	0	23			13			10		
	Total	32	30	2	0	64	96.9	92.5	34	94.1	86.0	30	100.0	100,0
All products		86	100	6	10	202	92,1	88.3	92	93.5	88.3	110	90.9	85.4

Appendix 4 – Relative detection levels

Ground beef **Protocol 1**
Salmonella Infantis 128

Level	Inoculation level	I.C.	Method	Negative	Positive	Total
0	/	/	Reference	6	0	6
			Alternative	6	0	6
			Total	*12*	*0*	*12*
1	0.2	[0;1]	Reference	6	0	6
			Alternative	6	0	6
			Total	*12*	*0*	*12*
2	0.3	[0;2]	Reference	6	0	6
			Alternative	5	1	6
			Total	*11*	*1*	*12*
3	0.6	[0;2]	Reference	2	4	6
			Alternative	5	1	6
			Total	*7*	*5*	*12*
4	0.6	[0;2]	Reference	4	2	6
			Alternative	5	1	6
			Total	*9*	*3*	*12*
5	1.5	[0;4]	Reference	3	3	6
			Alternative	2	4	6
			Total	*5*	*7*	*12*
6	3.1	[0;7]	Reference	3	3	6
			Alternative	0	6	6
			Total	*3*	*9*	*12*

Raw milk **Protocol 1**
Salmonella Montevideo 510

Level	Inoculation level	I.C.	Method	Negative	Positive	Total
0	/	/	Reference	6	0	6
			Alternative	6	0	6
			Total	*12*	*0*	*12*
1	0.3	[0;2]	Reference	3	3	6
			Alternative	4	2	6
			Total	*7*	*5*	*12*
2	0.7	[0;3]	Reference	4	2	6
			Alternative	4	2	6
			Total	*8*	*4*	*12*
3	1.3	[0;4]	Reference	1	5	6
			Alternative	4	2	6
			Total	*5*	*7*	*12*
4	6.6	[2;12]	Reference	0	6	6
			Alternative	0	6	6
			Total	*0*	*12*	*12*

Produces **Protocol 1**
Salmonella Virchow F276

Level	Inoculation level	I.C.	Method	Negative	Positive	Total
0	/	/	Reference	6	0	6
			Alternative	6	0	6
			Total	*12*	*0*	*12*
1	0.4	[0;2]	Reference	3	3	6
			Alternative	3	3	6
			Total	*6*	*6*	*12*
2	0.9	[0;3]	Reference	2	4	6
			Alternative	2	4	6
			Total	*4*	*8*	*12*
3	1.7	[0;5]	Reference	1	5	6
			Alternative	1	5	6
			Total	*2*	*10*	*12*
4	4.3	[1;9]	Reference	0	6	6
			Alternative	0	6	6
			Total	*0*	*12*	*12*

Ready-to-reheat food **Protocol 2**
Salmonella Typhimurium 4874

Level	Inoculation level	I.C.	Method	Negative	Positive	Total
0	/	/	Reference	6	0	6
			Alternative	6	0	6
			Total	*12*	*0*	*12*
1	0.5	[0;2]	Reference	4	2	6
			Alternative	4	2	6
			Total	*8*	*4*	*12*
2	1.0	[0;3]	Reference	2	4	6
			Alternative	2	4	6
			Total	*4*	*8*	*12*
3	1.9	[0;5]	Reference	0	6	6
			Alternative	0	6	6
			Total	*0*	*12*	*12*
4	3.7	[1;8]	Reference	0	6	6
			Alternative	0	6	6
			Total	*0*	*12*	*12*

Appendix 5 – Inclusivity and exclusivity

INCLUSIVITY									
Strain					inoculation level (cfu/225ml BPW + supplement)	NEOGEN ANSR *Salmonella* method	Confirmation		
Strain			Reference	Origin			RVS/XLD	RVS/ASAP	Latex
1	*Salmonella*	Agona	A00V38	Feedstuff	12	+	+	+	+
2	*Salmonella*	Anatum	6140	Bœuf Bourguignon	8	+	+	+	+
3	*Salmonella*	*arizonae* SIIIa 51:z4,z23:-	CIP 5523	Turkey	5	+	+	+	+
4	*Salmonella*	*arizonae* SIIIa 50 ;z4 ;z23	CIP 5526	Egg powder	6	-	-	-	/
					4 (+25ml milk)	+	+	+	+
5	*Salmonella*	*diarizonae SIIIb 38:IV:z53*	Ad451	Raw milk cheese	10	+	+	+	+
6	*Salmonella*	*diarizonae SIIIb 61:-:,1,5,7*	Ad1280	Raw milk cheese	2	+	+	+	+
7	*Salmonella*	Blockley	Ad 923	Chicken	5	+	+	+	+
8	*Salmonella*	*bongori 48:z35*	Ad598	Environmental sample	8	+	+	White, pink colonies	+
9	*Salmonella*	Bovismorbificans	728	Agar	11	+	+	+	+
10	*Salmonella*	Braenderup	178	Food product	4	+	+	+	+
11	*Salmonella*	Brandenburg	Ad 351	Seafood	2	+	+	+	+
12	*Salmonella*	Bredeney	396	Ground beef	4	+	+	+	+
13	*Salmonella*	Cerro	Ad 689	Dehydrated proteins	8	+	+	+	+
14	*Salmonella*	Cremieu	230	Hare	4	+	+	+	+
15	*Salmonella*	Derby	Ad 1093	Frozen fish fillet	13	+	+	+	+
16	*Salmonella*	Dublin	Ad 528	Beef meat	9	+	+	White colonies	+
17	*Salmonella*	Enteritidis	Ad 926	Raw veal meat	6	+	+	+	+
18	*Salmonella*	Gallinarum	Ad 300	Poultry slaughterhouse	2	+	Small colourless colonies	Small pink colonies	+
19	*Salmonella*	Give	436		4	+	+	+	+
20	*Salmonella*	Hadar	35		18	+	+	+	+
21	*Salmonella*	Havana	Ad 930	Poultry	5	-	-	-	/
					8 (+ 25ml milk)	+	+	+	+
22	*Salmonella*	Heidelberg	A00E005	Dairy industry environmental sample	4	+	+	+	+
23	*Salmonella*	*houtenae* (sub-group IV) 43:z4z32	Ad 597	Fish	8	+	+	+	+
24	*Salmonella*	Indiana	2	Fish flour	2	+	+	+	+
25	*Salmonella*	*indica* (sub-group VI) 1,26,14,25:a: enx	Ad 600	Environmental sample	3	+	+	+	+

INCLUSIVITY									
Strain					inoculation level (cfu/225ml BPW + supplement)	NEOGEN ANSR *Salmonella* method	Confirmation		
Strain			Reference	Origin			RVS/XLD	RVS/ASAP	Latex
26	*Salmonella*	Infantis	12	Ready-to-eat	6	+	+	+	+
27	*Salmonella*	Kedougou	Ad 929	Environmental sample (slaughterhouse)	3	+	+	+	+
28	*Salmonella*	Kottbus	1	Environmental sample (slaughterhouse)	10	+	+	+	+
29	*Salmonella*	Livingstone	E1	Egg white powder	7	+	+	+	+
30	*Salmonella*	London	326	Ham	5	+	+	+	+
31	*Salmonella*	Manhattan	900	Dairy environmental sample	12	+	+	+	+
32	*Salmonella*	Mbandaka	Ad 914	Mayonnaise	8	+	+	+	+
33	*Salmonella*	Montevideo	Ad 912	Raw milk	3	+	+	+	+
34	*Salmonella*	Napoli	Ad 928	Bovine	2	+	+	+	+
35	*Salmonella*	Newport	540	Toulouse sausage	4	+	+	+	+
36	*Salmonella*	Panama	195	Ground beef	9	+	+	+	+
37	*Salmonella*	Paratyphi A	ATCC 9150		6	+	White colonies	+	+
38	*Salmonella*	Paratyphi B	Ad 301	Clinical	9	+	+	+	+
39	*Salmonella*	Paratyphi C	ATCC 13428		4	+	+	+	+
40	*Salmonella*	Regent	328	Duck	4	+	+	+	+
41	*Salmonella*	Rissen	39	Poultry	4	+	+	+	+
42	*Salmonella*	Saintpaul	F31	Pilchard fillet	4	+	+	+	+
43	*Salmonella*	*salamae* (sub-group II) 42:b:enxz	Ad 593	Cereals	3	+	+	+	+
44	*Salmonella*	Senftenberg	Ad 355	Seafood	3	+	+	+	+
45	*Salmonella*	Typhi	Ad 302	Clinical	5	+	+	+	+
46	*Salmonella*	Typhimurium	305	Paella	8	+	+	+	+
47	*Salmonella*	Typhimurium S1 1,4 [5], 12 :- :-	Ad 1333	Tiramisu	4	+	+	+	+
48	*Salmonella*	Typhimurium S1 1,4 [5], 12 : i : -	Ad 1334	Ready-to-eat meal (meat)	4	+	+	+	+
49	*Salmonella*	Typhimurium S1	Ad1335	Environmental sample	8	+	+	+	+
50	*Salmonella*	Urbana	Ad 501	Food product	6	-	-	-	/
					9 (+ 25ml milk)	+	+	+	+
51	*Salmonella*	Virchow	F276	Curry	5	+	+	+	+

EXCLUSIVITY					
Strains				Inoculation level (cfu/ml)	NEOGEN ANSR *Salmonella* method
	Starin	Reference	Origin		
1.	*Citrobacter braakii*	Ad833	Raw beef meat	$4.1x10^5$	-
2.	*Citrobacter Diversus*	adria 140	Raw milk	$3.7x10^5$	-
3.	*Citrobacter freundii*	adria 23	Raw pork sausage	$2.9x10^5$	-
4.	*Citrobacter freundii*	adria 175	Raw duck meat	$3.2x10^5$	-
5.	*Citrobacter koseri*	adria 71	Frozen vegetables	$4.6x10^5$	-
6.	*Enterobacter agglomerans*	adria 11	Cheese	$1.8x10^5$	-
7.	*Enterobacter amnigenus*	A00C068	Raw poultry meat	$1.6x10^5$	-
8.	*Enterobacter cloacae*	adria 10	Raw milk	$1.2x10^5$	-
9.	*Enterobacter intermedius*	adria 60	Bean	$9.2x10^4$	-
10.	*Enterobacter kobei*	Ad 342	Ham	$1.6x10^5$	-
11.	*Enterobacter sakazakii*	adria 95	Fermented milk	$3.6x10^5$	-
12.	*Erwinia carotovora*	CIP 8283	Potatoes	$1.2x10^4$	-
13.	*Escherichia coli*	adria 19	Greated carrots	$3.7x10^5$	-
14.	*Escherichia hermanii*	Ad 461	Dessert	$1.0x10^5$	-
15.	*Escherichia vulneris*	adria 127	Raw milk	$3.8x10^5$	-
16.	*Hafnia alvei*	adria 167	Raw pork sausage	$3.4x10^5$	-
17.	*Klebsiella oxytoca*	57	Food product	$3.4x10^5$	-
18.	*Klebsiella pneumoniae*	47	Raw turkey meat	$2.0x10^5$	-
19.	*Kluyvera spp*	adria 41	Raw milk	$1.3x10^5$	-
20.	*Morganella morganii*	CIP A236	/	$1.7x10^5$	-
21.	*Pantoea agglomerans*	adria 86	Frozen vegetables	$5.3x10^5$	-
22.	*Proteus mirabilis*	Ad639	Mayonnaise	$9.8x10^5$	-
23.	*Proteus vulgaris*	adria 43	Sliced ham	$5.0x10^4$	-
24.	*Providencia rettgeri*	adria 112	White liquid egg	$1.8x10^5$	-
25.	*Rhanella aquatilis*	adria 69	Molluscs	$4.3x10^4$	-
26.	*Serratia liquefaciens*	26	Egg product	$6.2x10^4$	-
27.	*Serratia proteomaculans*	A00C056	Ham	$5.6x10^4$	-
28.	*Shigella flexneri*	CIP 8248	/	$1.3x10^5$	-
29.	*Shigella sonnei*	CIP 8249T (ATCC 29930)	/	$1.7x10^5$	-
30.	*Yersinia enterocolotica*	adria 32	Bacon	$1.1x10^5$	-

Appendix 6 – Paired results of the alternative and reference methods for each level

L0	Reference method		Total
Alternative method	+	-	
+	0	0	0
-	3	109	112
Total	3	109	112

PPNA=5

AC= 97.3 LCL 94.3

L1	Reference method		Total
Alternative method	+	-	
+	109	3	112
-	0	0	0
Total	109	3	112

AC= 97.3 LCL 94.3

L2	Reference method		Total
Alternative method	+	-	
+	106	3	109
-	2	1	3
Total	108	4	112

PPNA=1

PPND=2

AC= 95.5 LCL 91.6

L1+L2	Reference method		Total
Alternative method	+	-	
+	215	6	221
-	2	1	3
Total	217	7	224

AC= 96.4 LCL 93.9

L0+L1+L2	Reference method		Total
Alternative method	+	-	
+	215	6	221
-	5	110	115
Total	220	116	336

PPNA=6

PPND=2

AC= 96.7 LCL 94.8