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ISO 16140-2:2016 validation of SureFast® Salmonella ONE method for the detection of *Salmonella* spp. in poultry meat, meat products, dairy products, vegetables, egg products and feed

MicroVal study number	2014LR43 mod
Method/Kit name	**SureFast® Salmonella ONE method**
Report version	**SUMMARY REPORT - Version 1** 18 December 2018
MicroVal Expert Laboratory	ADRIA Développement ZA Creac'h Gwen F-29196 QUIMPER Cedex (France)

This report consists of 63 pages, including 3 appendices.
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Competencies of the laboratory are certified by COFRAC accreditation for the analyses marked with the symbol◆.

Standardized report - Qualitative methods

This report is prepared in accordance with ISO 16140-2:2016 and MicroVal technical committee interpretation of ISO 16140-2 v.1.0.

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Expert Laboratory	**ADRIA Développement** ZA Creac'h Gwen F-29196 QUIMPER Cedex (France)
Method/Kit name	**SureFast® Salmonella ONE method**
Validation standard	NF EN ISO 16140-2 (June 2016) : Microbiology of the food chain - Method validation *Part 1: Vocabulary* *Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method*
Reference method♦	EN ISO 6579 (December 2002): Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp.
Scope of validation	☒ **Poultry meat** ☒ **Meat products** ☒ **Dairy products** ☒ **Vegetables** *(excluding sprouts)* ☒ **Egg products** ☒ **Feed**
Certification organization	Lloyd's Register

♦ Analyses performed according to the COFRAC accreditation

List of abbreviations

-	No typical colonies but presence of background microflora
(x)	Number of colonies in the plate
*	1/2 dilution
**	1/5 dilution
***	1/10 dilution
****	1/50 dilution
1/2	50% level of target analyte
AL	Acceptability Limit
Alt	Alternative method
Art. Cont.	Artificial contamination
BPW	Buffered Peptone Water
CFU	Colony Forming Units
d	Doubtful result
EL	Expert Laboratory
FP	False Positive
FPR	False Positive Ratio
g	Gram
h	Hour
ILS	Interlaboratory Study
m	Minoritary level of target analyte
M	Majoritary level of target analyte
MCS	Method Comparison Study
min	minute
MKTTn	Muller-Kauffmann Tetrathionate-novobiocin broth
ml	millilitre
MR	(MicroVal) Method Reviewer
MSRV	Modified Semi-solid Rappaport Vassiliadis medium
MVTC	MicroVal Technical Committee
NA	Negative agreement
NC	Non characteristic PCR curve
ND	Negative deviation
ni	Non isolated colonies
P	Pure culture level of target analyte
PA	Positive agreement
PD	Positive deviation
pos (+)	positive/growth/target detected
PPNA	Positive presumptive negative agreement
PPND	Presumptive Positive Negative Deviation (belongs to the False Positive results)
RLOD	Relative Level of Detection
RT	Relative Trueness
RTC	Ready to cook
RTE	Ready to eat
RTRH	Ready to reheat
RVS	Rappaport-Vassiliadis Soya broth
SE	Relative Sensitivity
SP	Relative Specificity
st	Plate without any colony
T	Late amplification curve
TP	True Positive
w	Weak reaction
XLD	Xylose Lysine Deoxycholate agar

Bold typing: artificially inoculated samples

1 INTRODUCTION

In this report, a MicroVal validation study, based on ISO 16140-2:2016, of alternative method for the detection of *Salmonella* spp. in five different (food) categories and in feed products was carried out by ADRIA Développement as the MicroVal Expert Laboratory.

The alternative method used is the **SureFast® Salmonella ONE method** for detection of *Salmonella* spp, the protocol of the alternative method is the following:

- Enrichment in Buffered Peptone Water at 37°C ± 1°C for 16 h to 20 h;
- Sample preparation and detection by the SureFast® Salmonella ONE Kit (No F5211) using BioRad CFX 96, Roche LightCycler® 480 II, Applied Biosystems 7500, MIC and Agilent AriaMx.

The reference method used is: the EN ISO 6579 (December 2002): Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp The scope of this ISO 6579 method covers Food and Feed.

Scope of the validation study is: a broad range of food (poultry meat, meat products, dairy products, vegetables (*excluding sprouts)*, egg products, and feed).

Criteria evaluated during the Method Comparison Study (MCS) have been:

- Sensitivity study;
- Relative level of detection study;
- Inclusivity and exclusivity study.

The summarized conclusions on the method comparison study are:

- The observed values for ND - PD and ND + PD for the 6 individual categories and for all categories for each of the thermocyclers tested meet the Acceptability Limits (observed values ≤ AL);

- The RLOD values (using the confirmed alternative method results) meet the Acceptability Limit which is 1.5 for a paired study design, for all categories tested;

- The SureFast® Salmonella ONE method is selective and specific.

It is possible to store the primary enrichment broth for 72 h at 5 ± 3°C.

Restriction for use: sprouts are excluded from the scope of the validation.

The summary of the conclusion on the Inter-laboratory study is: The data and interpretations comply with the EN ISO 16140-2:2016 requirements. **The SureFast® Salmonella ONE method is considered equivalent to the ISO 6579 standard.**

This report corresponds to the method comparison study and the Inter-laboratory study, and gathers the observed data and interpretations according to the EN ISO 16140- 2:2016 standard and the MicroVal technical rules v.1.0.

2 METHOD PROTOCOLS

2.1 Reference method♦

The reference method corresponds to the EN ISO 6579 (December 2002): Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp. See the flow diagram in **Annex A**.

♦ Analyses performed according to the COFRAC accreditation

2.2 Alternative method

See the flow diagram of the alternative method in Annex B.

The alternative method principle is based on Real-Time PCR. SureFast® Salmonella ONE method is a new method developed by CONGEN Biotechnologie.

The protocol is the following:

- Enrichment in Buffered Peptone Water at 37°C ± 1°C for 16 h to 20 h;

- Sample preparation and real-time PCR with the SureFast® Salmonella ONE, using 5 thermocyclers:
  - BioRad CFX 96 (CFX Manager ™ Software Version 3.1)
  - Roche LightCycler® 480 II (LCS 480 1.5.1.6.2)
  - Applied Biosystems 7500 (7500 Software v2.0.6)
  - MIC (MIC PCR v2.2.0)
  - Agilent AriaMx (Software v1.4)

The software programs of all thermocyclers included in this study offer both an automatic and a manual evaluation tool.
For result analysis the automatic evaluation tool was always used first.
When samples only show a weak positive fluorescence increase, the automatic evaluation might not be able to detect these positive samples. In these cases it is a standard approach to apply a manual evaluation. The person analysing the PCR data (e.g. scientist) by eye screening identifies these wrong negative samples and sets the threshold manually to a value of 5 % of the maximal fluorescence of the positive control.

Some examples of curves are provided in **Annex C** for each cycler used.

- **Confirmation procedure:**
  - Running a sub-culture in RVS before streaking onto XLD or a chromogenic agar plate, followed by a direct latex test (Thermo Fisher Scientific) or a direct biochemical gallery on characteristic colonies without applying a purification step.

  OR

- Running a sub-culture in RVS and MKTTn before streaking onto XLD or a chromogenic agar plate. Typical colonies are confirmed using the tests described in the EN ISO 6579 (sub-culture on non selective agar, serological and biochemical tests).

During the validation study, note that:

- The minimum enrichment time was tested: 16 h;
- All positive and negative PCR results were confirmed using both confirmation procedures (RVS and RVS+MKTTn)
- The characteristic colonies were confirmed by:
    - Latex and biochemical tests without purification step;
    - The tests described in the reference method after purification step.

Enrichment storage of the primary enrichment for 72 h at 5°C ± 3°C was also evaluated in order to offer sufficient practicability to the users. This was done on the positive and discordant samples in the sensitivity study.

2.3 Study design

The method comparison study was carried out using 25 g portions of sample material. Unless otherwise stated, sample preparations were done according to ISO 6887 parts.
As the reference and the alternative method share the initial enrichment step the same test portion (Item) was used for the two methods. All resulting data were treated as paired data (EN-ISO 16140-2).

3 METHOD COMPARISON STUDY

The alternative method is dedicated to five thermocyclers:

- BioRad CFX 96;
- Roche LightCycler® 480 II;
- Applied Biosystems 7500;
- MIC;
- Agilent AriaMx.

The tests were done with ALL of them. One DNA extraction was run per sample. The lysates were then separated in two tubes: one was used in ADRIA to run the test on the BioRad CFX96; the other one was sent in frozen conditions with a specific carrier to CONGEN which run the tests on the other thermocyclers. After arrival, DNA extracts were stored at -20°C. Immediately after arrival, CONGEN started the runs on the respective cyclers. To avoid any further thaw/freeze cycles, only the amount of DNA samples analysed the same day was unfrozen. The samples were run in parallel using the different cyclers.

The DNA samples were tested according to the indications and cycler conditions given in the kit insert.

3.1 Sensitivity Study

The sensitivity study (SE) is the ability of the method selected to detect the analyte by either the reference or the alternative method.

3.1.1 *Categories and sample types*

A total of 6 Categories were included in this validation study.

A minimum of 60 Items for each Category were tested by both the reference method and the alternative method in the sensitivity study, with a minimum of 30 positive samples per Category.

Each Category was made up of 3 Types, with at least 20 Items representative for that Type.

The categories, the types and the number of samples analyzed are presented in Table 1.

Table 1 - Categories, types and number of samples analyzed

Category		Type		Alternative method protocol	Test portion size	Number of samples
1	Poultry meat products	a	Raw, frozen	BPW for 16 - 20 h at 37°C ± 1°C	25 g	24
		b	RTC		25 g	21
		c	RTE and RTRH		25 g	23
					Total	68
2	Meat Products	a	Raw, Frozen, Seasoned	BPW for 16 - 20 h at 37°C ± 1°C	25 g	20
		b	RTC		25 g	20
		c	RTE and RTRH		25 g	21
					Total	61
3	Dairy products	a	Pasteurized	BPW for 16 - 20 h at 37°C ± 1°C	25 g	20
		b	Raw		25 g	26
		c	Powder, ingredients		25 g	20
					Total	66
4	Vegetables	a	RTC	BPW for 16 - 20 h at 37°C ± 1°C	25 g	20
		b	Fresh herbs, spices		25 g	26
		c	RTE		25 g	26
					Total	72
5	Egg products	a	Pasteurized	BPW for 16 - 20 h at 37°C ± 1°C	25 g	22
		b	Powder		25 g	20
		c	Egg products		25 g	20
					Total	62
6	Feed products	a	Pet	BPW for 16 - 20 h at 37°C ± 1°C	25 g	21
		b	Cattle		25 g	21
		c	Raw material		25 g	20
					Total	62
All products						391

391 samples were analyzed. The distribution of positive and negative samples per tested category and type is given respectively in Table 2.

Table 2 - Distribution per tested category and type

Category		Type		Positive samples*	Negative samples	Total
1	Poultry meat products	a	Raw, frozen	10	14	24
		b	RTC	9	12	21
		c	RTE and RTRH	12	11	23
			Total	31	37	68
2	Meat Products	a	Raw, Frozen, Seasoned	10	10	20
		b	RTC	13	7	20
		c	RTE and RTRH	8	13	21
			Total	31	30	61
3	Dairy products	a	Pasteurized	13	7	20
		b	Raw	9	17	26
		c	Powder, ingredients	10	10	20
			Total	32	34	66
4	Vegetables	a	RTC	9	11	20
		b	Fresh herbs, spices	9	17	26
		c	RTE	13	13	26
			Total	31	41	72
5	Egg products	a	Pasteurized	11	11	22
		b	Powder	9	11	20
		c	Egg products	11	9	20
			Total	31	31	62
6	Feed products	a	Pet	11	10	21
		b	Cattle	9	12	21
		c	Raw material	12	8	20
			Total	32	30	62
All categories				**188**	**203**	**391**

*Positive by at least one of the methods

3.1.2 *Test sample preparation*

Naturally contaminated samples were preferentially analyzed. But artificially contaminated samples were run using seeding protocols (storage for 48 h at 5°C ± 3°C, storage for 7 days at - 18°C, inoculation with lyophilized strains and storage for 2 weeks at ambient temperature) and spiking protocols (strains stored for 7 days at 5°C ± 3°C, strains heat-treated for 8 min at 56°C).

When 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 TSYEA and XLD).

The same strain was not used to inoculate more than 6 samples.

53 samples were artificially contaminated by *spiking*, using 25 different strains and 2 injury protocols. 50 gave a positive result. Most of the spiking inoculations, after injury protocols on the inoculum, were lower or equal to 5 CFU/sample.

156 samples were artificially contaminated by *seeding*, using 47 different strains and 3 seeding protocols. 103 gave a positive result. Most of the seeding inoculations were lower or equal to 5 CFU/sample.

The number of positive naturally and artificially contaminated samples per inoculation level is provided in Table 3.

Table 3 - Number of positive naturally and artificially contaminated samples

	Naturally contaminated	Inoculated samples			Total
		≤ 5 CFU	5 < x ≤ 10	10 < x ≤ 15	
Number of samples	35	130	18	5	188
%	19	69	10	3	100

19 % of the samples were naturally contaminated.

3.1.3 *Confirmation protocols*

The negative and positive PCR tests were confirmed by:

- Running a sub-culture in RVS before streaking onto XLD and a chromogenic agar plate, followed by a direct latex test (Ref. DR1108A Latex *Salmonella* Oxoid Thermo Fisher Scientific) and a direct biochemical gallery (Ref. 20100 API 20E BioMérieux) on characteristic colonies without applying a purification step.

AND

- Running a sub-culture in RVS and MKTTn before streaking onto XLD and a chromogenic agar plate. Typical colonies were confirmed using the tests described in the ISO 6579 (sub-culture on non selective agar, serological and biochemical tests).

During the validation study, both protocols were tested on all samples.

3.1.4 *Sensitivity study results*

Sample numbers in **bold** indicate artificial inoculation of the sample.

Table 4 shows the summary of results of the reference method and the alternative methods for **all Categories and all thermocyclers tested**.

Table 5 shows the interpretation of sample results between the reference and alternative method (based on the confirmed alternative method).

Table 4 - Summary of sensitivity study results – *All categories*

Thermocycler	Response	Reference method positive (R+)	Reference method negative (R-)
Bio-Rad CFX 96	**Alternative method positive (A+)**	Positive agreement (R+/A+) **PA =179**	Positive deviation (R-/A+) **PD = 2**
	Alternative method negative (A-)	Negative deviation (R+/A-) **ND = 7**	Negative agreement (R-/A-) **NA =203**
Roche LightCycler® 480 II	**Alternative method positive (A+)**	Positive agreement (R+/A+) **PA = 181**	Positive deviation (R-/A+) **PD = 2**
	Alternative method negative (A-)	Negative deviation (R+/A-) **ND = 5**	Negative agreement (R-/A-) **NA = 203**
Applied Biosystems 7500	**Alternative method positive (A+)**	Positive agreement (R+/A+) **PA = 181**	Positive deviation (R-/A+) **PD = 2**
	Alternative method negative (A-)	Negative deviation (R+/A-) **ND = 5**	Negative agreement (R-/A-) **NA = 203**
MIC	**Alternative method positive (A+)**	Positive agreement (R+/A+) **PA = 181**	Positive deviation (R-/A+) **PD = 2**
	Alternative method negative (A-)	Negative deviation (R+/A-) **ND = 5**	Negative agreement (R-/A-) **NA = 203**
Agilent AriaMx	**Alternative method positive (A+)**	Positive agreement (R+/A+) **PA = 181**	Positive deviation (R-/A+) **PD = 2**
	Alternative method negative (A-)	Negative deviation (R+/A-) **ND = 5**	Negative agreement (R-/A-) **NA = 203**

Table 5 – Interpretation of sample results between the reference and alternative method (based on the confirmed alternative method)

Table 5a - Bio-Rad CFX96

Category		Type		PA	NA[1]	PD	ND[2]	PPND[3]	PPNA[3]	Total
1	Poultry meat products	a	Raw, frozen	10	14	0	0	0	0	24
		b	RTC	7	12	0	2	0	0	21
		c	RTE and RTRH	11	11	1	0	0	0	23
			Total	28	37	1	2	0	0	68
2	Meat Products	a	Raw, Frozen, Seasoned	9	10	0	1	0	0	20
		b	RTC	12	7	0	1	0	0	20
		c	RTE and RTRH	8	11	0	0	0	2	21
			Total	29	28	0	2	0	2	61
3	Dairy products	a	Pasteurized	13	6	0	0	0	1	20
		b	Raw	8	16	1	0	0	1	26
		c	Powder, ingredients	10	10	0	0	0	0	20
			Total	31	32	1	0	0	2	66
4	Vegetables	a	RTC	9	11	0	0	0	0	20
		b	Fresh herbs, spices	8	17	0	1	0	0	26
		c	RTE	13	13	0	0	0	0	26
			Total	30	41	0	1	0	0	72
5	Egg products	a	Pasteurized	11	10	0	0	0	1	22
		b	Powder	9	6	0	0	0	5	20
		c	Egg products	11	9	0	0	0	0	20
			Total	31	25	0	0	0	6	62
6	Feed products	a	Pet	11	9	0	0	0	1	21
		b	Cattle	7	12	0	2	0	0	21
		c	Raw material	12	8	0	0	0	0	20
			Total	30	29	0	2	0	1	62
All products				**179**	**192**	**2**	**7**	**0**	**11**	**391**

[1] NA: including PPNA, [2] ND: including PPND, [3] FP = PPNA + PPND

Table 5b - Roche LightCycler® 480 II

Category		Type		PA	NA	PD	ND	PPND	PPNA	Total
1	Poultry meat products	a	Raw, frozen	10	13	0	0	0	1	24
		b	RTC	8	10	0	1	0	2	21
		c	RTE and RTRH	11	11	1	0	0	0	23
			Total	29	34	1	1	0	3	68
2	Meat Products	a	Raw, Frozen, Seasoned	9	10	0	1	0	0	20
		b	RTC	13	7	0	0	0	0	20
		c	RTE and RTRH	8	12	0	0	0	1	21
			Total	30	29	0	1	0	1	61
3	Dairy products	a	Pasteurized	13	7	0	0	0	0	20
		b	Raw	8	16	1	0	0	1	26
		c	Powder, ingredients	10	10	0	0	0	0	20
			Total	31	33	1	0	0	1	66
4	Vegetables	a	RTC	9	11	0	0	0	0	20
		b	Fresh herbs, spices	8	17	0	1	0	0	26
		c	RTE	13	13	0	0	0	0	26
			Total	30	41	0	1	0	0	72
5	Egg products	a	Pasteurized	11	11	0	0	0	0	22
		b	Powder	9	10	0	0	0	1	20
		c	Egg products	11	9	0	0	0	0	20
			Total	31	30	0	0	0	1	62
6	Feed products	a	Pet	11	10	0	0	0	0	21
		b	Cattle	7	12	0	2	0	0	21
		c	Raw material	12	8	0	0	0	0	20
			Total	30	30	0	2	0	0	62
All categories				**181**	**197**	**2**	**5**	**0**	**6**	**391**

Table 5c - Applied Biosystems 7500

Category		Type		PA	NA	PD	ND	PPND	PPNA	Total
1	Poultry meat products	a	Raw, frozen	10	13	0	0	0	1	24
		b	RTC	8	10	0	1	0	2	21
		c	RTE and RTRH	11	11	1	0	0	0	23
			Total	29	34	1	1	0	3	68
2	Meat Products	a	Raw, Frozen, Seasoned	9	10	0	1	0	0	20
		b	RTC	13	7	0	0	0	0	20
		c	RTE and RTRH	8	12	0	0	0	1	21
			Total	30	29	0	1	0	1	61
3	Dairy products	a	Pasteurized	13	7	0	0	0	0	20
		b	Raw	8	16	1	0	0	1	26
		c	Powder, ingredients	10	10	0	0	0	0	20
			Total	31	33	1	0	0	1	66
4	Vegetables	a	RTC	9	11	0	0	0	0	20
		b	Fresh herbs, spices	8	17	0	1	0	0	26
		c	RTE	13	13	0	0	0	0	26
			Total	30	41	0	1	0	0	72
5	Egg products	a	Pasteurized	11	11	0	0	0	0	22
		b	Powder	9	10	0	0	0	1	20
		c	Egg products	11	9	0	0	0	0	20
			Total	31	30	0	0	0	1	62
6	Feed products	a	Pet	11	10	0	0	0	0	21
		b	Cattle	7	12	0	2	0	0	21
		c	Raw material	12	8	0	0	0	0	20
			Total	30	30	0	2	0	0	62
All categories				**181**	**197**	**2**	**5**	**0**	**6**	**391**

Table 5d - MIC

Category		Type		PA	NA	PD	ND	PPND	PPNA	Total
1	Poultry meat products	a	Raw, frozen	10	13	0	0	0	1	24
		b	RTC	8	10	0	1	0	2	21
		c	RTE and RTRH	11	11	1	0	0	0	23
			Total	29	34	1	1	0	3	68
2	Meat Products	a	Raw, Frozen, Seasoned	9	10	0	1	0	0	20
		b	RTC	13	7	0	0	0	0	20
		c	RTE and RTRH	8	12	0	0	0	1	21
			Total	30	29	0	1	0	1	61
3	Dairy products	a	Pasteurized	13	7	0	0	0	0	20
		b	Raw	8	16	1	0	0	1	26
		c	Powder, ingredients	10	10	0	0	0	0	20
			Total	31	33	1	0	0	1	66
4	Vegetables	a	RTC	9	11	0	0	0	0	20
		b	Fresh herbs, spices	8	17	0	1	0	0	26
		c	RTE	13	13	0	0	0	0	26
			Total	30	41	0	1	0	0	72
5	Egg products	a	Pasteurized	11	11	0	0	0	0	22
		b	Powder	9	10	0	0	0	1	20
		c	Egg products	11	9	0	0	0	0	20
			Total	31	30	0	0	0	1	62
6	Feed products	a	Pet	11	10	0	0	0	0	21
		b	Cattle	7	12	0	2	0	0	21
		c	Raw material	12	8	0	0	0	0	20
			Total	30	30	0	2	0	0	62
All categories				**181**	**197**	**2**	**5**	**0**	**6**	391

Table 5e - Agilent AriaMx

Category		Type		PA	NA	PD	ND	PPND	PPNA	Total
1	Poultry meat products	a	Raw, frozen	10	13	0	0	0	1	24
		b	RTC	8	10	0	1	0	2	21
		c	RTE and RTRH	11	11	1	0	0	0	23
			Total	29	34	1	1	0	3	68
2	Meat Products	a	Raw, Frozen, Seasoned	9	10	0	1	0	0	20
		b	RTC	13	7	0	0	0	0	20
		c	RTE and RTRH	8	12	0	0	0	1	21
			Total	30	29	0	1	0	1	61
3	Dairy products	a	Pasteurized	13	7	0	0	0	0	20
		b	Raw	8	16	1	0	0	1	26
		c	Powder, ingredients	10	10	0	0	0	0	20
			Total	31	33	1	0	0	1	66
4	Vegetables	a	RTC	9	11	0	0	0	0	20
		b	Fresh herbs, spices	8	17	0	1	0	0	26
		c	RTE	13	13	0	0	0	0	26
			Total	30	41	0	1	0	0	72
5	Egg products	a	Pasteurized	11	11	0	0	0	0	22
		b	Powder	9	10	0	0	0	1	20
		c	Egg products	11	9	0	0	0	0	20
		Total		31	30	0	0	0	1	62
6	Feed products	a	Pet	11	10	0	0	0	0	21
		b	Cattle	7	12	0	2	0	0	21
		c	Raw material	12	8	0	0	0	0	20
		Total		30	30	0	2	0	0	62
All categories				**181**	**197**	**2**	**5**	**0**	**6**	**391**

3.1.5 *Sensitivity study calculations*

The sensitivity study parameters, as specified in Table 6, were calculated for all Categories and Types, and the overview for each thermocycler is given in Table 7.

Table 6 – Formula to calculate the sensitivity parameters

Sensitivity for the alternative method	$SE_{alt} = \frac{(PA+PD)}{(PA+ND+PD)} \times 100\%$
Sensitivity for the reference method	$SE_{ref} = \frac{(PA+ND)}{(PA+ND+PD)} \times 100\%$
Relative trueness	$RT = \frac{(PA+NA)}{N} \times 100\%$
False positive ratio for the alternative method	$FPR = \frac{(FP)}{NA} \times 100\%$

Table 7 - Overview calculated sensitivity parameters per Category, Type and Thermocycler

Table 7a - Bio-Rad CFX96

Category		Type		PA	NA[1]	PD	ND[2]	FP[3]	SE alt %	SE ref %	RT %	FPR %
1	Poultry meat products	a	Raw, frozen	10	14	0	0	0	100,0	100,0	100,0	0,0
		b	RTC	7	12	0	2	0	77,8	100,0	90,5	0,0
		c	RTE and RTRH	11	11	1	0	0	100,0	91,7	95,7	0,0
			Total	28	37	1	2	0	93,5	96,8	95,6	0,0
2	Meat Products	a	Raw, Frozen, Seasoned	9	10	0	1	0	90,0	100,0	95,0	0,0
		b	RTC	12	7	0	1	0	92,3	100,0	95,0	0,0
		c	RTE and RTRH	8	11	0	0	2	100,0	100,0	100,0	18,2
			Total	29	28	0	2	2	93,5	100,0	96,7	6,7
3	Dairy products	a	Pasteurized	13	6	0	0	1	100,0	100,0	100,0	16,7
		b	Raw	8	16	1	0	1	100,0	88,9	96,2	6,3
		c	Powder, ingredients	10	10	0	0	0	100,0	100,0	100,0	0,0
			Total	31	32	1	0	2	100,0	96,9	98,5	5,9
4	Vegetables	a	RTC	9	11	0	0	0	100,0	100,0	100,0	0,0
		b	Fresh herbs, spices	8	17	0	1	0	88,9	100,0	96,2	0,0
		c	RTE	13	13	0	0	0	100,0	100,0	100,0	0,0
			Total	30	41	0	1	0	96,8	100,0	98,6	0,0
5	Egg products	a	Pasteurized	11	10	0	0	1	100,0	100,0	100,0	10,0
		b	Powder	9	6	0	0	5	100,0	100,0	100,0	83,3
		c	Egg products	11	9	0	0	0	100,0	100,0	100,0	0,0
			Total	31	25	0	0	6	100,0	100,0	100,0	19,4
6	Feed products	a	Pet	11	9	0	0	1	100,0	100,0	100,0	11,1
		b	Cattle	7	12	0	2	0	77,8	100,0	90,5	0,0
		c	Raw material	12	8	0	0	0	100,0	100,0	100,0	0,0
			Total	30	29	0	2	1	93,8	100,0	96,8	3,3
All products				**179**	**192**	**2**	**7**	**11**	**96,3**	**98,9**	**97,7**	**5,4**

[1] NA: including PPNA, [2] ND: including PPND, [3] FP = PPNA + PPND

Table 7b - Roche LightCycler® 480 II

Category		Type		PA	NA	PD	ND	FP	SE alt %	SE ref %	RT %	FPR %
1	Poultry meat products	a	Raw, frozen	10	13	0	0	1	100,0	100,0	100,0	7,7
		b	RTC	8	10	0	1	2	88,9	100,0	95,2	20,0
		c	RTE and RTRH	11	11	1	0	0	100,0	91,7	95,7	0,0
			Total	29	34	1	1	3	96,8	96,8	97,1	8,1
2	Meat Products	a	Raw, Frozen, Seasoned	9	10	0	1	0	90,0	100,0	95,0	0,0
		b	RTC	13	7	0	0	0	100,0	100,0	100,0	0,0
		c	RTE and RTRH	8	12	0	0	1	100,0	100,0	100,0	8,3
			Total	30	29	0	1	1	96,8	100,0	98,4	3,3
3	Dairy products	a	Pasteurized	13	7	0	0	0	100,0	100,0	100,0	0,0
		b	Raw	8	16	1	0	1	100,0	88,9	96,2	6,3
		c	Powder, ingredients	10	10	0	0	0	100,0	100,0	100,0	0,0
			Total	31	33	1	0	1	100,0	96,9	98,5	2,9
4	Vegetables	a	RTC	9	11	0	0	0	100,0	100,0	100,0	0,0
		b	Fresh herbs, spices	8	17	0	1	0	88,9	100,0	96,2	0,0
		c	RTE	13	13	0	0	0	100,0	100,0	100,0	0,0
			Total	30	41	0	1	0	96,8	100,0	98,6	0,0
5	Egg products	a	Pasteurized	11	11	0	0	0	100,0	100,0	100,0	0,0
		b	Powder	9	10	0	0	1	100,0	100,0	100,0	10,0
		c	Egg products	11	9	0	0	0	100,0	100,0	100,0	0,0
			Total	31	30	0	0	1	100,0	100,0	100,0	3,2
6	Feed products	a	Pet	11	10	0	0	0	100,0	100,0	100,0	0,0
		b	Cattle	7	12	0	2	0	77,8	100,0	90,5	0,0
		c	Raw material	12	8	0	0	0	100,0	100,0	100,0	0,0
			Total	30	30	0	2	0	93,8	100,0	96,8	0,0
All categories				**181**	**197**	**2**	**5**	**6**	**97,3**	**98,9**	**98,2**	**3,0**

Table 7c - Applied Biosystems 7500

Category		Type		PA	NA	PD	ND	FP	SE $_{alt}$ %	SE $_{ref}$ %	RT %	FPR %
1	Poultry meat products	a	Raw, frozen	10	13	0	0	1	100,0	100,0	100,0	7,7
		b	RTC	8	10	0	1	2	88,9	100,0	95,2	20,0
		c	RTE and RTRH	11	11	1	0	0	100,0	91,7	95,7	0,0
			Total	29	34	1	1	3	96,8	96,8	97,1	8,1
2	Meat Products	a	Raw, Frozen, Seasoned	9	10	0	1	0	90,0	100,0	95,0	0,0
		b	RTC	13	7	0	0	0	100,0	100,0	100,0	0,0
		c	RTE and RTRH	8	12	0	0	1	100,0	100,0	100,0	8,3
			Total	30	29	0	1	1	96,8	100,0	98,4	3,3
3	Dairy products	a	Pasteurized	13	7	0	0	0	100,0	100,0	100,0	0,0
		b	Raw	8	16	1	0	1	100,0	88,9	96,2	6,3
		c	Powder, ingredients	10	10	0	0	0	100,0	100,0	100,0	0,0
			Total	31	33	1	0	1	100,0	96,9	98,5	2,9
4	Vegetables	a	RTC	9	11	0	0	0	100,0	100,0	100,0	0,0
		b	Fresh herbs, spices	8	17	0	1	0	88,9	100,0	96,2	0,0
		c	RTE	13	13	0	0	0	100,0	100,0	100,0	0,0
			Total	30	41	0	1	0	96,8	100,0	98,6	0,0
5	Egg products	a	Pasteurized	11	11	0	0	0	100,0	100,0	100,0	0,0
		b	Powder	9	10	0	0	1	100,0	100,0	100,0	10,0
		c	Egg products	11	9	0	0	0	100,0	100,0	100,0	0,0
			Total	31	30	0	0	1	100,0	100,0	100,0	3,2
6	Feed products	a	Pet	11	10	0	0	0	100,0	100,0	100,0	0,0
		b	Cattle	7	12	0	2	0	77,8	100,0	90,5	0,0
		c	Raw material	12	8	0	0	0	100,0	100,0	100,0	0,0
			Total	30	30	0	2	0	93,8	100,0	96,8	0,0
All categories				**181**	**197**	**2**	**5**	**6**	**97,3**	**98,9**	**98,2**	**3,0**

Table 7d - MIC

Category		Type		PA	NA	PD	ND	FP	SE alt %	SE ref %	RT %	FPR %
1	Poultry meat products	a	Raw, frozen	10	13	0	0	1	100,0	100,0	100,0	7,7
		b	RTC	8	10	0	1	2	88,9	100,0	95,2	20,0
		c	RTE and RTRH	11	11	1	0	0	100,0	91,7	95,7	0,0
			Total	29	34	1	1	3	96,8	96,8	97,1	8,1
2	Meat Products	a	Raw, Frozen, Seasoned	9	10	0	1	0	90,0	100,0	95,0	0,0
		b	RTC	13	7	0	0	0	100,0	100,0	100,0	0,0
		c	RTE and RTRH	8	12	0	0	1	100,0	100,0	100,0	8,3
			Total	30	29	0	1	1	96,8	100,0	98,4	3,3
3	Dairy products	a	Pasteurized	13	7	0	0	0	100,0	100,0	100,0	0,0
		b	Raw	8	16	1	0	1	100,0	88,9	96,2	6,3
		c	Powder, ingredients	10	10	0	0	0	100,0	100,0	100,0	0,0
			Total	31	33	1	0	1	100,0	96,9	98,5	2,9
4	Vegetables	a	RTC	9	11	0	0	0	100,0	100,0	100,0	0,0
		b	Fresh herbs, spices	8	17	0	1	0	88,9	100,0	96,2	0,0
		c	RTE	13	13	0	0	0	100,0	100,0	100,0	0,0
			Total	30	41	0	1	0	96,8	100,0	98,6	0,0
5	Egg products	a	Pasteurized	11	11	0	0	0	100,0	100,0	100,0	0,0
		b	Powder	9	10	0	0	1	100,0	100,0	100,0	10,0
		c	Egg products	11	9	0	0	0	100,0	100,0	100,0	0,0
			Total	31	30	0	0	1	100,0	100,0	100,0	3,2
6	Feed products	a	Pet	11	10	0	0	0	100,0	100,0	100,0	0,0
		b	Cattle	7	12	0	2	0	77,8	100,0	90,5	0,0
		c	Raw material	12	8	0	0	0	100,0	100,0	100,0	0,0
			Total	30	30	0	2	0	93,8	100,0	96,8	0,0
All categories				**181**	**197**	**2**	**5**	**6**	**97,3**	**98,9**	**98,2**	**3,0**

Table 7e - Agilent AriaMx

Category		Type		PA	NA	PD	ND	FP	SE $_{alt}$ %	SE $_{ref}$ %	RT %	FPR %
1	Poultry meat products	a	Raw, frozen	10	13	0	0	1	100,0	100,0	100,0	7,7
		b	RTC	8	10	0	1	2	88,9	100,0	95,2	20,0
		c	RTE and RTRH	11	11	1	0	0	100,0	91,7	95,7	0,0
			Total	29	34	1	1	3	96,8	96,8	97,1	8,1
2	Meat Products	a	Raw, Frozen, Seasoned	9	10	0	1	0	90,0	100,0	95,0	0,0
		b	RTC	13	7	0	0	0	100,0	100,0	100,0	0,0
		c	RTE and RTRH	8	12	0	0	1	100,0	100,0	100,0	8,3
			Total	30	29	0	1	1	96,8	100,0	98,4	3,3
3	Dairy products	a	Pasteurized	13	7	0	0	0	100,0	100,0	100,0	0,0
		b	Raw	8	16	1	0	1	100,0	88,9	96,2	6,3
		c	Powder, ingredients	10	10	0	0	0	100,0	100,0	100,0	0,0
			Total	31	33	1	0	1	100,0	96,9	98,5	2,9
4	Vegetables	a	RTC	9	11	0	0	0	100,0	100,0	100,0	0,0
		b	Fresh herbs, spices	8	17	0	1	0	88,9	100,0	96,2	0,0
		c	RTE	13	13	0	0	0	100,0	100,0	100,0	0,0
			Total	30	41	0	1	0	96,8	100,0	98,6	0,0
5	Egg products	a	Pasteurized	11	11	0	0	0	100,0	100,0	100,0	0,0
		b	Powder	9	10	0	0	1	100,0	100,0	100,0	10,0
		c	Egg products	11	9	0	0	0	100,0	100,0	100,0	0,0
			Total	31	30	0	0	1	100,0	100,0	100,0	3,2
6	Feed products	a	Pet	11	10	0	0	0	100,0	100,0	100,0	0,0
		b	Cattle	7	12	0	2	0	77,8	100,0	90,5	0,0
		c	Raw material	12	8	0	0	0	100,0	100,0	100,0	0,0
			Total	30	30	0	2	0	93,8	100,0	96,8	0,0
All categories				**181**	**197**	**2**	**5**	**6**	**97,3**	**98,9**	**98,2**	**3,0**

3.1.6 *Confirmations*

Two confirmation protocols were applied during the study: by running a subculture only in RVS broth before streaking onto selective agar plates or by applying the whole protocol of the reference method (subculture in RVS and MKTTn prior streaking). The same results were observed with both confirmation procedures.

For 11 samples which gave positive PCR results using the Bio-Rad CFX96, it was impossible to confirm the presence of *Salmonella* spp. in the enrichment broth even when applying additional confirmatory tests: inoculation of 5 RVS,

5 MKTTn broths and 5 MSRV plates. These samples are listed in Table 8. These samples were treated as false positive since their viability could not be confirmed.

These results explain the high percentage value obtained for the false positive ratio for the egg products category using the **CFX96 from Bio-Rad** (FPR = 19.4 %).

Table 8 - Positive presumptive non confirmed samples

Sample N°	Product	CFX96 Bio-Rad	ROCHE LightCycler 480II	Applied Biosystems 7500	MIC	Agilent AriaMx
6402	**Pâté**	+(36,13)/+(34,73)/+(34,22)	+(37,8)	+(32,9)	+(36,5)	+(34,9)
6403	**Pâté**	+(33,51)/+(34,06)/+(34,53)	-	-	-	-
8306	Pasteurized milk	+(36,45)/-/-	-	-	-	-
7414	**Raw milk cheese**	+(42,45)/-/-	-	-	-	-
6305	**Pasteurized liquid whole egg**	+(39,21)/+(39,32)/+(37,43)	-	-	-	-
82	White egg powder	+(37,14)/+(35,71)/-	-	-	-	-
83	White egg powder	+(43,40)/+(35,39)/-	-	-	-	-
84	White egg powder	+(35,57)/+(38,64)/-	-	-	-	-
524	Whole egg powder	+(33,61)/+(33,45)/+(35,27)	+(39,69)	+(37,1)	+(34,2)	+(35,3)
1999	Whole egg powder	+(38,16)/+(39,25)/+(38,32)	-	-	-	-
6421 bis	**Pellets for dog (beef)**	+(39,8)/-/-	-	-	-	-
7473	Raw poultry meat	-	+(35,2)	+(28,8)	+(33,7)	+(36,4)
7899	Seasoned turkey meat	-	+(36,7)	+(39,9)	+(33,9)	+(39,2)
7902	Turkey meat	-	+(34,4)	+(37,3)	+(34,9)	+(41,2)
8123	Ewe milk	-	+(35,1)	+(35,1)	+(33,4)	+(38,8)

Note that for 4 samples (7473, 7899, 7902 and 8233), positive PCR tests were obtained only when DNA extracts were tested by CONGEN; this was perhaps due to a cross contamination linked to the preparation of DNA extracts for shipment.

The protocol described in the kit insert (section 5.1) offers the option to assess whether a process of bacterial growth has occurred or not. It is recommended to compare the samples at the beginning and at the end of the culturing. Bacterial growth can be assumed at a Cp-value difference > 3.

When enough matrix was still available, the protocol described above was chosen to be applied in case of non-confirmation positive PCR results. In this study, this was done for 3 samples; the results were the following (See Table 9).

The protocol used was: 25 g + 225 ml BPW (without incubation step), lysis step and PCR.

This protocol is applied only to check whether or not bacterial growth has occurred and is only recommended in the case of non confirmation of positive PCR results and was not applied for all samples.

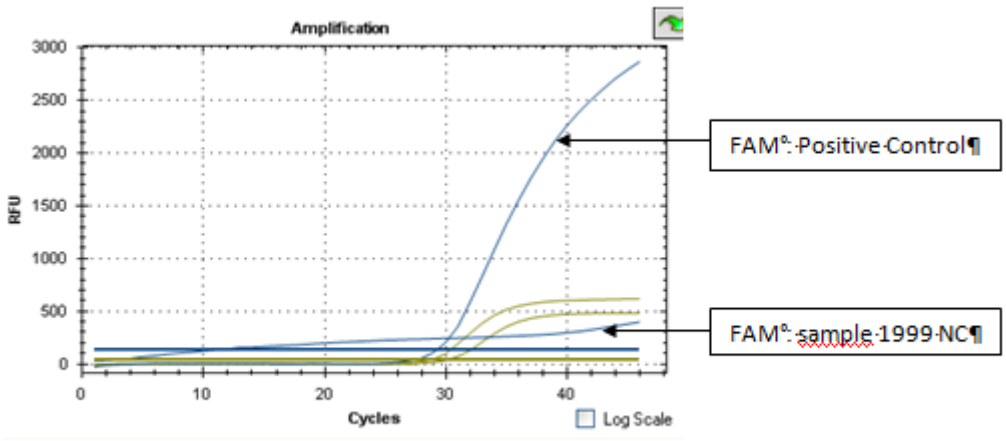
The test was done at T_0 (before enrichment) and at T_1 (after enrichment for 16 h at 37°C).

Table 9 - Growth control protocol described in the kit insert

Sample N°	Product	PCR result before enrichment step (Bio-Rad CFX96)	PCR result after enrichment step (16 h at 37°C)					PCR result after enriched BPW storage (72 h at 5°C ± 3°C)				
			Bio-Rad CFX 96	Roche LightCycler 480II	Applied Biosystems 7500	MIC	Agilent AriaMx	Bio-Rad CFX 96	Roche LightCycler 480II	Applied Biosystems 7500	MIC	Agilent AriaMx
82	White egg powder	+(30,04)	+(37,14)/+(35,71)/-	-	-	-	-	+(39,02)/-/+(37,74)	-	-	-	-
524	Whole egg powder	-	+(33,61)/+(33,45)/+(35,27)	+(39,7)	+(37,1)	+(34,1)	+(35,2)	+(40,14)/+(36,58)/+(37,99)	-	-	-	-
1999	Whole egg powder	+(12,01) NC	+(38,16)/+(39,25)/+(38,32)	-	-	-	-	+(35,49)	-	-	-	-

NC: non characteristic curve (See Figure 1)

Figure 1: sample 1999 PCR result before enrichment step (CFX96 BIO-RAD)



For 2 samples (82 and 1999), a positive PCR test was obtained without applying the enrichment step. For one of them (1999), an atypical amplification curve was obtained.
The result differences on the different thermocyclers may be due to the geographic testing arrangement. ADRIA ran the tests directly after the enrichment step on the BioRad CFX96, while CONGEN received the same samples in frozen condition and analyzed them on Roche LightCycler 480II, ABI 7500, MIC and Agilent AriaMx.

The comparison of the PCR results after 16 h of incubation and 72 h of storage shows that even after storage a positive PCR result was observed. For these tests two independent DNA extractions were prepared, one after 16 h and the other one after 72 h. For this reason, the obtained positive PCR results were probably not due to cross contamination during the extraction or the PCR step.

3.1.7 *PCR inhibitions*

PCR inhibitions in undiluted samples were observed for 48 samples; they are listed in Table 10.
This concerns following products: Dairy products, fresh vegetable ready to cook, Cocoa products, Aromatic herbs or spices, Egg products and Feed products. Some of these products are well known to potentially inhibit the

PCR reaction. According to the recommendations in the user manual, in these cases, dilutions were applied. Here, for 16 of the 48 samples, PCR results were obtained after a 1/2 dilution

In total, PCR inhibitions were observed, even after a 1/2 dilution, in 32 samples (in pink color in Table 10): 2 Dairy products, 7 Cocoa products, 12 Aromatic herbs or spices, 9 Egg products, 2 Feed products.

Table 10 - PCR inhibitions

Sample N°	Product	CFX96 BIO-RAD			ROCHE LightCycler 480II			Applied Biosystems 7500			MIC			Agilent AriaMX			Category	Type
		Result	Ratio before DNA dilution	Ratio after DNA dilution (1/2*) applied	Result	Ratio before DNA dilution	Ratio after DNA dilution (1/2*) applied	Result	Ratio before DNA dilution	Ratio after DNA dilution (1/2*) applied	Result	Ratio before DNA dilution	Ratio after DNA dilution (1/2*) applied	Result	Ratio before DNA dilution	Ratio after DNA dilution (1/2*) applied		
89	Infant formula	i/i*/-**	2/20	1/20	-	1/20	0/20	i/-*	3/20	0/20	-	0/20	0/20	i/-*	2/20	1/20	3	c
7442	**Infant formula**	i/+(20,03)*			i/21,5*			i/22,3*			24,8			i/24**				
8108	**Milk powder**	29,28			31,3			i/25,9*			30,3			33,5				
7836	**Mushrooms**	28,87	0/20	0/20	29,3	0/20	0/20	29,7	0/20	0/20	29,1	0/20	0/20	i/29,7*	1/20	0/20		a
6129	**Curry**	-	18/26	13/26	i/-*	19/26	10/26	i/-*	20/26	11/26	i/-**	14/26	11/26	i/-**	19/26	16/26	4	b
6130	**Chilli pepper**	i/-*/-**			-			i/-*			-			i/-*				
6131	**Turmeric**	NC/NC*/-**			i/-*			i/-**			-			i/-*				
6132	**Cocoa powder**	NC/-*			i/-*			i/-*			i/-**			i/-**				
6133	**Cocoa powder**	NC(i)/-*			i/-*			i/-*			-			i/-**				
6134	**Cocoa powder**	NC/i*/i**/NC***/-****			i/-*			i/-*			i/-**			i/-**				
6141	**Oregano**	NC(i)/i*/i**/-***			i/-*			i/-*			i/-*			i/-**				
6142	**Provence herbs**	NC(i)/i*/i**/i***/-****			i/-*			i/-*			i/-*			i/-**				
6143	**Tarragon**	NC(i)/-*			i/-*			i/-**			i/-**			i/-**				
7448	**Tarragon**	i/-*			i/-**			i/-*			i/-*			i/-**				
7449	**Cinnamon**	i/+(31,91)*			i/34,4*			i/35,6*			36,5			i/34,8*				
7451	**Cocoa powder**	i/i*/i**/+(24,68)***			i/24,2**			i/24,3**			i/23,1**			i/24**				
7452	**Cocoa powder**	i/i*/i**/+(23,84)***			i/24**			i/24,4**			i/23**			i/23,6**				
1726	**Cocoa powder 100%**	i/i*i/i**/i***/-****			i/-**			i/-**			i/-**			i/-**				
1727	**Cocoa powder**	i/-*			i/-**			i/-**			i/-**			i/-**				
1728	**Dehydrated oregano**	i/i*/-**			i/-**			i/-**			i/-**			i/-**				
1729	**Dehydrated Basil**	i/i*i/i**/i***/-****			i/-**			i/-****			i/-**			i/-**				
2599	**Basil**	i/i*/i**/-***/-****			i/-**			i/-****			i/-**			i/-**				
2600	**Thyme**	i/i*/i**/-***/-****/-****/-****			i/-**			i/-****			-			-				
2601	**Herbs of Provence**	i/i*/i**/i***/+(30,95)****			i/29***			i/32,6**			30,5			i/30,9***				
70	Pasteurized liquid egg yolk	NC/NC/NC*/-**	3/22	1/22	i/-**	9/22	4/22	i/-**	6/22	3/22	i/-**	7/22	3/22	i/-**	6/22	5/22	5	a
71	Pasteurized liquid egg yolk	NC/NC/NC*/-**			i/-**			i/-**			i/-**			i/-**				
72	Pasteurized liquid egg yolk	NC/NC/NC*/-**			i/-**			i/-**			i/-*			i/-**				
73	Pasteurized whole liquid egg	-			i/-*			-			i/-*			-				
74	Pasteurized whole liquid egg	NC/-			-			-			i/-*			-				
75	Pasteurized whole liquid egg	NC/-			i/-*			i/-*			i/-*			i/-*				
79	Pasteurized liquid egg yolk	i/i*/i**/-***			i/-**			-			i/-**			i/-**				
722	**Pasteurized liquid egg yolk**	i/+(41,79)/+(32,33)*/+(31,88)*			i/30,84**			i/29*			31,1			33,2				
6300	**Pasteurized liquid egg yolk**	19,62			i/21,15*			i/17,2*			27,1			i/20,91*				
6301	**Pasteurized liquid egg yolk**	23,34			i/24,06*			28,2			28,1			27,8				
2000	Egg yolk powder	i/-*	1/20	0/20	i/-**	4/20	4/20	i/-*	4/20	3/20	i/-**	1/20	1/20	i/-**	1/20	1/20		b
7282	**Egg yolk powder**	28,22			i/27,27**			i/27,7**			28,8			32,4				
7287	**Egg yolk powder**	24,65			i/23,24**			i/22,8**			27,1			32,6				
7290	**Egg yolk powder**	26,17			i/24,73**			i/24**			25,1			28,9				
6287	**Chocolate mousse**	-	0/20	0/20	i/-*	2/20	0/20	-	0/20	0/20	-	0/20	0/20	i/-*	1/20	0/20		c
7294	**Preparation for dessert**	24,18			i/26,2*			25			25,6			23,2				
86	Pellets for bovine	NC/-	0/21	0/21	i/-**	1/21	1/21	i/-*	1/21	0/21	i/-*	1/21	0/21	i/-*	2/21	0/21	6	b
1574	Pellets for cattles	-			-			-			-			i/-*				
6495	Soya	i/+(35,72)*	3/20	0/20	i/38,51*	4/20	0/20	i/34*	4/20	1/20	34,3	0/20	1/20	i/38,5*	4/20	0/20		c
6496	Dehydrated proteins	27,02			i/29,5*			i/25,4*			26,4			i/29,6*				
6497	Dehydrated proteins	-			i/-*			i/-*			-			i/-*				
6498	Dehydrated proteins	23,26			i/25,83*			i/28,9**			24			i/24,6*				
100	Dehydrated proteins	i/+(26,52)*			27,9			27,4			26,5			25,5				
101	Dehydrated proteins	i/+(22,75)*			24,4			24,6			24			23				
TOTAL		/	27	15	/	40	19	/	38	18	/	23	16	/	36	23	/	/

Dilution rate: *1/2, **1/5, ***1/10, ****1/50

For all thermocyclers (CFX96, Roche LightCycler 480II, Applied Biosystems 7500, MIC, Agilent AriaMx), the majority of inhibitions concern:

- Fresh herbs, cocoa, spices and aromates: 73.1 % of the samples tested showed inhibition on all cyclers tested;
- Egg products: 14.5 % of the analyzed samples showed inhibition on all cyclers included in this study.

3.1.8 *Discordant results*

Negative deviations are listed in Table 11.

Seven negative deviations were observed using the CFX96 from Bio-Rad. For 2 samples (723 and 6491), positive PCR results were observed with the 4 other thermocylers tested.

PCR replicates were run using the CFX96 from Bio-Rad for the 7 samples. For 2 of them, positive PCR results were then obtained (234 and 238).
5 samples were artificially contaminated and 2 naturally contaminated with *Salmonella* Typhimurium.

As the reference and the alternative methods have the same enrichment broths, the negative deviations were probably due to the fact that the detection level of the alternative method was not reached.

Positive deviations are listed in Table 12.

2 positive deviations were obtained with all the tested thermocyclers. The positive PCR results were confirmed by proceeding to inoculation of 5 MSRV plates before streaking onto selective agar plates. These samples concern one artificially contaminated sample (*Salmonella* Bredeney) and one naturally contaminated sample.

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Table 11 - Negative deviations

Sample N°	Product	Artificial contaminations		Reference method: ISO 6579♦	Alternative method : SureFast® Salmonella ONE method																	Category	Type
					PCR					Confirmation	Final result					Agreement ISO method / Alternative method							
		Strain	Inoculation level CFU/ test portion		CFX BIORAD	ROCHE LightCycler 480II	Applied Biosystems 7500	MIC	Agilent AriaMx		CFX96 BIORAD	ROCHE LightCycler 480II	Applied Biosystems 7500	MIC	Agilent AriaMx	CFX96 BIORAD	ROCHE LightCycler 480II	Applied Biosystems 7500	MIC	Agilent AriaMx		
723	**RTC (turkey meat)**	S. Derby Ad1337	5,4	+	-/-/-	35,7	31,3	38,1	36,8	+	-	+	+	+	+	**ND**	**PA**	**PA**	**PA**	**PA**	1	b
725	**RTC (chicken meat)**	S. Virchow 647	1,2	+	-/-/-	-	-	-	-	-	-	-	-	-	-	**ND**	**ND**	**ND**	**ND**	**ND**	1	b
6491	Ham	/	/	+ (S. Typhimurium)	-/-/-	36,2	37,4	34,9	37,3	+	-	+	+	+	+	**ND**	**PA**	**PA**	**PA**	**PA**	2	b
7477	Raw pork meat	/	/	+ (S. Typhimurium)	-(T)/-/-	-	-	-	-	-	-	-	-	-	-	**ND**	**ND**	**ND**	**ND**	**ND**	2	a
2600	**Thyme**	S.Caracas Ad2322	4,0	+	i/i*/i**/-***/-****/-****/-****	-	-	-	-	-	-	-	-	-	-	**ND**	**ND**	**ND**	**ND**	**ND**	4	b
234	**Feed for bovine**	S.Montevideo Ad2421	2,0	+	-/+(40,19)/+(35,09)	-	-	-	-	+	-	-	-	-	-	**ND**	**ND**	**ND**	**ND**	**ND**	6	b
238	**Feed for ducks**	S.Senftenberg Ad2418	3,2	+	-/+(35,95)/-	-	-	-	-	+	-	-	-	-	-	**ND**	**ND**	**ND**	**ND**	**ND**	6	b

Table 12 - Positive deviations

Sample N°	Product	Artificial contaminations		Reference method: ISO 6579♦	Alternative method : SureFast® Salmonella ONE method																	Category	Type
					PCR					Confirmation	Final result					Agreement ISO method / Alternative method							
		Strain	Inoculation level CFU/ test portion		CFX BIORAD	ROCHE LightCycler 480II	Applied Biosystems 7500	MIC	Agilent AriaMx		CFX BIORAD	ROCHE LightCycler 480II	Applied Biosystems 7500	MIC	Agilent AriaMx	CFX BIORAD	ROCHE LightCycler 480II	Applied Biosystems 7500	MIC	Agilent AriaMx		
7405	**RTE poultry (salad manchester)**	S.Bredeney Ad2042	1,2	-	+(24,91)	25,6	36,5	25,3	26,8	+ (MSRVx5)	+	+	+	+	+	**PD**	**PD**	**PD**	**PD**	**PD**	1	c
7068	Raw milk cheese	/	/	-	+(38,1)/+(36,24)/+(34,54)	39,4	37,8	35,9	36,3	+ (MSRVx5)	+	+	+	+	+	**PD**	**PD**	**PD**	**PD**	**PD**	3	b

♦ Analyses performed according to the COFRAC accreditation

The analysis of discordant results according to ISO 16140-2:2016 for a paired study is given in Table 13.

Table 13 - Interpretation of the sensitivity study results (paired study)

Table 13a - Bio-Rad CFX96

Category		Type		PD	ND [1]	ND-PD	AL	ND + PD	AL
1	Poultry meat products	a	Raw, frozen	0	0				
		b	RTC	0	2				
		c	RTE and RTRH	1	0				
			Total	1	2	1	3	3	6
2	Meat Products	a	Raw, Frozen, Seasoned	0	1				
		b	RTC	0	1				
		c	RTE and RTRH	0	0				
			Total	0	2	2	3	2	6
3	Dairy products	a	Pasteurized	0	0				
		b	Raw	1	0				
		c	Powder, ingredients	0	0				
			Total	1	0	-1	3	1	6
4	Vegetables	a	RTC	0	0				
		b	Fresh herbs, spices	0	1				
		c	RTE	0	0				
			Total	0	1	1	3	1	6
5	Egg products	a	Pasteurized	0	0				
		b	Powder	0	0				
		c	Egg products	0	0				
		Total		0	0	0	3	0	6
6	Feed products	a	Pet	0	0				
		b	Cattle	0	2				
		c	Raw material	0	0				
			Total	0	2	2	3	2	6
All categories				**2**	**7**	**5**	**6**	**9**	**16**

[1] ND: including PPND

Table 13b - Roche LightCycler® 480 II

Category		Type		PD	ND	ND-PD	AL	ND + PD	AL
1	Poultry meat products	a	Raw, frozen	0	0				
		b	RTC	0	1				
		c	RTE and RTRH	1	0				
			Total	1	1	0	3	2	6
2	Meat Products	a	Raw, Frozen, Seasoned	0	1				
		b	RTC	0	0				
		c	RTE and RTRH	0	0				
			Total	0	1	1	3	1	6
3	Dairy products	a	Pasteurized	0	0				
		b	Raw	1	0				
		c	Powder, ingredients	0	0				
			Total	1	0	-1	3	1	6
4	Vegetables	a	RTC	0	0				
		b	Fresh herbs, spices	0	1				
		c	RTE	0	0				
			Total	0	1	1	3	1	6
5	Egg products	a	Pasteurized	0	0				
		b	Powder	0	0				
		c	Egg products	0	0				
			Total	0	0	0	3	0	6
6	Feed products	a	Pet	0	0				
		b	Cattle	0	2				
		c	Raw material	0	0				
			Total	0	2	2	3	2	6
All categories				**2**	**5**	**3**	**6**	**7**	**16**

Table 13c - Applied Biosystems 7500

Category		Type		PD	ND	ND-PD	AL	ND + PD	AL
1	Poultry meat products	a	Raw, frozen	0	0				
		b	RTC	0	1				
		c	RTE and RTRH	1	0				
			Total	1	1	0	3	2	6
2	Meat Products	a	Raw, Frozen, Seasoned	0	1				
		b	RTC	0	0				
		c	RTE and RTRH	0	0				
			Total	0	1	1	3	1	6
3	Dairy products	a	Pasteurized	0	0				
		b	Raw	1	0				
		c	Powder, ingredients	0	0				
			Total	1	0	-1	3	1	6
4	Vegetables	a	RTC	0	0				
		b	Fresh herbs, spices	0	1				
		c	RTE	0	0				
			Total	0	1	1	3	1	6
5	Egg products	a	Pasteurized	0	0				
		b	Powder	0	0				
		c	Egg products	0	0				
			Total	0	0	0	3	0	6
6	Feed products	a	Pet	0	0				
		b	Cattle	0	2				
		c	Raw material	0	0				
			Total	0	2	2	3	2	6
All categories				**2**	**5**	**3**	**6**	**7**	**16**

Table 13d - MIC

Category		Type		PD	ND	ND-PD	AL	ND + PD	AL
1	Poultry meat products	a	Raw, frozen	0	0				
		b	RTC	0	1				
		c	RTE and RTRH	1	0				
			Total	1	1	0	3	2	6
2	Meat Products	a	Raw, Frozen, Seasoned	0	1				
		b	RTC	0	0				
		c	RTE and RTRH	0	0				
			Total	0	1	1	3	1	6
3	Dairy products	a	Pasteurized	0	0				
		b	Raw	1	0				
		c	Powder, ingredients	0	0				
			Total	1	0	-1	3	1	6
4	Vegetables	a	RTC	0	0				
		b	Fresh herbs, spices	0	1				
		c	RTE	0	0				
			Total	0	1	1	3	1	6
5	Egg products	a	Pasteurized	0	0				
		b	Powder	0	0				
		c	Egg products	0	0				
			Total	0	0	0	3	0	6
6	Feed products	a	Pet	0	0				
		b	Cattle	0	2				
		c	Raw material	0	0				
			Total	0	2	2	3	2	6
All categories				**2**	**5**	**3**	**6**	**7**	**16**

Table 13e - Agilent AriaMx

Category		Type		PD	ND	ND-PD	AL	ND + PD	AL
1	Poultry meat products	a	Raw, frozen	0	0				
		b	RTC	0	1				
		c	RTE and RTRH	1	0				
			Total	1	1	0	3	2	6
2	Meat Products	a	Raw, Frozen, Seasoned	0	1				
		b	RTC	0	0				
		c	RTE and RTRH	0	0				
			Total	0	1	1	3	1	6
3	Dairy products	a	Pasteurized	0	0				
		b	Raw	1	0				
		c	Powder, ingredients	0	0				
			Total	1	0	-1	3	1	6
4	Vegetables	a	RTC	0	0				
		b	Fresh herbs, spices	0	1				
		c	RTE	0	0				
			Total	0	1	1	3	1	6
5	Egg products	a	Pasteurized	0	0				
		b	Powder	0	0				
		c	Egg products	0	0				
			Total	0	0	0	3	0	6
6	Feed products	a	Pet	0	0				
		b	Cattle	0	2				
		c	Raw material	0	0				
			Total	0	2	2	3	2	6
All categories				**2**	**5**	**3**	**6**	**7**	**16**

The observed values for ND - PD and ND + PD for the 6 individual categories and for all categories for each of the thermocyclers tested meet the Acceptability Limits (observed values ≤ AL).

3.1.9 *Enrichment broth storage*

The initial enrichment (BPW) storage was done at 5°C ± 3°C for 72 hrs and tested again with the alternative method (PCR and confirmations).
219 positive samples were tested again; 12 changes were observed (see Table 14).

Table 14 - Observed changes in results before and after storage of the enrichment broth

Sample N°	Result before storage					Result after storage				
	Bio-Rad CFX96	Roche LightCycler® 480 II	Applied Biosystems 7500	MIC	Agilent AriaMx	Bio-Rad CFX96	Roche LightCycler® 480 II	Applied Biosystems 7500	MIC	Agilent AriaMx
6425 bis	PA	PA	PA	PA	PA	ND	ND	ND	ND	ND
6491	ND	PA	PA	PA	PA	PA	PA	PA	PA	PA
6750	PA	PA	PA	PA	PA	ND	PA	PA	PA	PA
7449	PA	PA	PA	PA	PA	PPND	PPND	PPND	PPND	PPND
7477	ND	ND	ND	ND	ND	PA	PA	PA	PA	PA
234	ND	ND	ND	ND	ND	PA	PA	PA	PA	PA
238	ND	ND	ND	ND	ND	PA	PA	PA	PA	PA
242	PA	PA	PA	PA	PA	PPND	ND	ND	ND	ND
722	PA	PA	PA	PA	PA	ND	PA	PA	PA	PA
723	ND	PA	PA	PA	PA	ND	ND	ND	ND	ND
724	PA	PA	PA	PA	PA	ND	ND	ND	ND	ND
2600	ND	ND	ND	ND	ND	PA	PA	PA	PA	PA

The analysis of discordant results regarding the storage of the enrichment broth is given in Table 15.

Table 15 - Interpretation of the sensitivity study results after storage of the enrichment broth (paired study)

Table 15a - BioRad CFX 96

Category		Positive deviations (PD)	Negative Deviations (ND[1])	ND-PD	Acceptability Limit (AL)	ND+PD	Acceptability Limit (AL)
1	Poultry meat products	1	3	2	3	4	6
2	Meat Products	0	1	1	3	1	6
3	Dairy products	1	0	-1	3	1	6
4	Vegetables	0	1	1	3	1	6
5	Egg products	0	1	1	3	1	6
6	Feed products	0	2	2	3	2	6
Total		**2**	**8**	**6**	**6**	**10**	**16**

[1] ND: including PPND

Table 15b - Roche LightCycler® 480 II

Category		Positive deviations (PD)	Negative Deviations (ND[1])	ND-PD	Acceptability Limit (AL)	ND+PD	Acceptability Limit (AL)
1	Poultry meat products	1	3	2	3	4	6
2	Meat Products	0	0	0	3	0	6
3	Dairy products	1	0	-1	3	1	6
4	Vegetables	0	1	1	3	1	6
5	Egg products	0	0	0	3	0	6
6	Feed products	0	3	3	3	3	6
Total		**2**	**7**	**5**	**6**	**9**	**16**

Table 15c - Applied Biosystems 7500

Category		Positive deviations (PD)	Negative Deviations (ND[1])	ND-PD	Acceptability Limit (AL)	ND+PD	Acceptability Limit (AL)
1	Poultry meat products	1	3	2	3	4	6
2	Meat Products	0	0	0	3	0	6
3	Dairy products	1	0	-1	3	1	6
4	Vegetables	0	1	1	3	1	6
5	Egg products	0	0	0	3	0	6
6	Feed products	0	3	3	3	3	6
Total		**2**	**7**	**5**	**6**	**9**	**16**

Table 15d - MIC

Category		Positive deviations (PD)	Negative Deviations (ND[1])	ND-PD	Acceptability Limit (AL)	ND+PD	Acceptability Limit (AL)
1	Poultry meat products	1	3	2	3	4	6
2	Meat Products	0	0	0	3	0	6
3	Dairy products	1	0	-1	3	1	6
4	Vegetables	0	1	1	3	1	6
5	Egg products	0	0	0	3	0	6
6	Feed products	0	3	3	3	3	6
Total		**2**	**7**	**5**	**6**	**9**	**16**

Table 15e - Agilent AriaMx

Category		Positive deviations (PD)	Negative Deviations (ND[1])	ND-PD	Acceptability Limit (AL)	ND+PD	Acceptability Limit (AL)
1	Poultry meat products	1	3	2	3	4	6
2	Meat Products	0	0	0	3	0	6
3	Dairy products	1	0	-1	3	1	6
4	Vegetables	0	1	1	3	1	6
5	Egg products	0	0	0	3	0	6
6	Feed products	0	3	3	3	3	6
Total		**2**	**7**	**5**	**6**	**9**	**16**

The observed values for ND - PD and ND + PD for the 6 individual categories and for all categories for each of the thermocyclers tested meet the Acceptability Limits (observed values ≤ AL).

3.2 Relative level of detection study

The relative level of detection is the level of detection at P = 0,50 (LOD_{50}) of the alternative method divided by the level of detection at P = 0,50 (LOD_{50}) of the reference method.

3.2.1 *Categories, sample types and strains*

One sample type and one relevant target micro-organism for this sample type was chosen for each of the Categories in this validation study, as shown in Table 16.

Table 16 - List of selected types and strains per category, as tested within the relative level of detection study

Category		Matrix	Strain and reference number	Strain origin	Storage conditions prior to analysis	Seeding or spiking procedure
1	Poultry meat products	Frozen poultry meat	*S.* Derby Ad 1337	Chicken meat	2 weeks a -20°C	Seeding
2	Meat Products	Ground beef	*S.* Typhimurium A00C060	Ground beef	48 h at 2 – 8°C	Seeding
3	Dairy products	Raw milk	*S.* Ohio Ad 1482	Raw milk	48 h at 2 – 8°C	Seeding
4	Vegetables	Fresh spinach	*S.* Virchow Ad 1721	Cereals	48 h at 2 – 8°C	Seeding
5	Egg products	Pasteurized whole egg	*S.* Enteritidis 657	Whole egg	Heat stressed strain and 48 h at 2 – 8°C	Spiking
6	Feed products	Pet food (croquettes)	*S.* Derby Ad 1500	Feed product	Lyophilisation, storage 2 weeks at ambient temperature	Seeding

3.2.2 *Test sample preparations*

Three levels of artificial contamination were prepared for each type:

- Negative control level: One non-inoculated in order to get 5 test portions,
- Low level: One inoculated between 0.4 and 1 CFU/sample in order to get 20 test portions providing fractional recovery,
- Higher level: One inoculated between 0.7 and 2.8 CFU/sample in order to get 5 test portions contaminated at a higher level.

Test portions were bulk inoculated and kept at an appropriate time/temperature for stabilization before testing (see Table 16).

3.2.3 *RLOD study results*

The RLOD calculations were performed using the Excel spread sheet (clause 5-1-4-2 Calculation and interpretation of RLOD) version 06.07.2015) of the international standard as described in ISO 16140-2: 2016.

The RLOD per category is given in Table 17.

Table 17 - RLOD

	Name	RLOD	RLODL	RLODU	b=ln (RLOD)	sd(b)	z-Test statistic	p-value
RLOD using the alternative method results	Frozen poultry meat	1,208	0,394	3,701	0,189	0,560	0,337	0,736
	Ground beef	1,000	0,473	2,113	0,000	0,374	0,000	1,000
	Egg product	0,805	0,337	1,920	-0,217	0,435	0,499	1,382
	Pellets	0,719	0,243	2,131	-0,329	0,543	0,606	1,456
	Fresh spinach	1,000	0,462	2,167	0,000	0,387	0,000	1,000
	Raw milk-CFX	1,136	0,526	2,452	0,128	0,385	0,332	0,740
	Raw milk-OTHER THERMOCYCLERS	1,241	0,563	2,739	0,216	0,396	0,546	0,585
	Combined	1,016	0,751	1,374	0,016	0,151	0,105	0,916
RLOD using the confirmed alternative method results	Frozen poultry meat	1,208	0,394	3,701	0,189	0,560	0,337	0,736
	Ground beef	1,000	0,473	2,113	0,000	0,374	0,000	1,000
	Egg product	1,000	0,406	2,463	0,000	0,451	0,000	1,000
	Pellets	1,000	0,363	2,751	0,000	0,506	0,000	1,000
	Fresh spinach	1,000	0,462	2,167	0,000	0,387	0,000	1,000
	Raw milk-CFX BIORAD	1,427	0,642	3,174	0,356	0,400	0,890	0,373
	Raw milk-OTHER THERMOCYCLERS	1,241	0,563	2,739	0,216	0,396	0,546	0,585
	Combined	1,109	0,816	1,507	0,103	0,153	0,672	0,502

For 2 matrices (pasteurised whole egg and pet food), positive PCR results were not confirmed. This explains the difference observed between the RLOD values before and after confirmation.

For raw milk matrix, the presence of *Salmonella* spp. in the enriched BPW was confirmed whereas PCR tests were negative using the CFX96 from Bio-Rad (samples n° 2863, 2871 and 3445). Note that for one sample (3445), positive PCR results were obtained using the other cyclers. This explains the different RLOD values obtained for Raw milk between the cyclers.

3.2.4 *Conclusion RLOD study*

The RLOD values (using the confirmed alternative method results) meet the acceptability limit, which is 1.5 for paired studies, for all the categories tested and for the 5 thermocyclers.

3.3 Inclusivity/exclusivity study

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 *Protocols*

Inclusivity: *Salmonella* strains cultures were performed in BHI medium at 37°C. Dilutions were made in order to inoculate 10 cells/225 ml in BPW broth. The enrichment broth was incubated for 16 h at 37°C ± 1°C. The alternative method protocol was then performed.

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

3.3.2 *Results inclusivity and exclusivity study*

A total of 100 strains were tested for **inclusivity**. All the strains showed the expected positive result.

A total of 30 strains were tested for **exclusivity**. All the strains showed the expected negative result.

3.3.3 *Conclusion inclusivity and exclusivity study*

The alternative SureFast® Salmonella ONE detection method is selective and specific.

3.4 Conclusions method comparison study

Overall, the conclusions for the Method Comparison Study are:

The observed values for ND - PD and ND + PD for the 6 individual categories and for all categories for each of the thermocyclers tested meet the Acceptability Limits (observed values ≤ AL).

Depending on the thermocycler used, the percentage values of false positive results are: 19.4% for the egg products category using the CFX 96 from BioRad and 8.1% for the meat category using the other cyclers.

For all thermo cyclers, the majority of inhibitions concern:

- Fresh herbs, cocoa, spices and aromates;
- Egg products.

The RLOD values (using the confirmed alternative method results) meet the acceptability limit, which is 1.5 for paired studies, for all the categories tested and the 5 thermocyclers tested.

The alternative SureFast® Salmonella ONE detection method is selective and specific.

It is possible to store the primary enrichment broth for 72 h at 5 ± 3°C.

4 INTER-LABORATORY STUDY

4.1 Study organisation

- *Collaborators number*

13 laboratories took part in the study. Lab K cancelled their participation due to illness and lack of staff.

- *Matrix and strain used*

Ground beef was contaminated by *Salmonella* Typhimurium A00C060 strain isolated from beef.

- *Samples*

Samples were prepared and individually inoculated on Monday 17 July 2017, as described below:

- 24 blind coded samples (25 g) for analysis of *Salmonella* by the SureFast® Salmonella ONE method and by the ISO 6579 (2002) reference method
- 1 sample labeled “Sample for total count enumeration” for aerobic mesophilic flora enumeration by ISO 4833-1 method,
- 1 water flask labelled “Temperature Control” with a temperature probe.

- *Inoculation*

The targeted inoculation levels were the following:

- Level 0: 0 CFU/25 g
- Level 1: inoculation level close to the RLOD in order to provide as much fractional positive recovery data as possible
- Level 2: 8 CFU/25 g

- *Labelling and shipping*

Blind coded 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 to 48 h to the participating laboratories. The temperature conditions had to stay lower or equal to 8°C during transport, and between 0°C – 8°C in the labs.

- *Analyses*

Collaborative study laboratories and the expert laboratory carried out the analyses on Wednesday 19 July 2017.

4.2 Experimental parameters controls

4.2.1 *Strain stability and background microflora stability*

Strain stability was checked by inoculating the matrix at 2.3 CFU/25 g and 1000 CFU/ g. Enumerations were performed for the high contamination level and detection analyses were performed for the low contamination level after 24 h and 48 h storage at 5°C ± 3°C. *Triplicates* were analysed. The aerobic mesophilic flora was also enumerated; the results are given in Table 18.

Table 18 - Sample stability

Day	Reference method (detection)			CFU/g			Aerobic mesophilic flora (CFU/g)
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3	
Day 0	+	+	+	1500	2100	3000	$2.4\ 10^3$
Day 1	+	+	+	1000	1500	1200	$4.6\ 10^4$
Day 2	+	+	+	1900	2300	900	$6.3\ 10^5$

No evolution was observed during storage at 5°C ± 3°C, except for the aerobic mesophilic flora.

4.2.2 *Contamination levels*

The enrichment of the inoculum was done the day of the inoculation.

The contamination levels and the sample codification were the following (see **Table 19**).

Table 19 - Contamination levels

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	3-5-7-12-13-17-19-24	/	/	/	/
Level 1	2-4-9-10-14-16-21-23	1.6	2.3	1.9	2.8
Level 2	1-6-8-11-15-18-20-22	8	11.1	9.2	13.3

4.2.3 *Logistic conditions*

Temperature conditions are given in Table 20.

Table 20 - Sample temperatures upon receipt

Collaborators	Temperature measured by the probe (°C)	Temperature measured at receipt (°C)	Receipt date and time	Analysis date
A	16.3	12.3	Day 2 9h45	Day 2
B	9.0	13.1	Day 2 9h39	Day 2
C	13.0	14.0	Day 2 12h30	Day 2
D	10.8	13.6	Day 2 10h32	Day 2
E	12.9	14.5	Day 2 13h00	Day 2
F	15.3	17.4	Day 2 13h45	Day 2
G	20.5	20.5	Day 3 8h08	Day 3
H	11.7	12.0	Day 2 8h57	Day 2
I	9.2	10.4	Day 2 11h20	Day 2
J	Probe failed	14.2	Day 2 12h54	Day 2
L	Probe failed	10.0	Day 2 9h50	Day 2
M	18.0	17.9	Day 2 15h00	Day 2

A problem was encountered during the transport for all the packages: the carrier was not able to deliver the packages in time or in good condition due to internal organizational problems (cyber attack).

The samples were received by the Labs on Wednesday 19 July and on Thursday 20 July for Lab G.

The temperature measured at receipt and by the probe was above 8°C for all the labs.

Table 21 shows the time during which the samples remained at a temperature above 8°C.

Table 21 – Package temperature

Collaborators	Storage time above 8°C (hours)
A	2.0
B	4.5
C	12.0
D	7.0
E	8.0
F	12.5
H	7.0
I	1.2
J	Probe failed
L	Probe failed
M	14.5

A growth simulation of the strain according to the time/temperature conditions encountered during transport was carried out with predictive software tool “Sym’Previus” https://symprevius.eu/fr/ for the lab G which received the samples in the worst conditions (Day 3 at 20.5°C)**.** Depending on the temperature, the target level rose from 2.0 CFU/25 g to 3.5 CFU/25 g.

Table 22 – Contamination level on Day 2

Time (Day)	Units	Median	Quantile 5%	Quantile 95%
2	Log CFU/g	-0.85	-1.04	-0.63
	CFU/g	0,14	0.09	0.23
	CFU/25g	3.5	2.25	5.75

For 2 labs (J and L) the probe failed during shipment but the temperatures measured at receipt were similar to those measured by the other labs, respectively 14.2 and 10°C. Based on the fact that fractional positive recovery was obtained for the low inoculation level as expected, it is proposed to keep the data and not run the inter-laboratory study again, even if the temperature was higher than expected.

4.3 Results analysis

4.3.1 *Expert laboratory results*

The results obtained by the expert laboratory are given in Table 23.

Table 23 – Expert laboratory results

Level	Reference method	Alternative method
L1	0/8	0/8
L2	7/8	7/8
L3	8/8	8/8

4.3.2 *Results observed by the collaborating laboratories*

Aerobic mesophilic flora enumeration

Depending on the Lab results, the enumeration levels varied from 4.6 10^4 CFU/g to 1.2 10^7 CFU/g.

Salmonella spp detection

12 collaborators participated in the study. The results obtained for the reference method are provided in Table 24 and for the alternative method in Table 25.

7 collaborators used the BioRad CFX 96 (A-B-C-E-G-I-M), 1 the MIC (L), 1 the AB 7500 Fast (F), 2 the Agilent Aria Mx (H-J) and one (D) the LightCycler 480 II.

Table 24 - Positive results by the reference method (ALL the collaborators)

Laboratory	Contamination level		
	L0	L1	L2
A	2	8	8
B	0	8	8
C	0	8	8
D	0	7	8
E	0	5	8
F	0	8	8
G*	0	7	8
H	0	8	8
I	0	8	8
J	0	8	8
L	0	7	8
M	0	8	8
Total	**2**	**90**	**96**

*Analysis at Day 3

Table 25 - Positive results (before and after confirmation) by the alternative methods (ALL the collaborators)

Laboratory	Contamination level					
	L0		L1		L2	
	Before confirmation	After confirmation	Before confirmation	After confirmation	Before confirmation	After confirmation
A	1	0	8	8	8	8
B	0	0	8	8	8	8
C	0	0	8	8	8	8
D	0	0	7	7	8	8
E	0	0	5	5	8	8
F	0	0	8	8	8	8
G*	0	0	7	7	8	8
H	0	0	8	8	8	8
I	0	0	8	8	8	8
J	0	0	8	8	8	8
L	0	0	7	7	8	8
M	0	0	8	8	8	8
Total	**1**	**0**	**90**	**90**	**96**	**96**

*Analysis at Day 3

Lab A obtained 1 positive PCR result not confirmed and two positive samples with the reference method on control samples. These results were probably due to cross-contaminations.
Lab G received the samples on Day 3, their results can't be kept for interpretation.
Lab D incubated the enrichment broth for 23 hours at 37°C ± 1°C.

It is proposed to keep the results from Lab D as the reference method and the alternative method are run on the same enrichment broth (paired study design) even if the bags were incubated longer than the maximum incubation time (20 h). We can consider that the maximum growth was reached before 20 hours incubation.

Based on the results observed, 2 Labs A and G were not retained for interpretation, the results from 10 collaborators were kept: B, C, D, E, F, H, I, J, L, M.

4.3.3 *Results of the collaborators retained for interpretation*

The results obtained with the 10 labs kept for interpretation are presented in Table 26 (reference method) and Table 27 (alternative method).

Table 26 - Positive results by the reference method
(Without Labs A and G)

Laboratory	Contamination level		
	L0	L1	L2
B	0	8	8
C	0	8	8
D	0	7	8
E	0	5	8
F	0	8	8
H	0	8	8
I	0	8	8
J	0	8	8
L	0	7	8
M	0	8	8
Total	**0**	**75**	**80**

Table 27 - Positive results (before and after confirmation)
by the alternative methods (Without Labs A and G)

Laboratory	Contamination level					
	L0		L1		L2	
	Before confirmation	After confirmation	Before confirmation	After confirmation	Before confirmation	After confirmation
B	0	0	8	8	8	8
C	0	0	8	8	8	8
D	0	0	7	7	8	8
E	0	0	5	5	8	8
F	0	0	8	8	8	8
H	0	0	8	8	8	8
I	0	0	8	8	8	8
J	0	0	8	8	8	8
L	0	0	7	7	8	8
M	0	0	8	8	8	8
Total	**0**	**0**	**75**	**75**	**80**	**80**

4.4 Calculation and interpretation

4.4.1 *Calculation of the specificity percentage (SP)*

The percentage specificities (SP) of the reference method and of the alternative method, using the data after confirmation, based on the results of level L0 are the following (See Table 28).

Table 28 - Percentage specificity

Specificity for the reference method	$SP_{ref} = \left(1 - \left(\frac{P_0}{N_-}\right)\right) x\ 100\ \% =$	100%
Specificity for the alternative method	$SP_{alt} = \left(1 - \left(\frac{CP_0}{N_-}\right)\right) x\ 100\ \% =$	100%

N: number of all L0 tests
P_0 = total number of false-positive results obtained with the blank samples before confirmation
CP_0 = total number of false-positive results obtained with the blank samples

4.4.2 *Calculation of the sensitivity (SE_{alt}), the sensitivity for the reference method (SE_{ref}), the relative trueness (RT) and the false positive ratio for the alternative method (FPR)*

Fractional positive results were obtained for the low inoculation levels (L1). This inoculation level was retained for calculation.

A summary of the results of the collaborators retained for interpretation, and obtained with the reference and the alternative methods for Level 1 is provided in Table 29.

Table 29 - Summary of the obtained results with the reference method and the alternative method for Level 1

Response	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement (A+/R+) **PA = 75**	Positive deviation (R-/A+) **PD = 0**
Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 0**	Negative agreement (A-/R-) **NA = 5**

Based on the data summarized in Table 29, the values of sensitivity of the alternative and reference methods, as well as the relative trueness and false positive ratio for the alternative method taking account the confirmations, are the following (See Table 30).

Table 30 - Sensitivity, relative trueness and false positive ratio percentages

Sensitivity for the alternative method:	$SE_{alt} = \frac{(PA+PD)}{(PA+PD+ND)} \; x \; 100\% =$	100 %
Sensitivity for the reference method:	$SE_{ref} = \frac{(PA+ND)}{(PA+PD+ND)} \; x \; 100\% =$	100 %
Relative trueness	$RT = \frac{(PA+NA)}{N} \; x \; 100\% =$	100 %
False positive ratio for the alternative method	$FPR = \frac{FP}{NA} \; x \; 100\% =$	0 %

4.4.3 *Interpretation of data*

There are no negative deviations and no positive deviations.

For a **paired study design**, the difference between (ND – PD) and the sum of (ND+PD) are calculated for the level where fractional recovery was obtained (L_1). The values found for (ND – PD) and (ND + PD) shall not be higher than the acceptability limits (AL).

The AL is not met when the observed value is higher than the AL.

The calculations are the following, according to the EN ISO 16140-2:2016 (See Table 31)

Table 31 - Calculations

	Calculated values	AL
(ND - PD)	0	3
(ND + PD)	0	4

4.4.4 *Evaluation of the RLOD between laboratories*

The RLOD was calculated using the EN ISO 16140-2:2016 Excel spreadsheet available at http://standards.iso.org/iso/16140 - RLOD (clause 5-1-4-2 Calculation and interpretation of RLOD) version 06.07.2015. The results are used only for information (see Table 32).

Table 32 - RLOD

Name	RLOD	RLODL	RLODU	b=ln(RLOD)	sd(b)	z-Test statistic	p-value
Inter-laboratory study	1,000	0,663	1,507	0,000	0,205	0,000	1,000

4.5 Conclusion of the Interlaboratory study

The data and interpretations comply with the EN ISO 16140-2:2016 requirements. **The SureFast® Salmonella ONE method is considered equivalent to the ISO standard.**

The observed values for ND – PD and ND + PD for the interlaboratory study (Level 1) meet the acceptability limits (observed values ≤ AL).

5 GENERAL CONCLUSIONS

The **method comparison study conclusions** are:

The observed values for ND - PD and ND + PD for the 6 individual categories and for all categories for each of the thermocyclers tested meet the Acceptability Limits (observed values ≤ AL).

Depending on the thermocycler used, the percentage values of false positive results are: 19.4% for the egg products category using the CFX 96 from BioRad and 8.1% for the meat category using the other cyclers.

For all thermocyclers, the majority of inhibitions concern:

- Fresh herbs, cocoa, spices and aromates;
- Egg products.

The RLOD values (using the confirmed alternative method results) meet the acceptability limit, which is 1.5 for paired studies, for all the categories tested and the 5 thermocyclers tested.

The alternative SureFast® Salmonella ONE detection method is selective and specific.

It is possible to store the primary enrichment broths for 72 h at 5 ± 3°C.

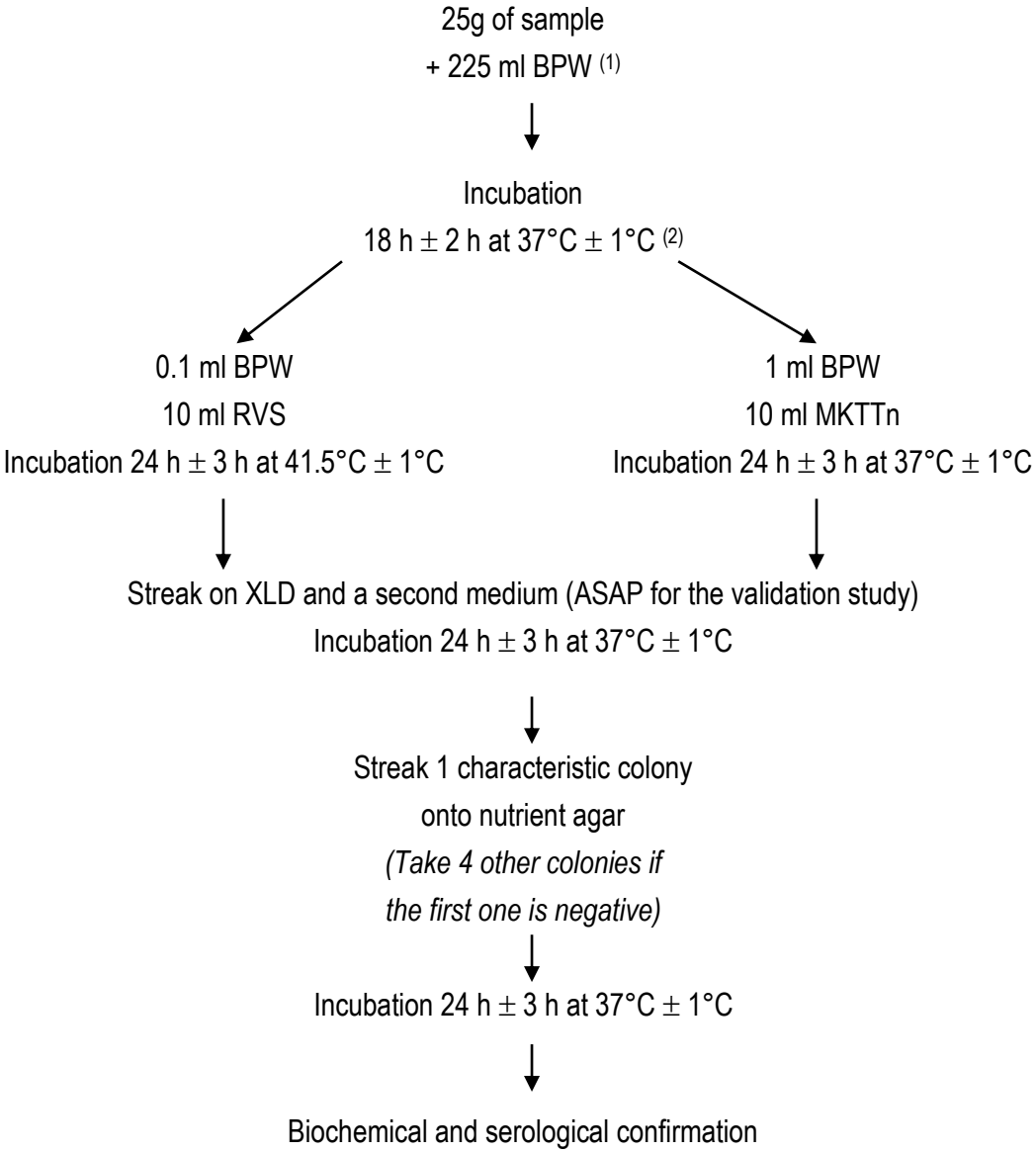
The **inter-laboratory study conclusions** are:

The data and interpretations comply with the EN ISO 16140-2:2016 requirements. **The SureFast® Salmonella ONE method is considered equivalent to the reference method ISO 6579 for the detection of *Salmonella* spp.**

Date, 18 December 2018

Maryse RANNOU
Project Manager
Validation of Alternative methods

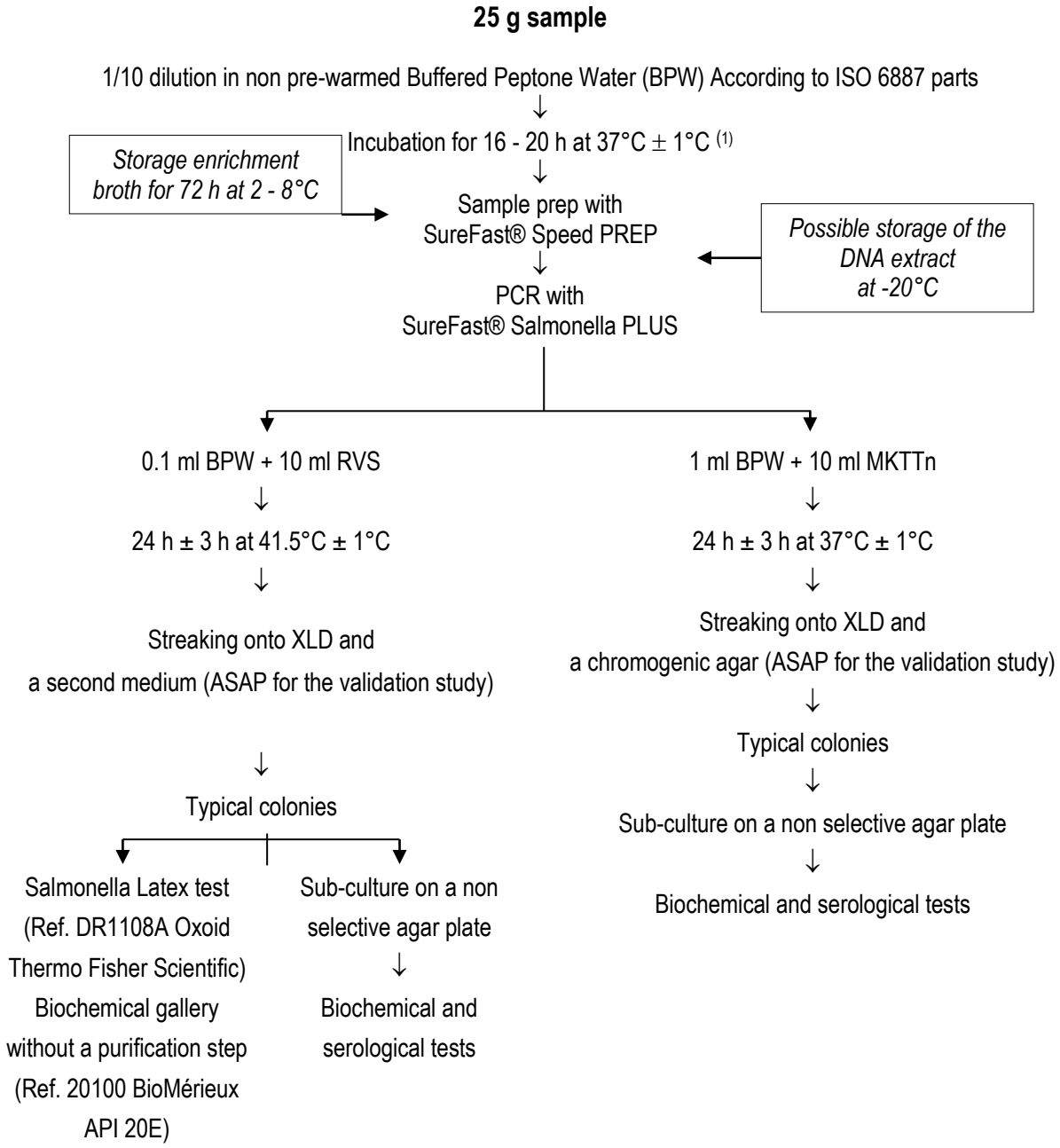
Annex A - Flow diagram of the reference method EN ISO 6579 (December 2002): Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp.



(1) For spices: BPW +5% K_2SO_3
For Milk powder with probiotics BPW 2x

(2) 16 h incubation time was tested during the validation study

Annex B - Flow diagram of the alternative method SureFast® Salmonella ONE method

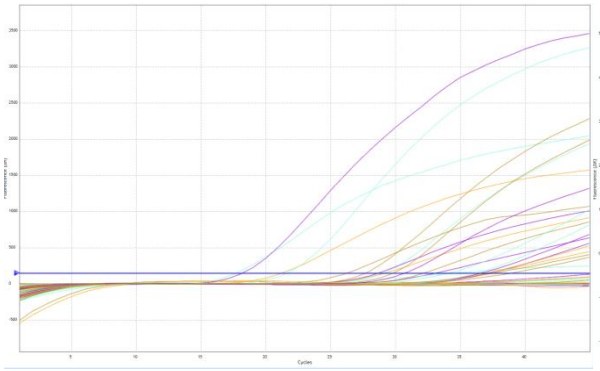


(1) 16 h incubation time was tested during the validation study

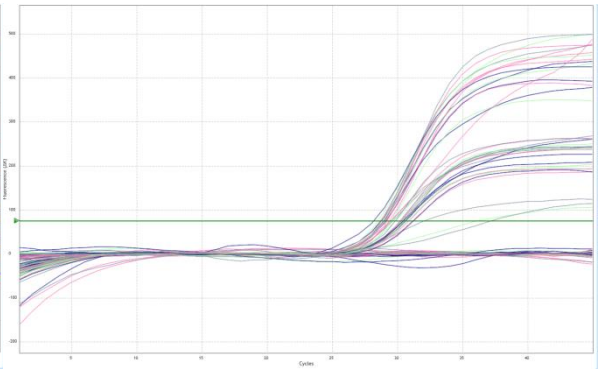
Annex C – Examples of curves for each cycler used

Cycler: Aria
Software version: Agilent Aria Software v1.4
Reference run (File name): 170614 Salmonella ONE WDH Lauf 2 Aria
Example curves:

FAM Chanel

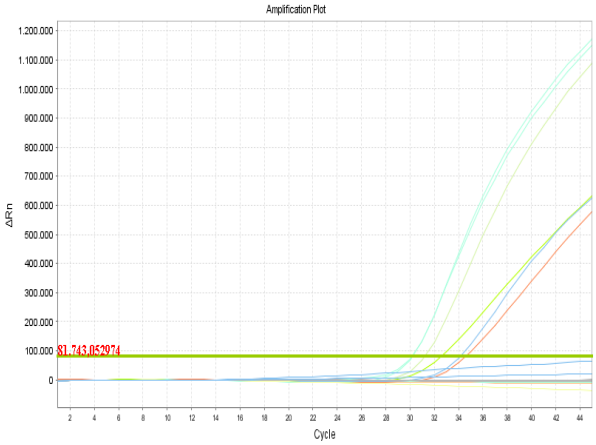


VIC Chanel

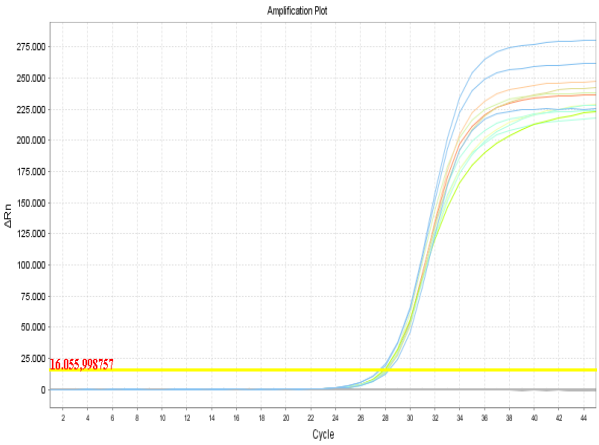


Cycler: ABI7500
Software version: 7500 Software v2.0.6
Reference run (File name): 170622 Salmo ONE Wdh ABI.eds
Example curves:

FAM Chanel



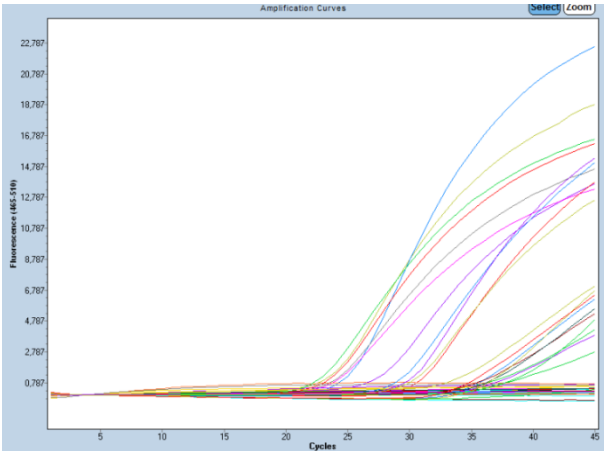
VIC Chanel



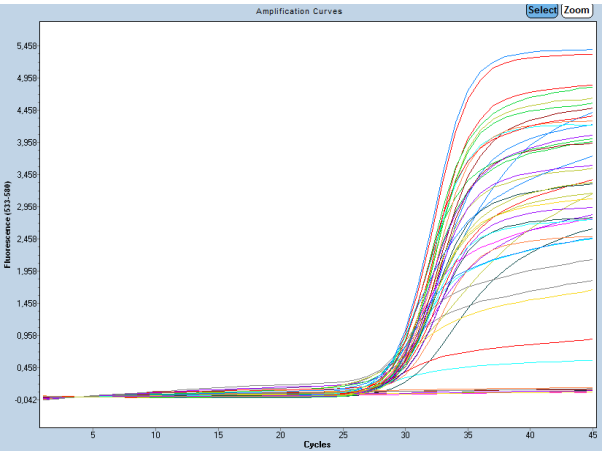
Cycler: ROCHE LC 480 II
Software version: LCS 480 1.5.1.6.2
Reference run (File name): 170620 Salmonella ONE Wdh LC480C

Example curves:

FAM Chanel



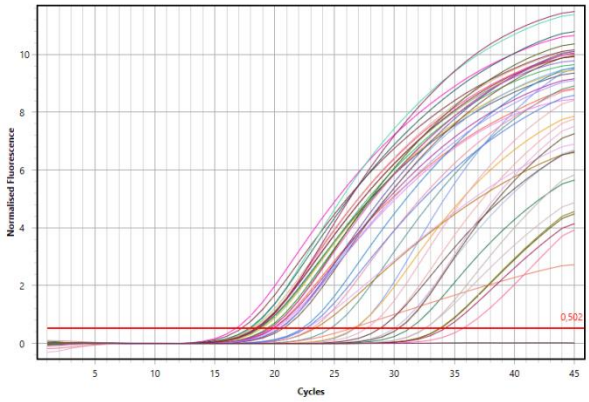
VIC Chanel



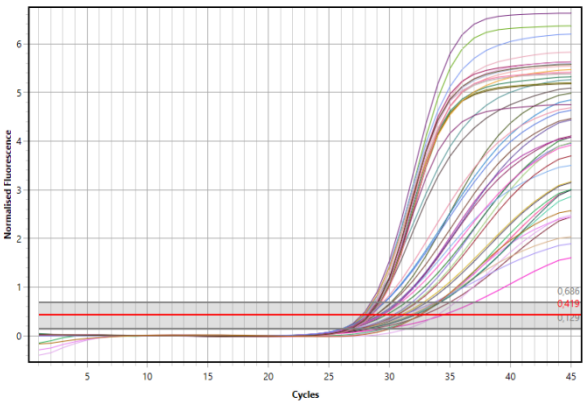
Cycler: MIC
Software version: micPCR v2.2.0
Reference run (File name): 170616 Salmo 397-440

Example curves:

FAM Chanel



VIC Chanel



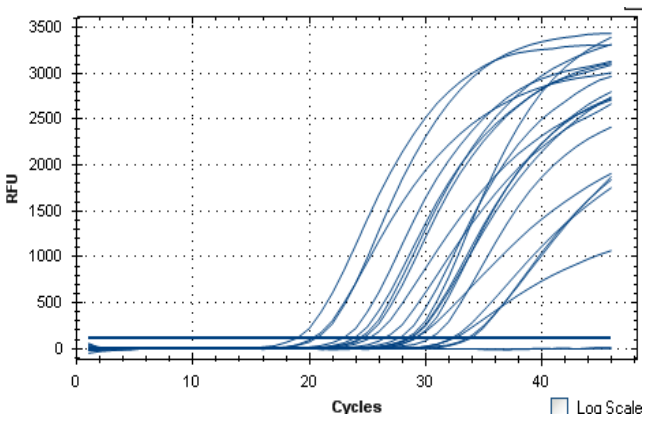
Cycler : CFX96 BIO-RAD

Software version : BIO-RAD CFX MANAGER 3.1

Reference run (File name): 20161209

Example curves:

FAM Chanel



VIC Chanel