ISO 16140-2:2016 validation study of foodproof® *Salmonella* spp. detection method for the detection of S*almonella* spp. in food, feed and primary production samples

- 2011LR40 | foodproof® *Salmonella* spp. automated protocol
- 2011LR42 | foodproof® *Salmonella* spp. method & vetproof® *Salmonella* spp. method, manual protocol

This document contains the following two validation reports:

- foodproof® *Salmonella* spp. detection method (foodproof *Salmonella* Detection Kit 5'Nuclease or hybridization probes)
- foodproof® *Salmonella* spp. Detection method - LyoKit - 5'Nuclease

Both reports contain the manual and the automated extraction.

ADRIA FOOD EXPERTISE

cofrac ESSAIS (TESTING)

ACCREDITATION
N°1-0144
scope available
on www.COFRAC.fr

ISO 16140-2:2016 validation study of foodproof® *Salmonella* spp. detection method for the detection of *Salmonella* spp. in food, feed and primary production samples

MicroVal study numbers	**2011LR40 and 2011LR42**
Method/Kit name	**foodproof® *Salmonella* spp. detection method (food**proof ***Salmonella*** **Detection Kit 5'Nuclease or hybridization probes)**
Study version	**Summary report – Version 1** 30 October 2019
MicroVal Expert Laboratory	ADRIA Développement ZA Creac'h Gwen F-29196 QUIMPER Cedex (France)

This report consists of 60 pages, including 2 appendices.
Only copies including the totality of this document are authorised.

Competencies of the laboratory are certified by COFRAC accreditation for the analyses marked with the symbol◆.

Standardized protocol - Qualitative methods

MICROVAL® NEN

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

Company	**BIOTECON Diagnostics GmbH** Hermannswerder 17 D-14473 Potsdam (Germany)
Expert Laboratory	**ADRIA Développement** ZA Creac'h Gwen F-29196 QUIMPER Cedex (France)
Method/Kit name	**foodproof® *Salmonella* spp. detection method (foodproof *Salmonella* Detection Kit 5'Nuclease or hybridization probes)**
Validation standard	☒ ISO 16140-1 (2016): Microbiology of the food chain — Method validation — *Part 1: Vocabulary* ☒ ISO 16140-2 (2016): Microbiology of the food chain — Method validation — *Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method*
Reference methods♦	☒ EN ISO 6579 (December 2002) - Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp. ☒ ISO 6579-1(February 2017) - Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* spp. - Part 1: detection of *Salmonella* spp
Scope of validation	☒ **Food categories including: Chocolate and bakery products, Meat and meat products, Milk and dairy products, Egg products, Beef meat 375 g** ☒ **Feed** ☒ **Primary production samples for manual extraction protocol**
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	Inter-laboratory Study
m	Minority level of target analyte
M	Majority level of target analyte
MCS	Method Comparison Study
min	minute
MKTTn	Muller-Kauffmann Tetrathionate-novobiocin broth
ml	Milliliter
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
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)
PPS	Primary production samples
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

1 INTRODUCTION

The **food**proof® *Salmonella* spp. detection method was validated on the 26th June 2013 for:

- Manual extraction protocol (**food**proof® StarPrep One Kit) (Certificate number: 2011LR42):
    - Chocolate and bakery products
    - Meat and meat products
    - Milk and dairy products
    - Egg products
    - Raw beef meat (375 g sample size)
    - Feed samples
    - Primary production samples

- Automated extraction protocol (**food**proof® Magnetic Preparation Kit I) (Certificate number: 2011LR40)
    - Meat and meat products
    - Milk and dairy products
    - Egg products
    - Raw beef meat (375 g sample size)
    - Feed samples.

The tests were run using 4 thermocyclers for the sensitivity study and RLOD. All the tests on the CFX 96 (Bio-Rad) were done at ADRIA. Blind-coded DNA extracts were sent to BIOTECON Diagnostics, which run the assays on the other thermocyclers. The results were analyzed by ADRIA. The following thermocycler / Detection kit pairs were tested:

- LightCycler 2.0 (Roche Diagnostics) / **food**proof® *Salmonella* Detection Kit - Hybridization Probes (LC 1.x, 2.0, 480 II) No R 310 27 (tests run by BIOTECON);
- LightCycler 480 (Roche Diagnostics) / **food**proof® *Salmonella* Detection Kit - Hybridization Probes (LC 1.x, 2.0, 480 II) No R 310 27 (tests run by BIOTECON);
- Stratagene Mx3005P (Agilent) / **food**proof® *Salmonella* Detection Kit - 5'Nuclease - No R 302 27 or R 302 27 L (test run by BIOTECON);
- CFX96 (Bio-Rad) / **food**proof® *Salmonella* Detection Kit - 5'Nuclease - No R 302 27 or R 302 27 L (tests run by ADRIA).

In May 2019, the renewal was obtained for the manual protocol, in September 2019, the renewal as well as an extension for the chocolate and bakery products category for the automated extraction protocol were obtained according to the ISO 16140-2:2016:

- **For the renewal study:**
  The protocol dedicated to primary production samples has changed; this category was tested again (sensitivity study and RLOD determination).
  Additional samples analyses were performed for the other categories in order to have the required number of samples in the sensitivity part.
  For the dairy products category, the RLOD was performed again as non-satisfying results were obtained with some cyclers for the initial validation study, results probably linked to the presence of DNAses in the lysates.

- **For the extension study:**
  The foodproof® ***Salmonella*** **Detection Kit - 5' Nuclease (CFX96), and the food**proof® ***Salmonella*** **Detection Kit - Hybridization Probes (LightCycler 480)** combined with an automated extraction protocol (**food**proof® Magnetic Preparation Kit I) was tested **for Chocolate and bakery products** in order to include this category in the scope for the automated extraction protocol. Note that for this study in agreement with the MVTC, only two thermocyclers were evaluated (CFX96 and Light cycler 480).

2 METHOD PROTOCOLS

2.1 Reference methods◆

The initial validation study was run using the ISO 6579 standard - Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp..

◆ Analysis performed according to the COFRAC accreditation

The renewal study was run using the ISO 6579-1 (February 2017): Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* spp. - Part 1: detection of *Salmonella* spp.

The same sample size was used for the reference method and the alternative method, i.e. 25 g or 375 g.

Samples preparation used in the reference and the alternative methods were done according to ISO6887 parts 1 to 5:

- Part 1: general rules for the preparation of the initial suspension and decimal dilutions;
- Part 2: specific rules for the preparation of meat and meat products;
- Part 3: specific rules for the preparation of fish and fishery products;
- Part 4: specific rules for the preparation of miscellaneous products;
- Part 5: specific rules for the preparation of milk and milk products.

The flow diagrams are given in **Annex A**.

2.2 Alternative method

The kit inserts are:

- **food**proof® **StarPrep One Kit: manual extraction protocol** No S 400 07 or S 400 07 L;
- **food**proof® **Magnetic Preparation Kit I: automated extraction protocol** No S 400 11 L;
- **food**proof® ***Salmonella*** **Detection Kit - 5'Nuclease -** No R 302 27 or R 302 27 L;
- **food**proof® ***Salmonella*** **Detection Kit - Hybridization Probes** (LC 1 .x, 2.0, 480 II) No R 310 27.

The **food**proof® *Salmonella* spp. method is a real-time PCR kit for *Salmonella* spp. detection in foods and feeds, as well as primary production samples (for manual extraction protocol).

After DNA extraction with the **food**proof® StarPrep One Kit (manual protocol), or the **food**proof® Magnetic Preparation Kit I (automated protocol), specific fluorescent oligonucleotide probes are used to detect target DNA during the amplification, by hybridizing to the amplicons. These fluorescent probes are linked to a fluorophore which fluoresces only when hybridized to the target sequence.

The protocols are described below (See **Table 1**).

Table 1 - Categories, protocols, extraction and PCR kits

	Protocol N°	②	**PPS**	⑦
DNA extraction procedure		Manual: StarPrep One, Standard protocol	Manual: StarPrep One, 500 µl/ protocol PPS	Automated: MagPrep I Standard protocol flex
DNA extraction kit		**Manual food**proof® **StarPrep One kit**	**Manual food**proof® **StarPrep One kit**	**Automated food**proof® **Magnetic Preparation kit**
Enrichment step		**18 - 22 h BPW 37°C ± 1 °C + 3 h pre-warmed BHI 37°C**	**16-20 h BPW 37°C ± 1 °C + 16-24 h RVS 41.5°C ± 1 °C**	**19 h - 20 h BPW 37°C ± 1 °C**
Categories	Chocolate and bakery product (25 g)	X	/	X
	Meat and meat product (25 g)	X	/	X
	Milk and dairy product (25 g)	X	/	X
	Egg products (25 g)	X	/	X
	Raw beef meat (375 g)	X	/	X
	Feed samples (25 g)	X	/	X
	Primary production samples (25 g)	/	X	/
Enrichment volume for extraction		100µL	500µL	200µL
PCR kit	CFX96	**food**proof® *Salmonella* Detection Kit - 5' Nuclease		
	LightCycler 480	**food**proof® *Salmonella* Detection Kit - Hybridization Probes		
	LightCycler 2.0			
	Stratagene Mx3005P	**food**proof® *Salmonella* Detection Kit - 5' Nuclease		
PCR Volume		5 µL	5 µl	5 µl

The positive PCR results are confirmed using the protocol described in the ISO 6579-1.

The flow diagram of the alternative method as well as the detailed protocol per tested category is provided in **Annex B**.

2.3 Study design

As the reference and the alternative method share the initial pre-enrichment step, the same test portion (Item) was used for the two methods. All resulting data were treated as paired data (EN ISO 16140-2:2016).

3 METHOD COMPARISON STUDY

The method comparison study is a study performed by the expert laboratory to compare the alternative method with the reference method.

3.1 Sensitivity study

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

3.1.1 Categories and sample types

A total of seven categories were included in this validation study.

For the initial validation study, 502 samples were tested using the manual extraction protocol and 347 samples using the automated extraction protocol.

For the extension study concerning the chocolate and bakery products, 60 samples were tested providing 30 positive and 30 negative results by both thermocyclers (CFX96 and LC480).

The Primary production samples category was tested again for the renewal study using the new protocol for this category. 17 additional samples have been tested covering the other categories in order to have 20 samples minimum per type, 7 positive samples minimum per type and 30 positive and 30 negative samples per category.

Taking into account the initial and renewal studies, the following samples were tested (See **Table 2** for the manual extraction protocol and **Table 3** for the automated extraction protocol).

- Manual extraction protocol:
  - For food and feed samples, 429 samples providing 195 positive and 234 negative samples.
  - For Primary production samples, 73 samples providing 31 positive and 42 negative results

- Automated extraction protocol: 416 samples providing 195 positive and 221 negative results.

Table 2 – Distribution of positive and negative samples per tested category and type - Manual extraction protocol

Category		Type		Positive samples	Negative samples	Total
1	Chocolate and bakery products	a	Dry and sugared low moisture(aw<0,85)	11	14	25
		b	Dry and sugared low moisture(aw<0,65)	11	15	26
		C	Pastry	9	13	22
			Total	31	42	73
2	Meat and meat products	a	Fresh meat	16	23	39
		b	Fermented and cured meat products	7	13	20
		C	Cooked meat products and cooked delicatessen	9	11	20
			Total	32	47	79
3	Milk and dairy products	a	Raw	11	16	27
		b	Heat processed	15	5	20
		C	Fermented	12	20	32
			Total	38	41	79
4	Egg products	a	Egg powders	9	11	20
		b	Liquid eggs	8	14	22
		C	Egg based products	13	12	25
			Total	30	37	67
5	Beef meat 375g	a	Fresh ground beef	10	12	22
		b	Frozen ground beef	11	9	20
		C	Fresh and frozen beef meat and beef meat preparation	11	10	21
			Total	32	31	63
6	Animal feed and pet food	a	Cattle feed	8	14	22
		b	Pet food and raw material for pet food (High moisture)	14	7	21
		C	Pet food and raw material for pet food (Low moisture)	10	15	25
			Total	32	36	68
All categories (food and feed)				**195**	**234**	**429**
7	PPS	a	Animal feces	16	14	30
		b	Environmental samples and non-feces	15	28	43
			Total	31	42	73
All categories (food, feed and PPS)				**226**	**276**	**502**

Table 3 – Distribution of positive and negative samples per tested category and type - Automated extraction protocol

Category		Type		Positive samples	Negative samples	Total
1	Chocolate and bakery products	a	Dry and sugared low moisture(aw<0,85)	10	10	20
		b	Dry and sugared low moisture(aw<0,65)	8	12	20
		c	Pastry	12	8	20
			Total	30	30	60
2	Meat and meat products	a	Fresh meat	16	23	39
		b	Fermented and cured meat products	7	13	20
		c	Cooked meat products and cooked delicatessen	9	11	20
			Total	32	47	79
3	Milk and dairy products	a	Raw	11	16	27
		b	Heat processed	15	5	20
		c	Fermented	12	20	32
			Total	38	41	79
4	Egg products	a	Egg powders	9	11	20
		b	Liquid eggs	9	13	22
		c	Egg based products	13	12	25
			Total	31	36	67
5	Beef meat 375g	a	Fresh ground beef	10	12	22
		b	Frozen ground beef	11	9	20
		c	Fresh and frozen beef meat and beef meat preparation	11	10	21
			Total	32	31	63
6	Animal feed and pet food	a	Cattle feed	8	14	22
		b	Pet food and raw material for pet food (High moisture)	14	7	21
		c	Pet food and raw material for pet food (Low moisture)	10	15	25
			Total	32	36	68
All categories (food and feed)				**195**	**221**	**416**

3.1.2 Test sample preparation

Artificial contaminations were done by spiking or seeding protocols.

When spiking, the strains were stressed using various injury protocols.

For food and feed samples, 241 samples were artificially contaminated; 160 gave positive results. At least, 17.9 % of the samples were naturally contaminated when using the manual extraction protocol or the automated extraction protocol.

For primary production samples, 49 samples were artificially contaminated; 25 gave positive results. 19.4 % of the samples were naturally contaminated.

For the manual extraction protocol, when all samples are taken into account the number of naturally contaminated samples represents 18.1%.

The repartition of the positive, naturally and artificially contaminated samples is given in **Table 4**.

Table 4 - Repartition of the positive, natural and artificial contamination samples

Protocol	Categories		Naturally contaminated	Artificially contaminated						Total
				Seeding protocol			Spiking protocol			
				≤3	3<x≤10	>10	≤5	5< x<10	>10	
Manual extraction protocol	Food and feed (except 7)	Positive samples	35	0	0	0	145	15	0	195
		%	17,9	0,0	0,0	0,0	74,4	7,7	0,0	100,0
	(PPS)	Positive samples	6	14	11	0	0	0	0	31
		%	19,4	45,2	35,5	0,0	0,0	0,0	0,0	100,0
	All categories	Positive samples	41	14	11	0	145	15	0	226
		%	18,1	6,2	4,9	0,0	64,2	6,6	0,0	100,0
Automated extraction protocol	Food and feed	Positive samples	35	8	8	0	130	14	0	195
		%	17,9	4,1	4,1	0,0	66,7	7,2	0,0	100,0

3.1.3 Protocols applied during the validation study

- **Incubation time**

The minimum incubation time was applied during the validation study as described in **Table 5**.

Table 5 - Incubation time applied for enrichment

	Category	Manual extraction protocol	Automated extraction protocol
Pre-enrichment step (BPW)	Food and feed	18 h	19 h
	Primary production samples	16 h	/
Enrichment step (BHI)	Food and feed	3 h	/
Enrichment step (RVS)	Primary production samples	16 h	/

Confirmations

During the validation study, the presumptive positive **food**proof® *Salmonella* tests were confirmed:

- Food and feed samples:

  Tests of the ISO 6579 method: subculture in RVS (0.1 ml + 10 ml, 24 h ± 3 h at 41.5°C± 1°C) and MKTTn (1 ml + 10 ml, 24 h ± 3 h at 37°C± 1°C) and streaking onto XLD and ASAP. Typical colonies were confirmed by biochemical and serological tests after a purification step;

- Primary production samples:

  Tests of the ISO 6579-1 method: subculture on MSRV (24 h to 48 h at 41.5°C ± 1°C) and into MKTTN (24 h at 41.5°C ± 1°C) and streaking onto XLD and ASAP. Typical colonies were confirmed by biochemical galleries and serological tests after purification step.

3.1.4 Sensitivity study results

Table 6 shows the summary of results of the reference method and the alternative methods for all Categories and both extraction protocols.

Table 6 - Summary of sensitivity study results –
All categories for both extraction protocols

		Reference method positive (R+)	Reference method negative (R-)
Manual extraction protocol	Alternative method positive (A+)	Positive agreement (R+/A+) **PA = 219**	Positive deviation (R-/A+) **PD = 2**
	Alternative method negative (A-)	Negative deviation (R+/A-) **ND = 5 (PPND = 0)**	Negative agreement (R-/A-) **NA = 275 (PPNA = 1)**
Automated extraction protocol	Alternative method positive (A+)	Positive agreement (R+/A+) **PA = 187**	Positive deviation (R-/A+) **PD = 2**
	Alternative method negative (A-)	Negative deviation (R+/A-) **ND = 6**	Negative agreement (R-/A-) **NA = 217 (PPNA = 4)**

Tables 7 and 8 show the interpretation of sample results between the reference and alternative method per category and type (based on the confirmed alternative method).

Table 7 – Interpretation of sample results between the reference and alternative method (based on the confirmed alternative):

Manual extraction protocol

Category		Type		PA	NA	PD	ND	Total
1	Chocolate and bakery products	a	Dry and sugared low moisture(aw<0,85)	10	14	0	1	25
		b	Dry and sugared low moisture(aw<0,65)	11	15	0	0	26
		c	Pastry	8	13	0	1	22
			Total	29	42	0	2	73
2	Meat and meat products	a	Fresh meat	16	23	0	0	39
		b	Fermented and cured meat products	7	13	0	0	20
		c	Cooked meat products and cooked delicatessen	8	11	0	1	20
			Total	31	47	0	1	79
3	Milk and dairy products	a	Raw	10	16	1	0	27
		b	Heat processed	14	5	0	1	20
		c	Fermented	12	20	0	0	32
			Total	36	41	1	1	79
4	Egg products	a	Egg powders	9	11	0	0	20
		b	Liquid eggs	8	14	0	0	22
		c	Egg based products	13	12	0	0	25
			Total	30	37	0	0	67
5	Beef meat 375g	a	Fresh ground beef	10	12	0	0	22
		b	Frozen ground beef	10	9	0	1	20
		c	Fresh and frozen beef meat and beef meat preparation	11	10	0	0	21
			Total	31	31	0	1	63
6	Animal feed and pet food	a	Cattle feed	8	14	0	0	22
		b	Pet food and raw material for pet food (High moisture)	14	7	0	0	21
		c	Pet food and raw material for pet food (Low moisture)	10	15	0	0	25
			Total	32	36	0	0	68
All categories (food and feed)				**189**	**234**	**1**	**5**	**429**
7	PPS	a	Animal feces	15	14	1	0	30
		b	Environmental samples and non-feces	15	28	0	0	43
			Total	30	42	1	0	73
All categories (food, feed and PPS)				**219**	**276**	**2**	**5**	**502**

with NA=NA+PPNA
ND = ND+PPND

Table 8 – Interpretation of sample results between the reference and alternative method (based on the confirmed alternative method):

Automated extraction protocol

Category		Type		PA	NA	PD	ND	Total
1	Chocolate and bakery products	a	Dry and sugared low moisture(aw<0,85)	10	10	0	0	20
		b	Dry and sugared low moisture(aw<0,65)	7	12	0	1	20
		c	Pastry	12	8	0	0	20
			Total	29	30	0	1	60
2	Meat and meat products	a	Fresh meat	16	23	0	0	39
		b	Fermented and cured meat products	7	13	0	0	20
		c	Cooked meat products and cooked delicatessen	8	11	0	1	20
			Total	31	47	0	1	79
3	Milk and dairy products	a	Raw	10	16	1	0	27
		b	Heat processed	14	5	0	1	20
		c	Fermented	12	20	0	0	32
			Total	36	41	1	1	79
4	Egg products	a	Egg powders	9	11	0	0	20
		b	Liquid eggs	7	13	1	1	22
		c	Egg based products	13	12	0	0	25
			Total	29	36	1	1	67
5	Beef meat 375g	a	Fresh ground beef	10	12	0	0	22
		b	Frozen ground beef	10	9	0	1	20
		c	Fresh and frozen beef meat and beef meat preparation	10	10	0	1	21
			Total	30	31	0	2	63
6	Animal feed and pet food	a	Cattle feed	8	14	0	0	22
		b	Pet food and raw material for pet food (High moisture)	14	7	0	0	21
		c	Pet food and raw material for pet food (Low moisture)	10	15	0	0	25
			Total	32	36	0	0	68
All categories (food and feed)				**187**	**221**	**2**	**6**	**416**

with NA=NA+PPNA
ND = ND+PPND

3.1.5 Sensitivity study calculations

The sensitivity study parameters as specified in **Table 9** were calculated for all Categories and Types, and the overview is given in **Table 10** for the manual extraction protocol and **Table 11** for the automated extraction protocol.

Table 9 – 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	$FPR = \frac{(FP)}{NA} \times 100\%$

with ND = ND+PPND
NA=NA+PPNA

Table 10 – Overview calculated sensitivity parameters per Category and Type - Manual extraction protocol

Category		Type		PA	NA	PD	ND	PPND	PPNA	SE alt %	SE ref %	RT %	FPR %
1	Chocolate and bakery products	a	Dry and sugared low moisture(aw<0,85)	10	14	0	1	0	0	90,9	100,0	96,0	0
		b	Dry and sugared low moisture(aw<0,65)	11	15	0	0	0	0	100,0	100,0	100,0	0,0
		c	Pastry	8	13	0	1	0	0	88,9	100,0	95,5	0,0
			Total	29	42	0	2	0	0	93,5	100,0	97,3	0,0
2	Meat and meat products	a	Fresh meat	16	23	0	0	0	0	100,0	100,0	100,0	0,0
		b	Fermented and cured meat products	7	13	0	0	0	0	100,0	100,0	100,0	0,0
		c	Cooked meat products and cooked delicatessen	8	11	0	1	0	0	88,9	100,0	95,0	0,0
			Total	31	47	0	1	0	0	96,9	100,0	98,7	0,0
3	Milk and dairy products	a	Raw	10	16	1	0	0	0	100,0	90,9	96,3	0,0
		b	Heat processed	14	5	0	1	0	0	93,3	100,0	95,0	0,0
		c	Fermented	12	20	0	0	0	0	100,0	100,0	100,0	0,0
			Total	36	41	1	1	0	0	97,4	97,4	97,5	0,0
4	Egg products	a	Egg powders	9	11	0	0	0	0	100,0	100,0	100,0	0,0
		b	Liquid eggs	8	14	0	0	0	0	100,0	100,0	100,0	0,0
		c	Egg based products	13	12	0	0	0	0	100,0	100,0	100,0	0,0
			Total	30	37	0	0	0	0	100,0	100,0	100,0	0,0
5	Beef meat 375g	a	Fresh ground beef	10	12	0	0	0	0	100,0	100,0	100,0	0,0
		b	Frozen ground beef	10	9	0	1	0	0	90,9	100,0	95,0	0,0
		c	Fresh and frozen beef meat and beef meat preparation	11	10	0	0	0	0	100,0	100,0	100,0	0,0
			Total	31	31	0	1	0	0	96,9	100,0	98,4	0,0
6	Animal feed and pet food	a	Cattle feed	8	14	0	0	0	0	100,0	100,0	100,0	0,0
		b	Pet food and raw material for pet food (High moisture)	14	7	0	0	0	0	100,0	100,0	100,0	0,0
		c	Pet food and raw material for pet food (Low moisture)	10	15	0	0	0	0	100,0	100,0	100,0	0,0
			Total	32	36	0	0	0	0	100,0	100,0	100,0	0,0
All categories (food and feed)				**189**	**234**	**1**	**5**	**0**	**0**	**97,4**	**99,5**	**98,6**	**0,0**
7	PPS	a	Animal feces	15	14	1	0	0	0	100,0	93,8	96,7	0,0
		b	Environmental samples and non faeces	15	27	0	0	0	1	100,0	100,0	100,0	3,6
			Total	30	41	1	0	0	1	100,0	96,8	98,6	2,4
All categories (food, feed and PPS)				**219**	**275**	**2**	**5**	**0**	**1**	**97,8**	**99,1**	**98,6**	**0,4**

Table 11 – Overview calculated sensitivity parameters per Category and Type - Automated extraction protocol

Category		Type		PA	NA	PD	ND	PPND	PPNA	SE alt %	SE ref %	RT %	FPR %
1	Chocolate and bakery products	a	Dry and sugared low moisture(aw<0,85)	10	10	0	0	0	0	100,0	100,0	100,0	0
		b	Dry and sugared low moisture(aw<0,65)	7	12	0	1	0	0	87,5	100,0	95,0	0,0
		c	Pastry	12	8	0	0	0	0	100,0	100,0	100,0	0,0
			Total	29	30	0	1	0	0	96,7	100,0	98,3	0,0
2	Meat and meat products	a	Fresh meat	16	22	0	0	0	1	100,0	100,0	100,0	4,3
		b	Fermented and cured meat products	7	13	0	0	0	0	100,0	100,0	100,0	0,0
		c	Cooked meat products and cooked delicatessen	8	11	0	1	0	0	88,9	100,0	95,0	0,0
			Total	31	46	0	1	0	1	96,9	100,0	98,7	2,1
3	Milk and dairy products	a	Raw	10	16	1	0	0	0	100,0	90,9	96,3	0,0
		b	Heat processed	14	4	0	1	0	1	93,3	100,0	95,0	20,0
		c	Fermented	12	20	0	0	0	0	100,0	100,0	100,0	0,0
			Total	36	40	1	1	0	1	97,4	97,4	97,5	2,4
4	Egg products	a	Egg powders	9	10	0	0	0	1	100,0	100,0	100,0	9,1
		b	Liquid eggs	7	13	1	1	0	0	88,9	88,9	90,9	0,0
		c	Egg based products	13	12	0	0	0	0	100,0	100,0	100,0	0,0
			Total	29	35	1	1	0	1	96,8	96,8	97,0	2,8
5	Beef meat 375g	a	Fresh ground beef	10	12	0	0	0	0	100,0	100,0	100,0	0,0
		b	Frozen ground beef	10	9	0	1	0	0	90,9	100,0	95,0	0,0
		c	Fresh and frozen beef meat and beef meat preparation	10	9	0	1	0	1	90,9	100,0	95,2	10,0
			Total	30	30	0	2	0	1	93,8	100,0	96,8	3,2
6	Animal feed and pet food	a	Cattle feed	8	14	0	0	0	0	100,0	100,0	100,0	0,0
		b	Pet food and raw material for pet food (High moisture)	14	7	0	0	0	0	100,0	100,0	100,0	0,0
		c	Pet food and raw material for pet food (Low moisture)	10	15	0	0	0	0	100,0	100,0	100,0	0,0
			Total	32	36	0	0	0	0	100,0	100,0	100,0	0,0
All categories (food and feed)				**187**	**217**	**2**	**6**	**0**	**4**	96,9	99,0	98,1	1,8

A summary of the results is given in **Table 12**.

Table 12 - Summary of results observed per extraction protocol

	Manual extraction protocol		All categories	Automated extraction protocol
	Food and feed	Primary production samples		Food and feed
Sensitivity for the alternative method (SE_{alt})	97.4 %	100 %	97.8%	96.9 %
Sensitivity for the reference method (SE_{ref})	99.5 %	96.8 %	99.1%	99.0 %
Relative trueness (RT)	98.6 %	98.6 %	98.6%	98.1 %
False positive ratio for the alternative method (FPR) * FP = PPNA + PPND	0.0 %	2.4 %	0.4%	1.8 %

With *ND = ND + PPND*
NA = NA + PPNA

3.1.6 Discordant results

The negative deviations are listed in **Table 13** and the positive deviations in **Table 14**.

5 negative deviations were obtained when using the manual extraction protocol among the 7 categories tested; they all concern food samples and were all confirmed positive by cultural methods. One of the samples was naturally contaminated (384). The PCR were repeated twice and for 3 samples, a positive PCR was then obtained once (5596) or twice (988-5791). The contamination level in the enrichment broths was probably just at the detection limit of the alternative method for all the samples.

For the automated extraction protocol, 6 negative deviations were also obtained; 4 concern artificially contaminated samples and 2 naturally contaminated samples. For 2 samples (5596 and 388) when PCR tests were repeated, one positive result was obtained. The contamination level in the enrichment broth probably did not reached the detection limit in these cases.

2 positive deviations were obtained with the manual extraction protocol (naturally contaminated samples) and 2 with the automated extraction protocol (1 naturally and 1 artificially contaminated sample).

Table 13 – Negative deviations

	N° sample	Product	Contamination (contamination level CFU/sample)	PCR	Confirmation	Agreement	Category
Manual Extraction protocol	981	Pastry	Artificial *Salmonella* Bareilly Ad1687 (0.6)	-/-/-	+	ND	1
	988	Chocolate cake	Artificial *Salmonella* Stanley Ad1688 (1.8)	-/+ (33.73)/ + (39.13)	+	ND	1
	5596	Ready to eat food (pork)	Artificial *Salmonella* Typhimurium Ad1334 (0.2)	-/+ (39.41)/-	+	ND	2
	384	Frozen ground meat	Natural	-/-/-	+	ND	5
	5791	Lemon and cream tart	Artificial *Salmonella* Livingstone Ad1170 (1.6)	-/+ -(39.05)/ + -41.45)	+	ND	3
Automated extraction protocol	2038	Cocoa beans	Artificial *Salmonella* Bareilly Ad1687 (2,8)	-/-/-	+	ND	1
	5544	Vanilla ice cream	Artificial *Salmonella* Montevideo 510 (2.8)	-/-/-	+	ND	3
	5386	Whole egg product	Natural	-/-/-	+	ND	4
	5596	Ready to eat food (pork)	Artificial *Salmonella* Typhimurium Ad1334 (0.2)	-/+ (39.61)/-	+	ND	4
	281	Frozen ground meat (375 g)	Artificial *Salmonella* Give 436 (3.0)	-/-/-	+	ND	5
	388	Beef meat (375 g)	Natural	-/+ (36.66)/-	+	ND	5

Table 14 – Positive deviations

	N° sample	Product	Contamination (contamination level CFU/sample)	Agreement	Category
Manual extraction protocol	34	Raw ewe milk	Natural	PD	3
	6949	Boot socks	Natural (*Salmonella* Djugu)	PD	7
Automated extraction protocol	34	Raw ewe milk	Natural	PD	3
	419	Egg yolk product	Artificial *Salmonella* Enteritidis 465 (4.8)	PD	4

The analyses of discordant results according to the EN ISO 16140-2:2016 for a paired study design is given in **Table 15** for the manual extraction protocol and **Table 16** for the automated extraction protocol.

Table 15 - Interpretation of the sensitivity study results (paired study) - Manual extraction protocol (paired study design)

Category		Type		N+	PD	ND	ND-PD	AL	ND+PD	AL
1	Chocolate and bakery products	a	Dry and sugared low moisture(aw<0,85)	11	0	1	1		1	
		b	Dry and sugared low moisture(aw<0,65)	11	0	0	0		0	
		c	Pastry	9	0	1	1		1	
			Total	31	0	2	2	3	2	6
2	Meat and meat products	a	Fresh meat	16	0	0	0		0	
		b	Fermented and cured meat products	7	0	0	0		0	
		c	Cooked meat products and cooked delicatessen	9	0	1	1		1	
			Total	32	0	1	1	3	1	6
3	Milk and dairy products	a	Raw	11	1	0	-1		1	
		b	Heat processed	15	0	1	1		1	
		c	Fermented	12	0	0	0		0	
			Total	38	1	1	0	3	2	6
4	Egg products	a	Egg powders	9	0	0	0		0	
		b	Liquid eggs	8	0	0	0		0	
		c	Egg based products	13	0	0	0		0	
			Total	30	0	0	0	3	0	6
5	Beef meat 375g	a	Fresh ground beef	10	0	0	0		0	
		b	Frozen ground beef	11	0	1	1		1	
		c	Fresh and frozen beef meat and beef meat preparation	11	0	0	0		0	
			Total	32	0	1	1	3	1	6
6	Animal feed and pet food	a	Cattle feed	8	0	0	0		0	
		b	Pet food and raw material for pet food (high moisture)	14	0	0	0		0	
		c	Pet food and raw material for pet food (low moisture)	10	0	0	0		0	
			Total	32	0	0	0	3	0	6
All categories (food and feed)				**195**	**1**	**5**	**4**	**6**	**6**	**16**
7	PPS ISO 6579/A1	a	Animal feces	16	1	0	-1		1	
		b	Environmental samples and non-feces	15	0	0	0		0	
			Total	31	1	0	-1	3	1	6
All categories (food, feed and PPS)				**226**	**2**	**5**	**3**	**6**	**7**	**18**

With ND=ND+PPND

Table 16 - Interpretation of the sensitivity study results (paired study) - Automated extraction protocol

Category		Type		N+	PD	ND	PPND	(ND+PPND)-PD	AL	ND+PPND+PD	AL
1	Chocolate and bakery products	a	Dry and sugared low moisture(aw<0,85)	10	0	0	0	0		0	
		b	Dry and sugared low moisture(aw<0,65)	8	0	1	0	1		1	
		c	Pastry	12	0	0	0	0		0	
			Total	30	0	1	0	1	3	1	6
2	Meat and meat products	a	Fresh meat	16	0	0	0	0		0	
		b	Fermented and cured meat products	7	0	0	0	0		0	
		c	Cooked meat products and cooked delicatessen	9	0	1	0	1		1	
			Total	32	0	1	0	1	3	1	6
3	Milk and dairy products	a	Raw	11	1	0	0	-1		1	
		b	Heat processed	15	0	1	0	1		1	
		c	Fermented	12	0	0	0	0		0	
			Total	38	1	1	0	0	3	2	6
4	Egg products	a	Egg powders	9	0	0	0	0		0	
		b	Liquid eggs	9	1	1	0	0		2	
		c	Egg based products	13	0	0	0	0		0	
			Total	31	1	1	0	0	3	2	6
5	Beef meat 375g	a	Fresh ground beef	10	0	0	0	0		0	
		b	Frozen ground beef	11	0	1	0	1		1	
		c	Fresh and frozen beef meat and beef meat preparation	11	0	1	0	1		1	
			Total	32	0	2	0	2	3	2	6
6	Animal feed and pet food	a	Cattle feed	8	0	0	0	0		0	
		b	Pet food and raw material for pet food (High moisture)	14	0	0	0	0		0	
		c	Pet food and raw material for pet food (Low moisture)	10	0	0	0	0		0	
			Total	32	0	0	0	0	3	0	6
All categories (food and feed)				**195**	**2**	**6**	**0**	**4**	**6**	**8**	**16**

The observed values for (ND - PD) and (ND+PD) meet the acceptability limit (AL) for the seven categories (food, feed and primary production samples).

3.1.7 PCR Instrument testing

The DNA extracts were analyzed by CFX96 by the expert lab, and by LC 480, LC 2.0, Mx3005P QPCR System by BIOTECON DIAGNOSTICS.

The results obtained with the different thermocyclers are given in **Table 17**.

Table 17 - Results obtained with the different thermocyclers

	Category	Thermocycler	Non tested[1]	PA	NA	PD	ND	Total	ND-PD	AL	Conclusion	ND+PD	AL	Conclusion
Manual Extraction protocol	Food and feed	LightCycler 480	0	189	234	1	5	429	4	6	ND-PD<AL	6	16	ND-PD<AL
		LightCycler 2.0	5	189	229	1	5	424	4	6	ND-PD<AL	6	16	ND-PD<AL
		Mx3005P QPCR	5	190	229	1	4	424	3	6	ND-PD<AL	5	16	ND-PD<AL
		CFX96	0	189	234	1	5	429	4	6	ND-PD<AL	6	16	ND-PD<AL
	PPS	LightCycler 480	0	30	42	1	0	73	-1	3	ND-PD<AL	1	6	ND-PD<AL
		LightCycler 2.0	0	30	42	1	0	73	-1	3	ND-PD<AL	1	6	ND-PD<AL
		Mx3005P QPCR	0	30	42	1	0	73	-1	3	ND-PD<AL	1	6	ND-PD<AL
		CFX96	0	30	42	1	0	73	-1	3	ND-PD<AL	1	6	ND-PD<AL
	All categories	LightCycler 480	0	219	276	2	5	502	3	6	ND-PD<AL	7	18	ND-PD<AL
		LightCycler 2.0	5	219	271	2	5	497	3	6	ND-PD<AL	7	18	ND-PD<AL
		Mx3005P QPCR	5	220	271	2	4	497	2	6	ND-PD<AL	6	18	ND-PD<AL
		CFX96	0	219	276	2	5	502	3	6	ND-PD<AL	7	18	ND-PD<AL
Automated extraction protocol	Food and feed	LightCycler 480	0	189	221	1	4	416	3	6	ND-PD<AL	5	16	ND-PD<AL
		LightCycler 2.0	5	159	187	1	4	351	3	5	ND-PD<AL	5	14	ND-PD<AL
		Mx3005P QPCR	5	160	187	1	3	351	2	5	ND-PD<AL	4	14	ND-PD<AL
		CFX96	0	187	221	2	6	416	4	6	ND-PD<AL	8	16	ND-PD<AL

NA = NA + PPNA ND = ND + PPND

The calculated values for (ND - PD) meet the acceptability limit for all the categories for both extraction protocols and for all the tested thermocyclers.
The calculated values for (ND+PD) meet the AL for all the categories for both extraction protocols and for all the tested thermocyclers.

[1] Some DNA extracts were no more available for testing with all the thermocyclers

3.1.8 PCR inhibition

No PCR inhibition was observed during the study.

3.1.9 Conclusion sensitivity study

The observed values for ND-PD and ND+PD for the individual categories and for all the categories meet the acceptability limits (observed values ≤ AL) for all the thermocyclers tested.

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 (proprietary) 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 were chosen for each of the Categories in this validation study, as shown in **Table 18**.

Table 18 - List of selected types and strains per category, as tested within the relative level of detection study

Category		Type	Strain	Reference number	Strain origin	Storage conditions after inoculation
1	Chocolate and bakery products	Cocoa powder	*Salmonella* Agona	Ad1483	Tiramisu	Lyophilized strain and storage 2 weeks at ambient temperature
			Salmonella Bareilly	Ad1687	Chocolate industry environment	
2	Meat and meat products	Ground beef (375 g sample size)	*Salmonella* Dublin	Ad530	Ground beef	72 h at 3°C ± 2°C
3	Milk and dairy products	Raw milk	*Salmonella* Anatum	Ad1166	Dairy product	48 h at 3°C ± 2°C
4	Egg products	Liquid egg	*Salmonella* Typhimurium	Ad1484	Liquid egg	72 h at 3°C ± 2°C
5	Beef meat (375g)	Fresh ground beef	*Salmonella* Dublin	Ad530	Ground beef	72 h at 3°C ± 2°C
6	Animal feed and pet food	Sausage for dog	*Salmonella* Cerro	Ad689	Dehydrated poultry protein	72 h at 3°C ± 2°C
7	Primary production samples	Boot socks	*Salmonella* Agona	Ad1306	Poultry slaughterhouse	24 h at ambient temperature

The RLOD determination for categories 1, 2, 4 and 6 was performed for the initial validation study. RLOD for categories 3 and 7 was run again for the renewal study.

3.2.2 Test sample preparations

Three levels of artificial contamination were prepared for each type:

- Negative control level: inoculation in order to get 5 test portions,
- Low level: inoculation in order to get 20 test portions providing fractional recovery,
- Higher level: inoculation in order to get 5 test portions contaminated at a higher level.

The DNA extracts were analyzed with the CFX 96 by the expert lab, and with the LC 480, LC 2.0, Mx3005P QPCR System by BIOTECON DIAGNOSTICS. Note that for raw milk (automated extraction protocol) the DNA extracts were sent twice as the RLOD values obtained with the first set of DNA extracts didn't meet the AL (RLOD varied from 1.609 to 3.244 depending on the cycler tested).This was probably due to the transport conditions. The second shipment was done by Express transport; this second set of data has been retained for RLOD calculation.

3.2.3 RLOD study results

The RLOD calculations were performed using the 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 RLOD are given in **Tables 19 and 20**.

Table 19 – Presentation of RLOD before and after confirmation of the alternative method results - Manual extraction protocol

CFX 96							
Name	RLOD	RLODL	RLODU	b=ln(RLOD)	sd(b)	z-Test statistic	p-value
Cocoa powder / S.Agona Ad1483	1,000	0,450	2,224	0,000	0,400	0,000	1,000
Sausages / S.Enteritidis Ad926	1,000	0,481	2,081	0,000	0,366	0,000	1,000
Raw milk / S.Anatum Ad1166	1,000	0,434	2,304	0,000	0,417	0,000	1,000
Eggs / S.Typhimurium Ad1484	1,000	0,345	2,901	0,000	0,532	0,000	1,000
Ground beef / S.Dublin Ad530	1,000	0,460	2,173	0,000	0,388	0,000	1,000
Sausages for dog / S.Cerro Ad689	1,000	0,481	2,081	0,000	0,366	0,000	1,000
Poultry feces / S.Agona Ad1306	0,861	0,392	1,894	-0,149	0,394	0,379	1,295
Combined	0,981	0,712	1,351	-0,020	0,160	0,122	1,097

LC 480							
Name	RLOD	RLODL	RLODU	b=ln(RLOD)	sd(b)	z-Test statistic	p-value
Cocoa powder / S.Agona Ad1483	1,000	0,450	2,224	0,000	0,400	0,000	1,000
Sausages / S.Enteritidis Ad926	1,000	0,481	2,081	0,000	0,366	0,000	1,000
Raw milk / S.Anatum Ad1166	1,000	0,434	2,304	0,000	0,417	0,000	1,000
Eggs / S.Typhimurium Ad1484	0,942	0,265	3,353	-0,060	0,635	0,094	1,075
Ground beef / S.Dublin Ad530	1,000	0,460	2,173	0,000	0,388	0,000	1,000
Sausages for dog / S.Cerro Ad689	1,000	0,481	2,081	0,000	0,366	0,000	1,000
Poultry feces / S.Agona Ad1306	1,000	0,478	2,092	0,000	0,369	0,000	1,000
Combined	0,988	0,717	1,363	-0,012	0,161	0,073	1,058

Mx3500P							
Name	RLOD	RLODL	RLODU	b=ln(RLOD)	sd(b)	z-Test statistic	p-value
Cocoa powder / S.Agona Ad1483	1,000	0,450	2,224	0,000	0,400	0,000	1,000
Sausages / S.Enteritidis Ad926	1,000	0,481	2,081	0,000	0,366	0,000	1,000
Raw milk / S.Anatum Ad1166	1,000	0,434	2,304	0,000	0,417	0,000	1,000
Eggs / S.Typhimurium Ad1484	0,942	0,265	3,353	-0,060	0,635	0,094	1,075
Ground beef / S.Dublin Ad530	1,000	0,460	2,173	0,000	0,388	0,000	1,000
Sausages for dog / S.Cerro Ad689	1,000	0,481	2,081	0,000	0,366	0,000	1,000
Poultry feces / S.Agona Ad1306	0,861	0,392	1,894	-0,149	0,394	0,379	1,295
Combined	0,969	0,704	1,335	-0,031	0,160	0,195	0,155

LC 2.0							
Name	RLOD	RLODL	RLODU	b=ln(RLOD)	sd(b)	z-Test statistic	p-value
Cocoa powder / S.Agona Ad1483	1,000	0,450	2,224	0,000	0,400	0,000	1,000
Sausages / S.Enteritidis Ad926	1,000	0,481	2,081	0,000	0,366	0,000	1,000
Raw milk / S.Anatum Ad1166	1,000	0,434	2,304	0,000	0,417	0,000	1,000
Eggs / S.Typhimurium Ad1484	0,942	0,265	3,353	-0,060	0,635	0,094	1,075
Ground beef / S.Dublin Ad530	1,000	0,460	2,173	0,000	0,388	0,000	1,000
Sausages for dog / S.Cerro Ad689	1,000	0,481	2,081	0,000	0,366	0,000	1,000
Poultry feces / S.Agona Ad1306	0,861	0,392	1,894	-0,149	0,394	0,379	1,295
Combined	0,969	0,704	1,335	-0,031	0,160	0,195	1,155

Table 20 – Presentation of RLOD before and after confirmation of the alternative method results - Automated extraction protocol

CFX 96							
Name	RLOD	RLODL	RLODU	b=ln(RLOD)	sd(b)	z-Test statistic	p-value
Cocoa powder / S.Bareilly Ad1687	1,000	0,434	2,304	0,000	0,417	0,000	1,000
Ground beef / S.Dublin Ad530	1,000	0,460	2,173	0,000	0,388	0,000	1,000
Sausages / S.Enteritidis Ad926	1,000	0,481	2,081	0,000	0,366	0,000	1,000
Raw milk / S.Anatum Ad1166	1,159	0,455	2,953	0,148	0,467	0,316	0,752
Eggs / S.Typhimurium Ad1484	1,000	0,345	2,901	0,000	0,532	0,000	1,000
Sausages for dog / S.Cerro Ad689	1,151	0,519	2,553	0,141	0,398	0,354	0,723
Combined	1,047	0,733	1,498	0,046	0,179	0,259	0,795

LC 480							
Name	RLOD	RLODL	RLODU	b=ln(RLOD)	sd(b)	z-Test statistic	p-value
Cocoa powder / S.Bareilly Ad1687	1,000	0,434	2,304	0,000	0,417	0,000	1,000
Ground beef / S.Dublin Ad530	1,000	0,460	2,173	0,000	0,388	0,000	1,000
Sausages / S.Enteritidis Ad926	1,000	0,481	2,081	0,000	0,366	0,000	1,000
Raw milk / S.Anatum Ad1166	1,357	0,518	3,554	0,305	0,481	0,634	0,526
Eggs / S.Typhimurium Ad1484	0,942	0,265	3,353	-0,060	0,635	0,094	1,075
Sausages for dog / S.Cerro Ad689	1,000	0,481	2,081	0,000	0,366	0,000	1,000
Combined	1,034	0,723	1,479	0,034	0,179	0,188	0,851

Mx 3500P							
Name	**RLOD**	**RLODL**	**RLODU**	**b=ln(RLOD)**	**sd(b)**	**z-Test statistic**	**p-value**
Ground beef / S.Dublin Ad530	1,000	0,460	2,173	0,000	0,388	0,000	1,000
Sausages / S.Enteritidis Ad926	1,000	0,481	2,081	0,000	0,366	0,000	1,000
Raw milk / S.Anatum Ad1166	1,000	0,434	2,304	0,000	0,417	0,000	1,000
Eggs / S.Typhimurium Ad1484	0,942	0,265	3,353	-0,060	0,635	0,094	1,075
Sausages for dog / S.Cerro Ad689	1,000	0,481	2,081	0,000	0,366	0,000	1,000
Combined	0,984	0,669	1,448	-0,016	0,193	0,082	1,065

LC 2.0							
Name	**RLOD**	**RLODL**	**RLODU**	**b=ln(RLOD)**	**sd(b)**	**z-Test statistic**	**p-value**
Ground beef / S.Dublin Ad530	1,000	0,460	2,173	0,000	0,388	0,000	1,000
Sausages / S.Enteritidis Ad926	1,000	0,481	2,081	0,000	0,366	0,000	1,000
Raw milk / S.Anatum Ad1166	1,159	0,455	2,953	0,148	0,467	0,316	0,752
Eggs / S.Typhimurium Ad1484	0,942	0,265	3,353	-0,060	0,635	0,094	1,075
Sausages for dog / S.Cerro Ad689	1,000	0,481	2,081	0,000	0,366	0,000	1,000
Combined	1,012	0,687	1,491	0,012	0,194	0,062	0,951

3.2.4 LOD_{50}

The $LOD_{50\ \%}$ calculations according to Wilrich & Wilrich POD-LOD calculation program - version 9, 2017-09-23 test are given **Table 21, Table 22** and **Table 23**.

Table 21 - LOD_{50} results - Reference method ISO 6579-1

CFX96/LC 480/Mx 3005P/LC 2.0			
Name	LOD 50	Lower conf. Limit	Upper conf. Limit
Cocoa powder - S. Agona Ad1483 (initial validation)	0,713	0,416	1,219
Cocoa powder- S. Bareilly Ad1687 (extension)	1.354	0.776	2.362
Sausages - S. Enteritidis Ad926	0,650	0,372	1,136
Raw milk - S. Anatum Ad1166	0,787	0,456	1,359
Egg - S. Typhimurium Ad1484	0,658	0,313	1,383
Ground beef (375g) - S. Dublin Ad530	0,454	0,250	0,825
Sausages for dog - S. cerro Ad689	0,693	0,403	1,192
Boot socks (PPS) - S. Agona Ad1306	0,511	0,308	0,849

Table 22 - LOD_{50} results - Manual extraction protocol

CFX96			
Name	LOD 50	Lower conf. Limit	Upper conf. Limit
Cocoa powder - S. Agona Ad1483	0,713	0,416	1,219
Sausages - S. Enteritidis Ad926	0,650	0,372	1,136
Raw milk - S. Anatum Ad1166	0,787	0,456	1,359
Egg - S. Typhimurium Ad1484	0,658	0,313	1,383
Ground beef (375g) - S. Dublin Ad530	0,454	0,250	0,825
Sausages for dog - S. cerro Ad689	0,693	0,403	1,192
Boot socks (PPS) - S. Agona Ad1306	0,448	0,268	0,748

LC 480			
Name	LOD 50	Lower conf. Limit	Upper conf. Limit
Cocoa powder - S. Agona Ad1483	0,713	0,416	1,219
Sausages - S. Enteritidis Ad926	0,650	0,372	1,136
Raw milk - S. Anatum Ad1166	0,787	0,456	1,359
Egg - S. Typhimurium Ad1484	0,628	0,295	1,335
Ground beef (375g) - S. Dublin Ad530	0,454	0,250	0,825
Sausages for dog - S. cerro Ad689	0,693	0,403	1,192
Boot socks (PPS) - S. Agona Ad1306	0,511	0,308	0,849

Mx 3005P			
Name	LOD 50	Lower conf. Limit	Upper conf. Limit
Cocoa powder - S. Agona Ad1483	0,713	0,416	1,219
Sausages - S. Enteritidis Ad926	0,650	0,372	1,136
Raw milk - S. Anatum Ad1166	0,787	0,456	1,359
Egg - S. Typhimurium Ad1484	0,628	0,295	1,335
Ground beef (375g) - S. Dublin Ad530	0,454	0,250	0,825
Sausages for dog - S. cerro Ad689	0,693	0,403	1,192
Boot socks (PPS) - S. Agona Ad1306	0,448	0,268	0,748

LC 2.0			
Name	LOD 50	Lower conf. Limit	Upper conf. Limit
Cocoa powder - S. Agona Ad1483	0,713	0,416	1,219
Sausages - S. Enteritidis Ad926	0,650	0,372	1,136
Raw milk - S. Anatum Ad1166	0,787	0,456	1,359
Egg - S. Typhimurium Ad1484	0,628	0,295	1,335
Ground beef (375g) - S. Dublin Ad530	0,454	0,250	0,825
Sausages for dog - S. cerro Ad689	0,693	0,403	1,192
Boot socks (PPS) - S. Agona Ad1306	0,448	0,268	0,748

Table 23 - LOD_{50} results - Automated extraction protocol

CFX96			
Name	LOD 50	Lower conf. Limit	Upper conf. Limit
Cocoa powder - S. Bareilly Ad1687	1,354	0,776	2,362
Ground beef (375g) - S. Dublin Ad530	0,454	0,250	0,825
Sausages - S. Enteritidis Ad926	0,650	0,372	1,136
Raw milk - S. Anatum Ad1166	0,881	0,505	1,539
Egg - S. Typhimurium Ad1484	0,658	0,313	1,383
Sausages for dog - S. cerro Ad689	0,791	0,460	1,360

LC 480			
Name	LOD 50	Lower conf. Limit	Upper conf. Limit
Cocoa powder / S. Bareilly Ad1687	1,354	0,776	2,362
Ground beef (375g) - S. Dublin Ad530	0,454	0,250	0,825
Sausages - S. Enteritidis Ad926	0,650	0,372	1,136
Raw milk - S. Anatum Ad1166	0,988	0,558	1,749
Egg - S. Typhimurium Ad1484	0,628	0,295	1,335
Sausages for dog - S. cerro Ad689	0,693	0,403	1,192

Mx 3005P			
Name	LOD 50	Lower conf. Limit	Upper conf. Limit
Ground beef (375g) - S. Dublin Ad530	0,454	0,250	0,825
Sausages - S. Enteritidis Ad926	0,650	0,372	1,136
Raw milk - S. Anatum Ad1166	0,787	0,456	1,359
Egg - S. Typhimurium Ad1484	0,628	0,295	1,335
Sausages for dog - S. cerro Ad689	0,693	0,403	1,192

LC 2.0			
Name	LOD 50	Lower conf. Limit	Upper conf. Limit
Ground beef (375g) - S. Dublin Ad530	0,454	0,250	0,825
Sausages - S. Enteritidis Ad926	0,650	0,372	1,136
Raw milk - S. Anatum Ad1166	0,881	0,505	1,539
Egg - S. Typhimurium Ad1484	0,628	0,295	1,335
Sausages for dog - S. cerro Ad689	0,693	0,403	1,192

3.2.5 Conclusion RLOD study

The RLOD values are lower than the Acceptability limit (AL) fixed at 1.5 for a paired study design for all the tested matrix/strain pairs, both protocols tested and all the thermocyclers tested.

3.3 Inclusivity / exclusivity study

The inclusivity is the ability of the alternative method to detect the target analyte from a wide range of strains. The 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 done in BPW in order to inoculate 10-100 cells/225 ml BPW incubated for 18 h at 37°C. The two alternative method protocols were performed (manual and automated).
  50 target strains were tested for the initial validation study, 50 additional strains were tested in 2018 for the renewal study.

- **Exclusivity**: non-target strains cultures were done in BHI incubated at 37°C. Dilutions were realized in order to inoculate 10^5 cell/ml BPW incubated for 18 h at 37°C. The two alternative method protocols were realized. 30 non-target strains were tested for the initial validation study.

3.3.2 Results

100 *Salmonella* strains were tested; all gave PCR positive results with the **food**proof® *Salmonella* Detection Kit (5' Nuclease).
Among the 30 non-target strains tested, none gave a positive result with the test kit.

3.3.3 Conclusion inclusivity and exclusivity study

The **food**proof® *Salmonella* spp. detection method for the detection of *Salmonella* spp. in food, feed and primary production samples is specific and selective.

4 INTER-LABORATORY STUDY

The inter-laboratory study is a study performed by multiple laboratories testing identical samples at the same time, the results of which are used to estimate alternative-method performance parameters.

4.1 Study organization

- *Collaborators number*

Samples were sent to 15 laboratories. In each lab, one technician run the manual and extraction protocol.

- *Matrix and strain used*

The study was carried out on ground beef contaminated by *Salmonella* Typhimurium A00C060.

- *Samples*

Samples were inoculated on Monday 7 of May 2012, as described below:

- 24 codified samples for *Salmonella* research by **food**proof® *Salmonella* method and by the reference method (ISO 6579:2002),
- 1 ground beef sample for aerobic mesophilic flora enumeration by ISO 4833 method,
- 1 water flask labeled “Temperature Control” with a temperature probe.

All the samples were already weighted in filter stomacher bags.

The analyses were started on Wednesday 9 of May 2012.

- *Inoculation*

The targeted inoculation levels were:

- Level 0: 0 CFU/g,
- Level 1: 5 CFU/g,
- Level 2: 25 CFU/g.

At least, each laboratory received 24 samples of 25 g, i.e. 8 samples per inoculation level and method.

- *Labeling and shipping*

Blind samples (code is only known by the expert laboratory) were placed in isothermal boxes, which contained cooling blocks, and express-shipped to the different laboratories.
A temperature control flask containing temperature probe was added to the package in order to register the temperature profile during the transport, package delivery.
Samples were shipped in 24 h to 48 h to the different laboratories. Sample temperature had to stay lower or equal to 8.4°C during transport, and between 0°C – 8.4°C at arrival.

- *Analyses*

Collaborative study laboratories and the expert laboratory carried out the analyses with the alternative and reference methods at day 2.

4.2 Experimental parameters controls

4.2.1 Strain stability and background microflora stability

Strain stability was checked by inoculating the matrix at 100 CFU/g and 5 CFU/25 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 3°C ± 2°C. Three samples were analyzed. The aerobic mesophilic flora was also enumerated; the results are given in **Table 24**.

Table 24 - *Salmonella* stability in the matrix

Day	Reference method (detection)			CFU/g (XLD)			Aerobic mesophilic flora (CFU/g)
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3	
Day 0	+	+	+	100	70	120	$3{,}2.10^3$
Day 1	+	+	+	90	160	150	$8{,}2.10^3$
Day 2	+	+	+	150	120	190	$8{,}2.10^3$

No evolution was observed during storage at 3°C ± 2°C.

4.2.2 Contamination levels

The contamination levels and the sample codification were the following (see **Table 25**).

Table 25 - 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	2 – 8 – 11 – 15 – 18 – 20 – 22 - 24	No inoculation	/	/	/
Low level	1 – 6 – 7 – 10 - 12 – 14 – 19 – 23	2	2,8	2,4	3,3
High level	3 – 4 – 5 – 9 - 13 – 16 – 17 - 21	20	19,9	17,2	23,0

4.2.3 Logistic conditions

The temperatures measured at reception by the collaborators, the temperatures registered by the thermo-probe, and the receipt and analysis dates are given in **Table 26**.

Table 26 - Sample temperatures at receipt

Collaborators	Temperature measured by the probe (°C)	Temperature measured at reception (°C)	Receipt date	Day of analysis
A	3,5	4,6	Day 1 (Tuesday 8th May 2012)	Day 2 (Wednesday 9th May 2012)
B	2,0	4,2	Day 1 (Tuesday 8th May 2012)	Day 2 (Wednesday 9th May 2012)
C	4,0	9,0	Day 1 (Tuesday 8th May 2012)	Day 2 (Wednesday 9th May 2012)
D	4,5	7,0	Day 1 (Tuesday 8th May 2012)	Day 2 (Wednesday 9th May 2012)
E	3,5	4,0	Day 1 (Tuesday 8th May 2012)	Day 2 (Wednesday 9th May 2012)
F	3,5	3,5	Day 1 (Tuesday 8th May 2012)	Day 2 (Wednesday 9th May 2012)
G	3,0	5,5	Day 1 (Tuesday 8th May 2012)	Day 2 (Wednesday 9th May 2012)
H	3,0	5,0	Day 1 (Tuesday 8th May 2012)	Day 2 (Wednesday 9th May 2012)
I	6,0	6,0	Day 1 (Tuesday 8th May 2012)	Day 2 (Wednesday 9th May 2012)
J	3,5	1,6	Day 1 (Tuesday 8th May 2012)	Day 2 (Wednesday 9th May 2012)
K	2,5	4,5	Day 1 (Tuesday 8th May 2012)	Day 2 (Wednesday 9th May 2012)
L	2,0	8,7	Day 1 (Tuesday 8th May 2012)	Day 2 (Wednesday 9th May 2012)
M	4,0	6,0	Day 1 (Tuesday 8th May 2012)	Day 2 (Wednesday 9th May 2012)
N	4,0	10,5	Day 1 (Tuesday 8th May 2012)	Day 2 (Wednesday 9th May 2012)
O	4,0	6,2	Day 1 (Tuesday 8th May 2012)	Day 2 (Wednesday 9th May 2012)

No problem was encountered during the transport or at receipt for the 15 collaborators. All the samples were delivered on time and in appropriate conditions.

Three collaborators (C, L and N) measured a temperature at receipt above 8.5°C. The temperature curves clearly indicate that all the packages arrived below 5°C.

4.3 Calculation and summary of data

4.3.1 MicroVal Expert laboratory results

The results obtained by the expert laboratory are given in **Table 27**.

Table 27 – 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

4.3.2 Results observed by the collaborative laboratories

- ***Aerobic mesophilic flora enumeration***

Depending on the Lab results, the enumeration levels varied from 3 500 to 160 000 CFU/g.

- ***Salmonella* spp. *detection***

15 collaborators participated to the study. The Lab A tested only the automated protocol and two technicians were involved (noted A1 and A2).

The results obtained are provided in **Table 28** and **Table 29** (reference method), in **Table 30** for the manual extraction protocol and **Table 31** for the automated extraction protocol.

Table 28 - Positive results by the reference method (ALL the collaborators)

Manual extraction protocol

Laboratory	Reference method		
	Level contamination		
	L0	*L1*	*L2*
B	2	8	8
C	0	8	8
D	0	8	8
E	0	6	8
F	0	8	8
G	0	8	8
H	1	6	8
I	2	8	8
J	0	8	8
K	0	8	8
L	0	8	8
M	0	8	8
N	1	8	8
O	4	8	8
Total	**P_0 =10**	**P_1 =108**	**P_2 =112**

Table 29 - Positive results (before and after confirmation) by the alternative method (ALL the collaborators)

Manual extraction protocol

Laboratory	Alternative method-Manual extraction protocol									
	Contamination level									
	L0				*L1*			*L2*		
	First PCR result	*Second PCR result*	*Confirmation result*	*Final result*	*PCR result*	*Confirmation result*	*Final result*	*PCR result*	*Confirmation result*	*Final result*
B	3	2	2	1	8	8	8	8	8	8
C	0	/	0	0	8	8	8	8	8	8
D	0	/	0	0	8	8	8	8	8	8
E	1	1	0	0	6	6	6	8	8	8
F	0	/	0	0	8	8	8	8	8	8
G	0	/	0	0	8	8	8	8	8	8
H	5	4	1	1	7	6	6	8	8	8
I	5	3	1	2	7	8	7	8	8	8
J	0	/	0	0	8	8	8	8	8	8
K	4	3	0	0	8	8	8	8	8	8
L	0	/	0	0	8	8	8	8	8	8
M	0	/	0	0	8	8	8	8	8	8
N	1	1	1	0	8	8	8	8	8	8
O	3	2	4	2	8	8	8	8	8	8
Total	**P_0 = 3**	**16**	**9**	**CP_0 = 3**	**P_1 = 3**	**108**	**CP_1 = 3**	**P_2 = 3**	**112**	**CP_2 = 3**

Table 30 - Positive results by the reference method (ALL the collaborators)

Automated extraction protocol

Laboratory	Reference method		
	Level contamination		
	L0	*L1*	*L2*
A1	0	8	8
A2	0	8	8
B	2	8	8
C	0	8	8
D	0	8	8
E	0	6	8
F	0	8	8
G	0	8	8
H	1	6	8
I	2	8	8
J	0	8	8
K	0	8	8
L	0	8	8
M	0	8	8
N	1	8	8
O	4	8	8
Total	**P_0 = 10**	**P_1 = 108**	**P_2 = 112**

Table 31 - Positive results (before and after confirmation) by the alternative method (ALL the collaborators) - Automated extraction protocol

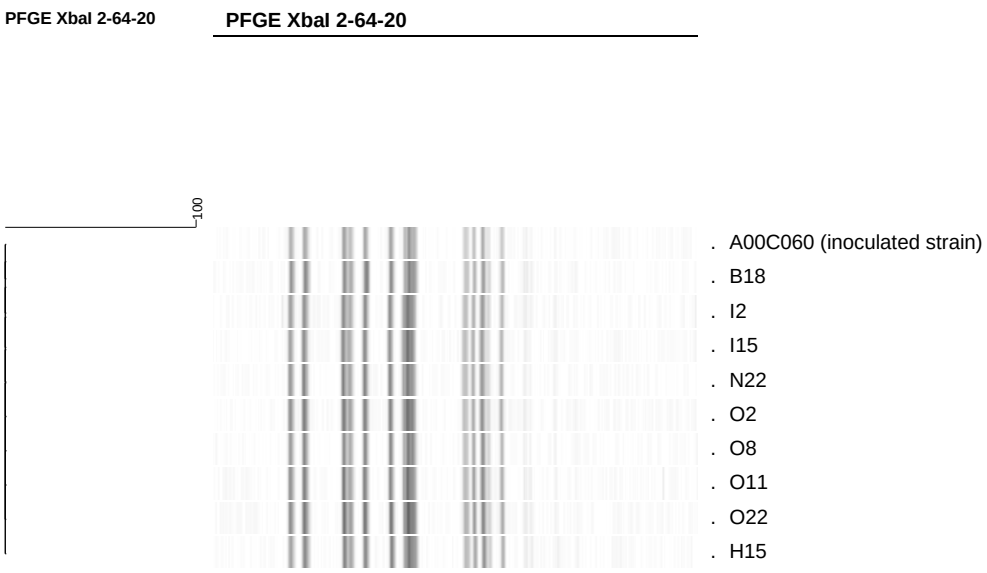
Laboratory	Alternative method-Automated extraction protocol									
	Contamination level									
	L0				*L1*			*L2*		
	First PCR result	*Second PCR result*	*Confirmation result*	*Final result*	*PCR result*	*Confirmation result*	*Final result*	*PCR result*	*Confirmation result*	*Final result*
A1	0	/	0	0	8	8	8	8	8	8
A2	0	/	0	0	8	8	8	8	8	8
B	4	2	2	1	8	8	8	8	8	8
C	0	/	0	0	8	8	8	8	8	8
D	7	7	0	0	8	8	8	8	8	8
E	0	/	0	0	6	6	6	8	8	8
F	2	1	0	0	8	8	8	8	8	8
G	0	/	0	0	8	8	8	8	8	8
H	5	4	1	0	7	6	6	8	8	8
I	0	/	2	0	7	8	7	8	8	8
J	0	/	0	0	8	8	8	8	8	8
K	8	8	0	0	8	8	8	8	8	8
L	0	/	0	0	8	8	8	8	8	8
M	0	/	0	0	8	8	8	8	8	8
N	2	2	1	0	8	8	8	8	8	8
O	4	3	4	2	8	8	8	8	8	8
Total	**P_0 = 32**	**27**	**10**	**CP_0 = 3**	**P_1 = 108**	**108**	**CP_1 = 3**	**P_2 = 112**	**112**	**CP_2 = 3**

Many presumed cross-contaminations occurred, enhancing discordant results between the compared methods. It was thus asked to the labs to run again the analyses **carefully**.

The second series of analyses didn't provide the same results, confirming that the already observed results were probably due to cross-contaminations.

To clearly confirm the cross-contaminations, molecular fingerprinting was done on the isolates provided by the collaborators to ADRIA**. The observed fingerprints (See Figure 1) fit perfectly with the inoculated strain fingerprint, confirming thus the cross contaminations.**

Figure 1 - Molecular fingerprints comparison



A00C060: fingerprint of the inoculated strain
B18 to O22: fingerprints of the isolates from the related samples B18 to O22, with B to O, the lab codes.

At least, two types of contaminations are observed:

- At the sub-culture step in the reference method protocol;
- At the DNA extraction or PCR steps in the alternative method protocol.

4.3.3 Results of the collaborators retained for interpretation

For the initial validation study, all the Labs with cross contamination at the subculture step were excluded for interpretation:

- Manual extraction protocol: Labs B, H, I, N and O;
- Automated extraction protocol: Labs B, H, I, N and O.

ADRIA data were taken into account in the interpretation; 10 data sets were thus used with the Manual extraction protocol and 12 with the automated protocol. This decision was stated with the MicroVal Referees and Technical Committee.

According to the ISO 16140-2:2016, the results of the organizing laboratory are excluded from interpretation. It is thus no more possible to use these data; it is proposed to keep the results from lab N which obtained only one positive result at Level 0 with the reference method for the Manual extraction protocol.

The results obtained with the labs kept for interpretation are presented in **Table 32** (reference method) and **Table 33** (alternative method) for the manual extraction protocol (10 Labs) and in **Tables 34 and 35** for the automated extraction protocol (11 Labs).

Table 32 - Positive results by the reference method (Without labs which had confirmed positive results on blank samples (10) except Lab N)

Manual extraction protocol

Laboratory	Reference method		
	Level contamination		
	L0	*L1*	*L2*
C	0	8	8
D	0	8	8
E	0	6	8
F	0	8	8
G	0	8	8
J	0	8	8
K	0	8	8
L	0	8	8
M	0	8	8
N	1	8	8
Total	**P_0 = 1**	**P_1 = 78**	**P_2 = 80**

Table 33 - Positive results (before and after confirmation) by the alternative method (Without labs which had confirmed positive results on blank samples (10) except Lab N) - Manual extraction protocol

Laboratory	Alternative method-Manual extraction protocol								
	Contamination level								
	L0			*L1*			*L2*		
	First PCR result	*Confirmation result*	*Final result*	*PCR result*	*Confirmation result*	*Final result*	*PCR result*	*Confirmation result*	*Final result*
C	0	0	0	8	8	8	8	8	8
D	0	0	0	8	8	8	8	8	8
E	1	0	0	6	6	6	8	8	8
F	0	0	0	8	8	8	8	8	8
G	0	0	0	8	8	8	8	8	8
J	0	0	0	8	8	8	8	8	8
K	4	0	0	8	8	8	8	8	8
L	0	0	0	8	8	8	8	8	8
M	0	0	0	8	8	8	8	8	8
N	1	1	0	8	8	8	8	8	8
Total	**P_0 = 6**	**1**	**CP_0 = 0**	**P_1 = 78**	**78**	**CP_1 = 78**	**P_2 = 80**	**80**	**CP_2 = 80**

Table 34 - Positive results by the reference method (Without labs which had confirmed positive results on blank samples (11))

Automated extraction protocol

Laboratory	Reference method		
	Level contamination		
	L0	***L1***	***L2***
A1	0	8	8
A2	0	8	8
C	0	8	8
D	0	8	8
E	0	6	8
F	0	8	8
G	0	8	8
J	0	8	8
K	0	8	8
L	0	8	8
M	0	8	8
Total	**P_0 = 0**	**P_1 = 86**	**P_2 = 88**

Table 35 - Positive results (before and after confirmation) by the alternative method (Without labs which had confirmed positive results on blank samples (11) -

Automated extraction protocol

Laboratory	Alternative method-Automated extraction protocol								
	Contamination level								
	L0			***L1***			***L2***		
	First PCR result	*Confirmation result*	*Final result*	*PCR result*	*Confirmation result*	*Final result*	*PCR result*	*Confirmation result*	*Final result*
A1	0	0	0	8	8	8	8	8	8
A2	0	0	0	8	8	8	8	8	8
C	0	0	0	8	8	8	8	8	8
D	7	0	0	8	8	8	8	8	8
E	0	0	0	6	6	6	8	8	8
F	2	0	0	8	8	8	8	8	8
G	0	0	0	8	8	8	8	8	8
J	0	0	0	8	8	8	8	8	8
K	8	0	0	8	8	8	8	8	8
L	0	0	0	8	8	8	8	8	8
M	0	0	0	8	8	8	8	8	8
Total	**P_0 = 17**	**0**	**CP_0 = 0**	**P_1 = 86**	**86**	**CP_1 = 86**	**P_2 = 88**	**88**	**CP_2 = 88**

4.3.4 *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 36**).

Table 36 - Percentage specificity

		Manual extraction protocol	Automated extraction protocol
Specificity for the reference method	$SP_{ref} = \left(1 - \left(\frac{P_0}{N\_}\right)\right) x\ 100\ \% =$	98.8 %	100.0 %
Specificity for the alternative method	$SP_{alt} = \left(1 - \left(\frac{CP_0}{N\_}\right)\right) x\ 100\ \% =$	100.0 %	100.0 %

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

4.3.5 *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 level (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 method (Manual and automated extraction protocols) for Level 1 is provided in **Table 37**.

Table 37 - 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-)
Manual extraction protocol	Alternative method positive (A+)	Positive agreement (A+/R+) **PA = 78**	Positive deviation (R-/A+) **PD = 0**
	Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 0 (PPND = 0)**	Negative agreement (A-/R-) **NA = 2 (PPNA = 0)**
Automated extraction protocol	Alternative method positive (A+)	Positive agreement (A+/R+) **PA = 86**	Positive deviation (R-/A+) **PD = 0**
	Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 0 (PPND = 0)**	Negative agreement (A-/R-) **NA = 2 (PPNA = 0)**

Based on the data summarized in **Table 37**, 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 38**).

Table 38 - Sensitivity, relative trueness and false positive ratio percentages

		Manual extraction protocol	Automated extraction protocol
Sensitivity for the alternative method:	$SE_{alt} = \frac{(PA+PD)}{(PA+PD+ND)} \; x \; 100\% =$	100.0 %	100.0 %
Sensitivity for the reference method:	$SE_{ref} = \frac{(PA+ND)}{(PA+PD+ND)} \; x \; 100\% =$	100.0 %	100.0 %
Relative trueness	$RT = \frac{(PA+NA)}{N} \; x \; 100\% =$	100.0 %	100.0 %
False positive ratio for the alternative method	$FPR = \frac{FP}{NA} \; x \; 100\% =$	0 %	0 %

4.3.6 Interpretation of data

No negative and positive deviations were observed for Level 1 during this study.

For a **paired study design**, the difference between (ND – PD) and the addition (ND + PD) are calculated for the level(s) where fractional recovery is obtained (L_1). The observed value found for (ND – PD) and (ND + PD) shall not be higher than the AL.

The limits are the following (See **Table 39**).

Table 39

	Manual extraction protocol (10 Labs)			Automated extraction protocol (11 Labs)		
	Calculated values	AL	Conclusion	Calculated values	AL	Conclusion
ND - PD	0	3	ND - PD < AL	0	3	ND - PD < AL
ND + PD	0	4	ND - PD < AL	0	4	ND - PD < AL

The EN ISO 16140-2:2016 requirements are fulfilled as (ND - PD) and (ND + PD) are below the AL.

There is indeed no difference between the sensitivity of the compared methods, and the alternative method complies with the reproducibility conditions.

4.3.7 Evaluation of the RLOD between laboratories

The RLOD was calculated with the data from the collaborators kept for interpretation (detailed in 4.3.3) 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 40**).

Table 40 - RLOD

Name	RLOD	RLODL	RLODU	b=ln(RLOD)	sd(b)	z-Test statistic	p-value
Manual extraction protocol	1.036	0.609	1.762	0.035	0.265	0.133	0.894
Automated extraction protocol	1.000	0.625	1.600	0.000	0.235	0.000	1.000

5 GENERAL CONCLUSION

The **method comparison study conclusions** are:

☒ The method comparison study scheme corresponds to a PAIRED STUDY design as the alternative and reference methods have a common enrichment procedure.

☒ In the sensitivity study, 7 categories were tested for the Manual extraction protocol: 5 food categories, one feed category and one primary production samples category. The protocol of the alternative method shows 2 positive deviations (PD) and 5 negative deviations (ND) for the overall categories.
For the automated extraction protocol, 6 categories were tested: 5 food categories and feed. The protocol of the alternative method shows 2 positive deviations and 5 negative deviations.
The ND - PD and ND + PD meet the acceptability limits (AL) for each individual category and all the categories; the ND + PD meet the AL for all the categories

☒ The RLOD meet the AL fixed at 1.5 for a paired study design for all the tested matrix/strain pairs for the manual and automated extraction protocols for the four thermocyclers tested.

☒ The inclusivity and exclusivity testing did give the expected results for the 100 target strains and the 30 non-target strains.

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

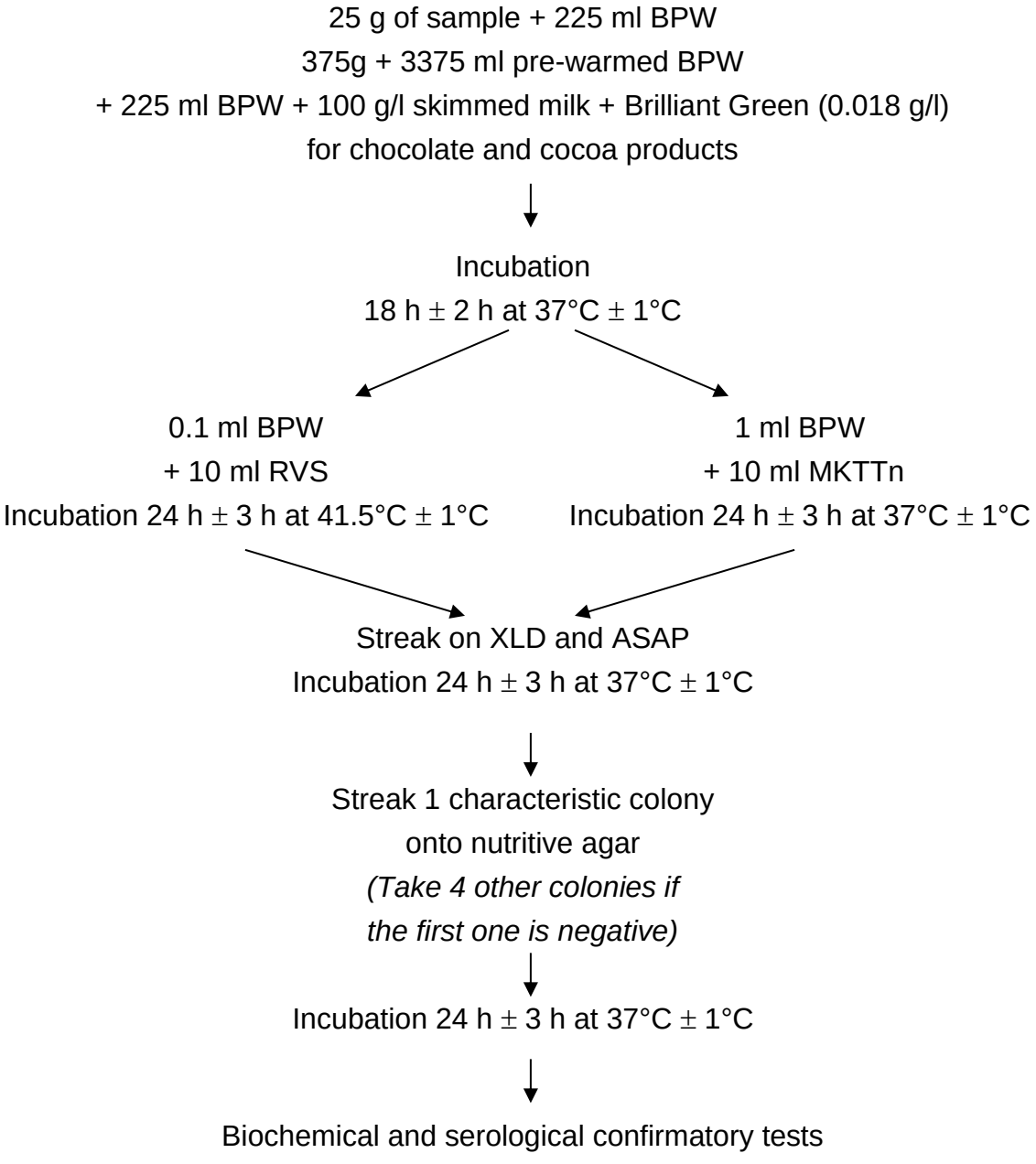
☒ The data and interpretations comply with the EN ISO 16140-2:2016 requirements. **The food**proof® ***Salmonella spp.*** **method is considered equivalent to the ISO standard.**

Quimper, 30 October 2019

Sarah PERON	Maryse RANNOU
Technical Study Manager	Project Manager
Validation of Alternative methods	Validation of Alternative methods
Food Safety & Quality	*Food Safety & Quality*
I hereby attest to the validation of the results of the analyses carried out under the COFRAC accreditation.	I hereby attest to the validation of the verification of the conformity of the report (opinion and interpretation).

Annex A – Flow diagrams of the reference methods EN ISO 6579:2002 and EN ISO 6579/A1 (2007) used for the validation study

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



EN ISO 6579/A1 (2007) reference method: Horizontal method for the detection of *Salmonella* spp. - Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage

25 g sample + 225 ml BPW

↓

Incubation at 37°C ±1°C for 18 h ± 2 h

↓

Inoculation of MSRV with 3 drops of BPW (a total of 0.1 ml)

↓

Incubation at 41.5°C ± 1°C for 24 h ± 3 h

↓

Screening for suspect growth of *Salmonella* spp.
Incubation for 24 h ± 3 h in case of negative test results

↓

Streak on XLD and ASAP

↓

Incubation at 37°C ±1°C for 24 h ± 3 h

↓

Streak 1 colony
onto nutritive agar
(Take 4 other colonies if
the first one is negative)

↓

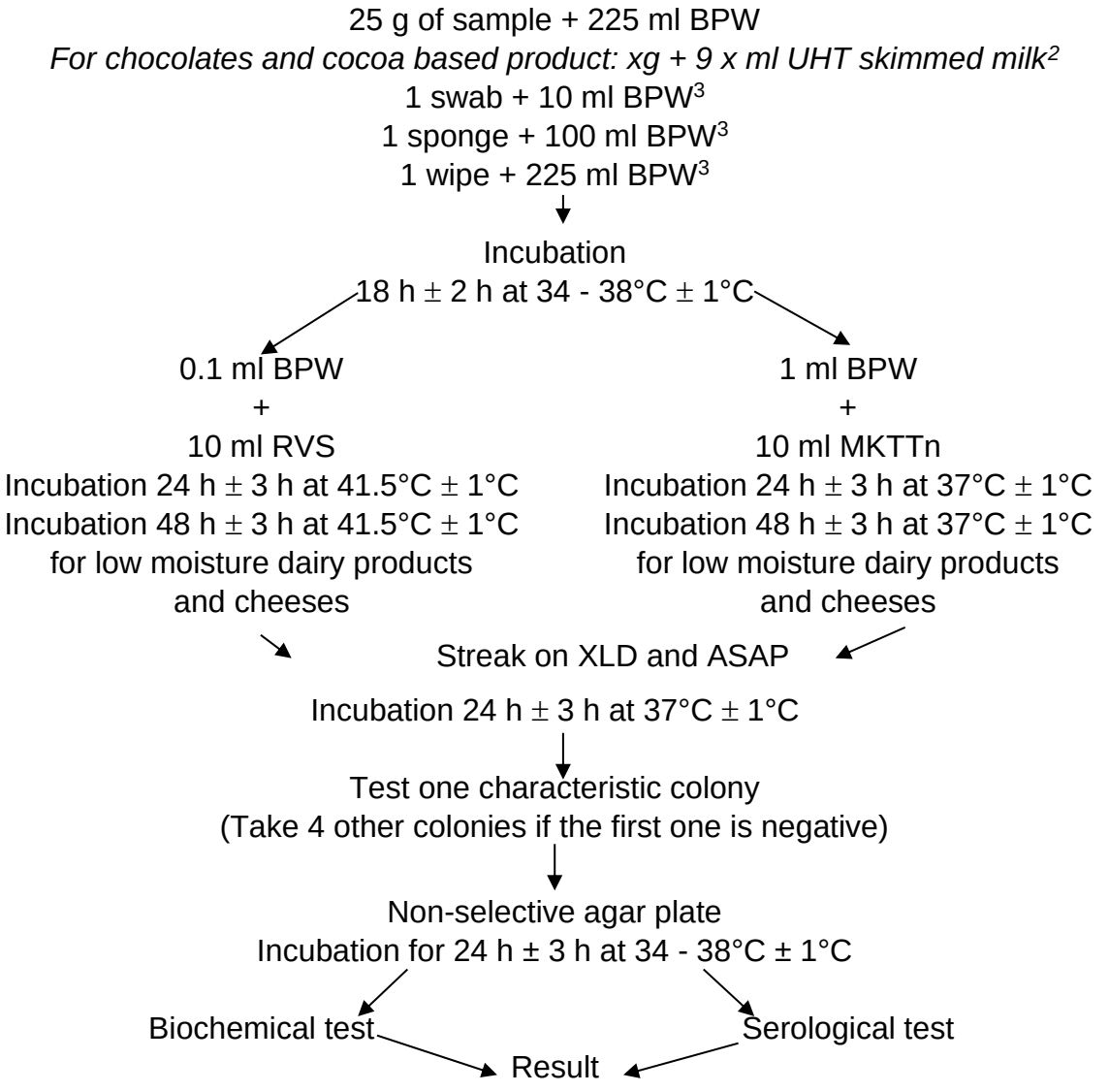
Incubation 24 h ± 3 h at 37°C ± 1°C

↓

Biochemical and serological confirmation

ISO 6579-1 (February 2017): Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* - Part 1: detection of *Salmonella* spp.

Food, feed and environmental samples: Preparation according to ISO 6887-1 to 5
Chocolate and cocoa based products: Cf. ISO 6887-4

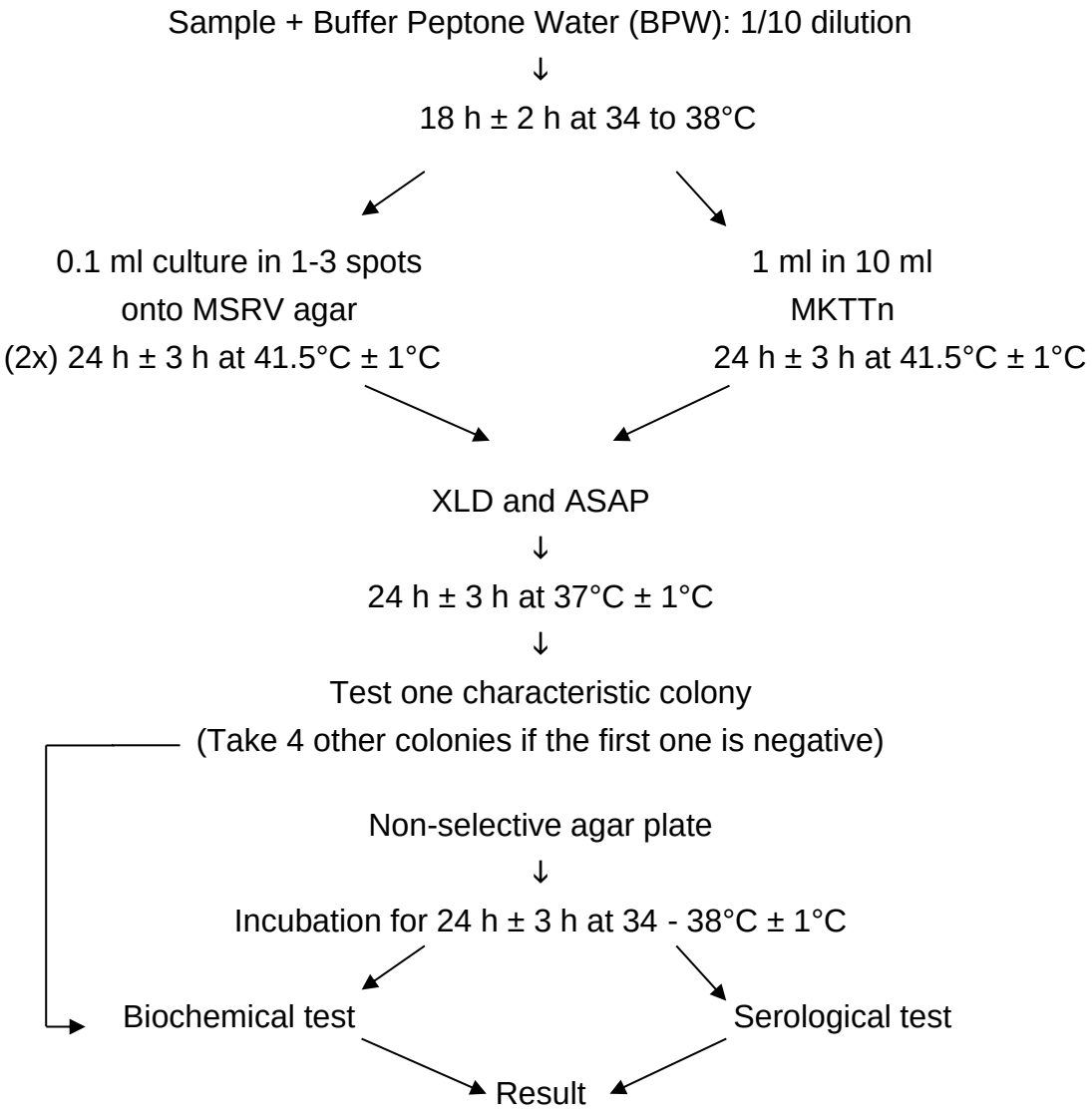


2 For chocolates products containing > 20 % fat, unless the products already contain sufficient emulsifier, add Tween 80. Optional: Brilliant Green 0.018 g/l

3 For sampling after cleaning process pre-moisten
- 1 swab + 1 ml broth universal neutralizing (+ 9 ml BPW)
- 1 sponge + 10 ml broth universal neutralizing (+ 90 ml BPW)
- 1 wipe + BPW + 10 % neutralizing agent (+ 225 ml BPW)

Primary production samples: feces and environmental samples

Preparation according to ISO 6877-6



Annex B – Flow diagrams of the alternative method: **food**proof® ***Salmonella*** **spp.**

Protocol ②

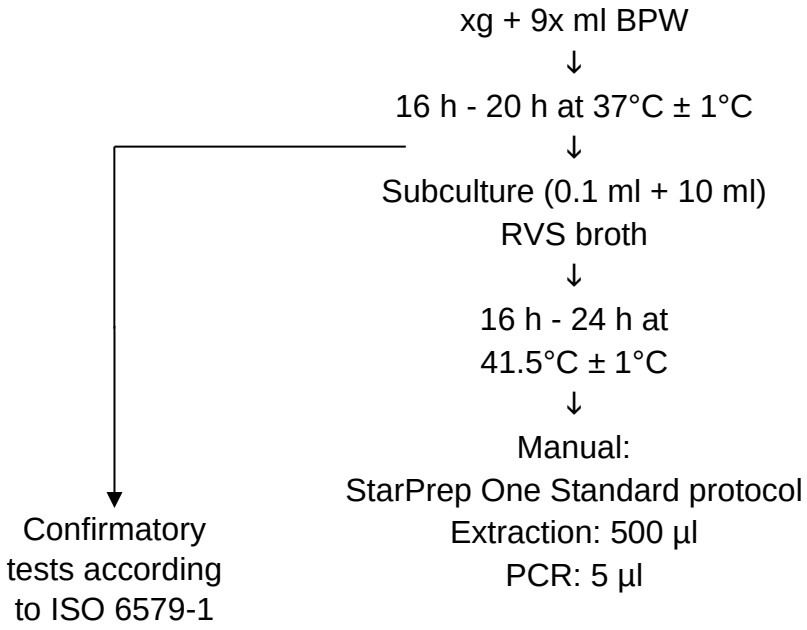
Chocolate and bakery products (25 g)
Meat and meat products (25 g or 375 g)
Milk and dairy products (25 g)
Egg products (25 g)
Feed samples (25 g

xg + 9x ml BPW
↓
20 h ± 2 h at 37°C ± 1°C
↓
Subculture (1 ml + 9 ml)
pre-warmed BHI at 37°C
3h
↓
Manual:
StarPrep One Standard protocol
Extraction: 100 µl
PCR: 5 µl

→ Confirmatory tests according to ISO 6579-1

Protocol PPS

Primary production samples (25 g or sampling device)



Protocol ⑦

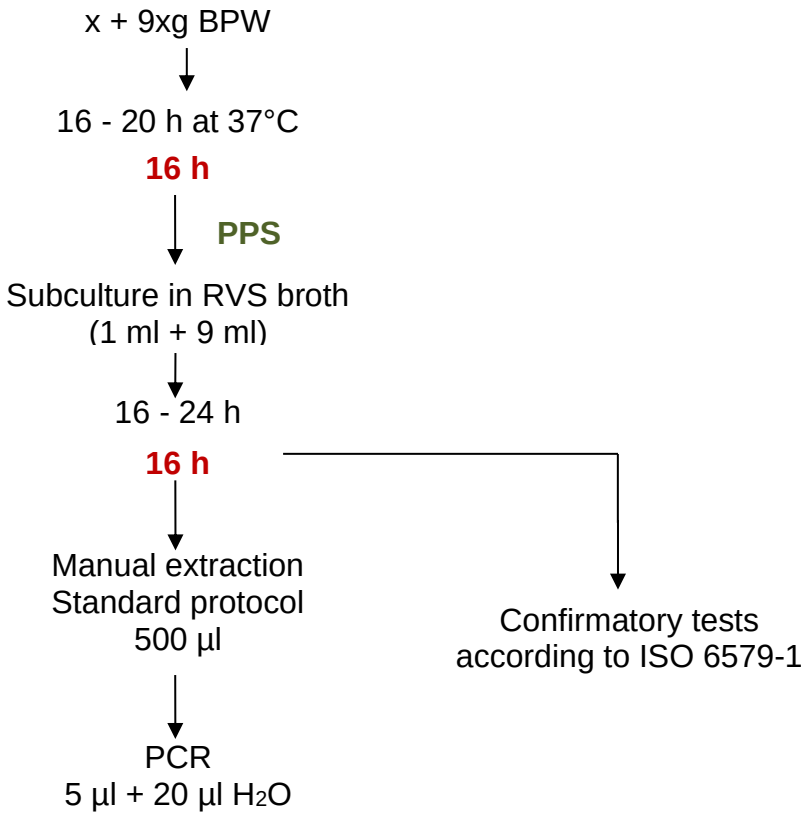
Chocolate and bakery products (25 g)
Meat and meat products (25 g or 375 g)
Milk and dairy products (25 g)
Egg products (25 g)
Feed samples (25 g

xg + 9x ml BPW
↓
19 h - 20 h at 37°C ± 1°C
↓
Automated:
MagPrep I Standard protocol flex
Extraction: 200 µl
PCR: 5 µl

→ Confirmatory tests according to ISO 6579-1

Kits: foodproof® *Salmonella* Detection Kit - 5'Nuclease - Protocols per category
foodproof® *Salmonella* Detection Kit - Hybridization Probes (LC 1.x, 2.0, 480 II)

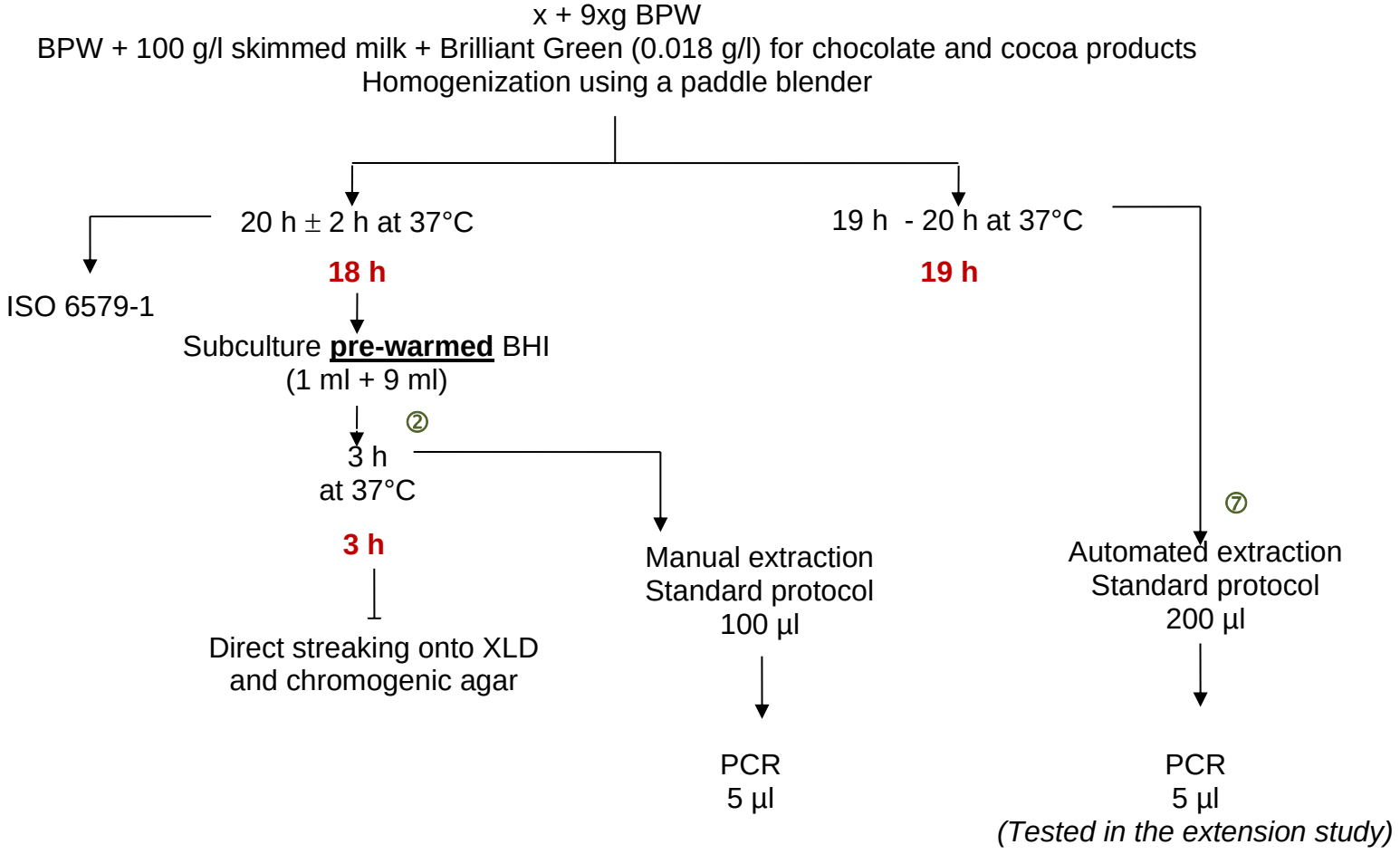
Primary production samples (Paired study design)



Possibility to store the DNA extracts at -20°C

Kits: foodproof® *Salmonella* Detection Kit - 5' Nuclease - Protocols per category
foodproof® *Salmonella* Detection Kit - Hybridization Probes (LC 1.x, 2.0, 480 II)

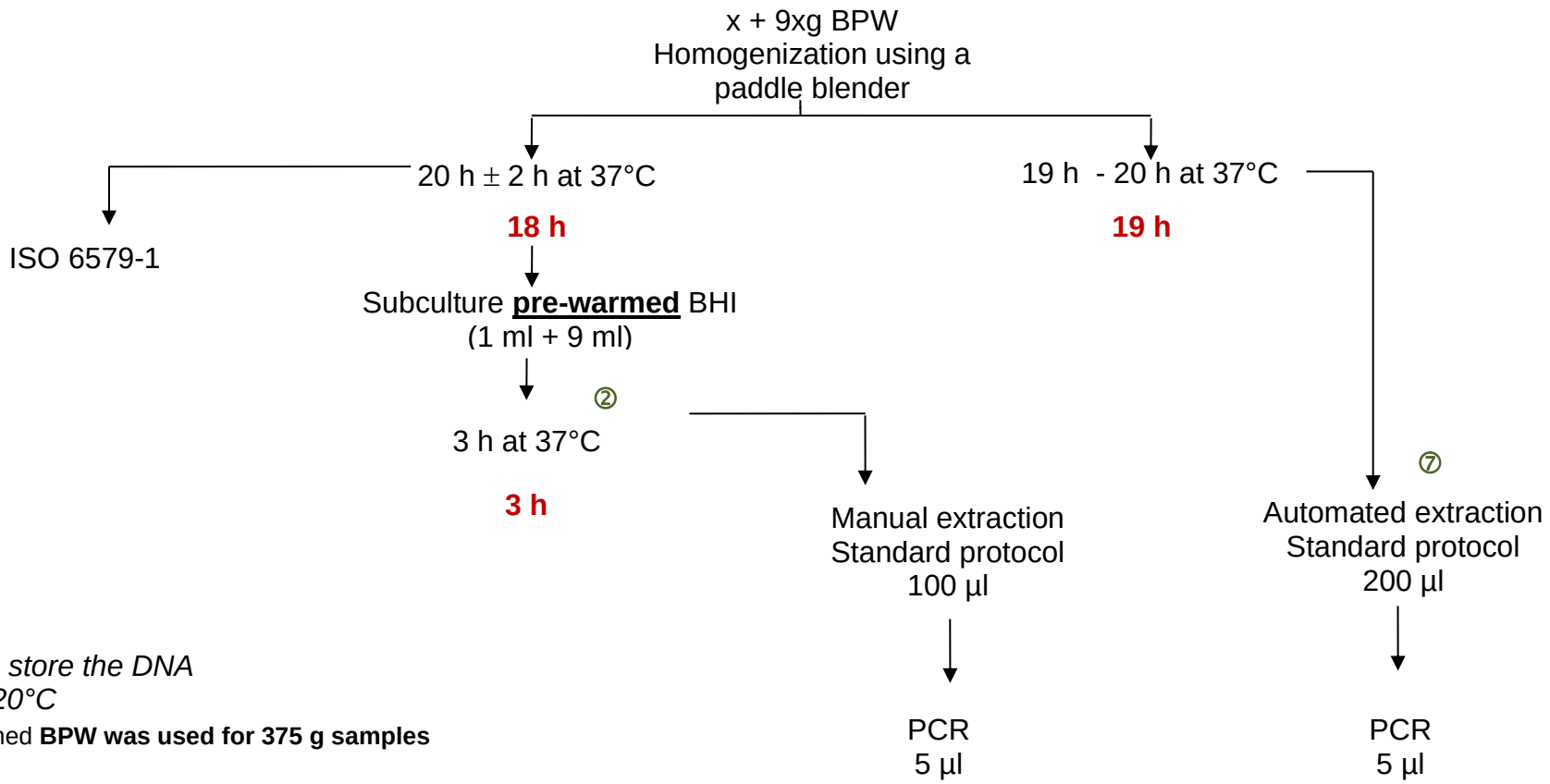
Chocolate and bakery products (25 g) (Paired study design)



Possibility to store the DNA extracts at -20°C

Kits: foodproof® *Salmonella* Detection Kit - 5'Nuclease - Protocols per category
foodproof® *Salmonella* Detection Kit - Hybridization Probes (LC 1.x, 2.0, 480 II)

Meat and meat products (25 g), Raw beef meat (375 g) (Paired study design)
Milk and dairy products (25 g) (Paired study design)
Egg products (25 g) (Paired study design)
Feed products (25 g) (Paired study design)



Possibility to store the DNA extracts at -20°C

Note: Pre-warmed **BPW was used for 375 g samples**

Extraction protocols

foodproof® StarPrep One Kit

Standard Manual protocol

Shake the enrichment culture gently and let settle
↓
Transfer 100 µl (supernatant) (500 µl for PPS) in a 1.5 ml reaction tube
↓
Centrifuge for 5 min at 8000 g
↓
Remove the supernatant
↓
Add 200 µl lysis buffer
↓
Resuspend the pellet by vortex or by pipetting gently up and down
↓
Heat treatment for 10 min at 95-100°C in a heating block
↓
Keep 1 min at 15 - 25°C
↓
Mix by vortexing for 2 sec
↓
Centrifuge for 2 min at 13000 g
↓
PCR on 5 µl DNA extract

foodproof® Magnetic Preparation Kit I

Automated protocol

Using the foodproof Magnetic Preparation Kit in combination
with the KingFisher® Flex workstation

Preparation of kit working solutions

Binding Buffer: add 80 ml absolute isopropanol
Wash Buffer I: add 154 ml absolute isopropanol
Wash Buffer II: add 164 ml absolute isopropanol

Resuspend the lysis buffer and the magnetic beads in the Binding Buffer
Place the Tip Comb 96 WH on a Tip Plate

Prefill:
Lysis plate: add 320 µl lysis buffer and 25 µl Reagent P (if necessary)[4]
Washing plate I: add 750 µl Wash Buffer I
Washing plate II: add 750 µl Wash Buffer II
Washing plate II: add 750 µl Wash Buffer II
Elution plate: add 300 µl Elution Buffer

Transfer 200 µl of the enriched sample into the lysis plate

Choose assay file “foodproof-MPK-I” on Instrument and press Start

After an elevated lysis step of 10 min, take out the plate and add 315 µl Binding Buffer. Reinsert the plate and press Start

PCR on 5 µl DNA extract

[4] For protein rich food samples (e.g. egg, pork, chicken salmon, cheese), addition of Reagent P to the Lysis Buffer is necessary.

ADRIA FOOD EXPERTISE

cofrac ESSAIS (TESTING)

ACCREDITATION
N°1-0144
scope available
on www.COFRAC.fr

ISO 16140-2:2016 validation study of foodproof® *Salmonella* spp. detection method for the detection of *Salmonella* spp. in food, feed and primary production samples

MicroVal study number	**2011LR40 and 2011LR42**
Method/Kit name	**foodproof® *Salmonella* spp. Detection method - LyoKit - 5'Nuclease**
Study Project version	**Summary Report - Version 1** 30 October 2019
MicroVal Expert Laboratory	ADRIA Développement ZA Creac'h Gwen F-29196 QUIMPER Cedex (France)

This project consists of 56 pages, including 2 appendices.
Only copies including the totality of this document are authorised.

Competencies of the laboratory are certified by COFRAC accreditation for the analyses marked with the symbol◆.

Confidential

Standardized protocol - Qualitative methods

MICROVAL® NEN

Foreword

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

Company	**BIOTECON Diagnostics GmbH** Hermannswerder 17 D-14473 Potsdam (Germany)
Expert Laboratory	**ADRIA Développement** ZA Creac'h Gwen F-29196 QUIMPER Cedex (France)
Method/Kit name	**foodproof® *Salmonella* spp. detection method** **LyoKit - 5'Nuclease**
Validation standard	▪ ISO 16140-1 (2016): Microbiology of the food chain - Method validation — *Part 1: Vocabulary* ▪ ISO 16140-2 (2016): Microbiology of the food chain - Method validation — *Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method*
Reference method♦	ISO 6579-1 (February 2017) - Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* - Part 1: detection of *Salmonella* spp
Scope of validation	☒ **Broad range of food** ☒ **Feed** ☒ **Primary production samples for manual extraction protocol**
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	Inter-laboratory Study
LC	LightCycler 480
m	Minority level of target analyte
M	Majority level of target analyte
MCS	Method Comparison Study
min	minute
MKTTn	Muller-Kauffmann Tetrathionate-novobiocin broth
ml	Milliliter
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
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)
PPS	Primary production samples
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

1 INTRODUCTION

The **food**proof® *Salmonella* spp. detection method was validated on the 26th June 2013 for:

- a manual extraction protocol (Certificate number: 2011LR42) using the **food**proof® StarPrep One Kit:
    - Chocolate and bakery products
    - Meat and meat products
    - Milk and dairy products
    - Egg products
    - Raw beef meat (375 g sample size)
    - Feed samples
    - Primary production samples

- An automated extraction protocol (Certificate number: 2011LR40) using the **food**proof® Magnetic Preparation Kit I:

    - Meat and meat products
    - Milk and dairy products
    - Egg products
    - Raw beef meat (375 g sample size)
    - Feed samples.

The following thermocycler / Detection kit pairs were tested:

- LightCycler 2.0 (Roche Diagnostics) / **food**proof® *Salmonella* Detection Kit - Hybridization Probes (LC 1.x, 2.0, 480 II) No R 310 27;
- LightCycler 480 (Roche Diagnostics) / **food**proof® *Salmonella* Detection Kit - Hybridization Probes (LC 1.x, 2.0, 480 II) No R 310 27;
- Stratagene Mx3005P (Agilent) / **food**proof® *Salmonella* Detection Kit - 5'Nuclease - No R 302 27 or R 302 27 L;
- CFX96 (Bio-Rad) / **food**proof® *Salmonella* Detection Kit - 5'Nuclease - No R 302 27 or R 302 27 L.

The renewal was obtained for these kits in May 2019 for the Manual extraction protocol and in September 2019 for the Automated extraction protocol with an extension for the Chocolate and Bakery products category.

In September 2019, an extension was obtained for a new kit: the foodproof® ***Salmonella*** **Detection LyoKit - 5' Nuclease**. This kit provides lyophilized components for the PCR assays but can be considered equivalent to **food**proof® *Salmonella* Detection Kit regarding the primers, probes, Taq Polymerase, temperature profile and performances (see attached declaration of equivalence). This extension study was carried out in combination with:

* **A manual extraction protocol** (**food**proof® StarPrep One Kit) for:
  - Chocolate and bakery products;
  - Meat and meat products;
  - Milk and dairy products;
  - Egg products;
  - Fish and seafood products;
  - Feed samples;
  - Primary production samples;

* **An automated extraction protocol** (**food**proof® Magnetic Preparation Kit I) for:
  - Chocolate and bakery products;
  - Meat and meat products;
  - Milk and dairy products;
  - Egg products;
  - Fish and seafood products
  - Feed samples;

In agreement with the MVTC only **two thermocyclers** have been tested for this extension study:

- CFX96 (Bio-Rad);
- LightCycler 480 (Roche Diagnostics);

both using the **food**proof® *Salmonella* Detection LyoKit - 5' Nuclease.

The following categories are claimed for the **food**proof® *Salmonella* Detection LyoKit - 5'Nuclease (See **Table 1**).

Table 1 - Scope of the foodproof® ***Salmonella* Detection LyoKit - 5' Nuclease**

Extraction		Manual foodproof StarPrep One Standard protocol		Automated MagPrep I Standard protocol	
Detection		**food**proof® *Salmonella* detection LyoKit (5' Nuclease)		**food**proof® *Salmonella* detection **Lyo**Kit (5' Nuclease)	
Thermocycler		LC 480	CFX96	LC 480	CFX96
Scope	Chocolate and bakery products	X	X	X	X
	Meat and meat products	X	X	X	X
	Milk and dairy products	X	X	X	X
	Egg products	X	X	X	X
	Fish and seafood products	X	X	X	X
	Feed samples	X	X	X	X
	PPS	X	X		

ADRIA Développement run the study with the Bio-Rad CFX96 thermocycler and sent **blind coded DNA extracts** to BIOTECON Diagnostics for testing with the LightCycler 480.

BIOTECON Diagnostics provided the results to ADRIA for interpretation.

2 METHOD PROTOCOLS

2.1 Reference methods◆

The reference method used for the extension study was the ISO 6579-1:2017 - Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* - Part 1: detection of *Salmonella* spp. (See **Annex A**).

◆ Analysis performed according to the COFRAC accreditation

Samples preparation used in the reference and the alternative method were done according to ISO 6887 parts 1 to 6:

- Part 1: General rules for the preparation of the initial suspension and decimal dilutions
- Part 2: Specific rules for the preparation of meat and meat products
- Part 3: Specific rules for the preparation of fish and fishery products
- Part 4: Specific rules for the preparation of miscellaneous products
- Part 5: Specific rules for the preparation of milk and milk products
- Part 6: Specific rules for the preparation of samples taken at the primary production stage

2.2 Alternative method

Principle

The **food**proof® *Salmonella* spp. method is a real-time PCR kit for *Salmonella* spp. detection in foods (categories depending on the extraction and detection protocols) and feeds, as well as primary production samples (for manual extraction protocol). Three PCR kits are available:

- **food**proof® *Salmonella* Detection Kit - 5' Nuclease, using real-time PCR instruments (liquid assay);
- **food**proof® *Salmonella* Detection Kit - Hybridization Probes, using the LightCycler 1.x 2.0 or 480 instruments (liquid assay);
- **food**proof® *Salmonella* Detection Lyokit - 5' Nuclease, using real-time PCR instruments (lyophilized assay).

This extension study concerns only the lyophilized kit.

After DNA extraction (See **Table 2**) with the **food**proof® StarPrep One Kit (manual protocol), **or** the **food**proof® Magnetic Preparation Kit I (automated protocol), specific fluorescent oligonucleotide probes are used to detect target DNA during the amplification, by hybridizing to the amplicons. These fluorescent probes are linked to a fluorophore which fluoresces only when hybridized to the target sequence.

Table 2 - Categories, extraction and PCR kits tested for the extension study

		DNA extraction kit	
		Manual foodproof® StarPrep One kit	**Automated foodproof® Magnetic Preparation kit**
Categories	Chocolate and bakery products	X	X
	Meat and meat products	X	X
	Milk and dairy products	X	X
	Egg products	X	X
	Fish and seafood products	X	X
	Feed samples	X	X
	Primary production samples	X	/
PCR kit	CFX96 [1]	**food**proof® *Salmonella* Detection Lyokit - 5' Nuclease	
	LightCycler 480 [1]		

Protocols

The kit inserts are:

- **food**proof® **StarPrep One Kit: manual extraction protocol** No S 400 07 or S 400 07 L;
- **food**proof® **Magnetic Preparation Kit I** No S 400 11 L;
- **food**proof® ***Salmonella*** **Detection LyoKit - 5'Nuclease** No R 602 27-1 / R 602 27-2 or No R 602 27-1 L / R 602 27-2 L.

Different protocols are available depending on the kits used and the tested categories. The protocols are described in Annex B, as well as the protocols to apply per category; they are summarized in Table 3.

[1] Only these two thermocyclers were tested for the extension study.

The different steps are the following:

- Enrichment in appropriate conditions according to the protocol tested
- Extraction
- Confirmation of positive PCR results by the tests described in the ISO 6579-1

Note that the final volume for PCR is 25 µl, this 25 µl final volume is obtained by using 25 µl of lysates with all protocols except for:

- cocoa samples (5 µl of lysates + 20 µl of H_2O PCR-grade)
- faeces and bootsocks samples (5 µl of lysates + 20 µl of H_2O PCR-grade)

Table 3 - Summary of the protocols used in the extension study depending on the tested categories and tested kits

		foodproof® *Salmonella* Detection LyoKit (5'Nuclease)			
Protocol N°		①	⑧	**PPS**	⑦
DNA extraction procedure		Manual: StarPrep One, Standard protocol		Manual: StarPrep One, 500 µl/ protocol PPS	Automated: MagPrep I Standard protocol flex
Enrichment step		**18 - 20 h BPW 37°C ± 1 °C**	**18 - 20 h BPW 37°C ± 1 °C + 3 h pre-warmed BHI 37°C**	**16 - 20 h BPW 37°C ± 1 °C + 16 - 24 h RVS 41.5°C ± 1 °C**	**19 h - 20 h BPW 37°C ± 1 °C**
Categories	Chocolate and bakery product 25 g	X	X	/	X
	Meat and meat product 25 g	X	X	/	X
	Milk and dairy product 25 g	X	X	/	X
	Egg products 25 g	X	X	/	X
	Fish and seafood products 25g	X	X	/	X
	Feed samples 25 g	X	X	/	X
	Primary production samples 25 g	/	/	X	/
Enrichment volume for extraction		100µL	100µL	500µL	200µL
DNA extract volume to use for PCR		25 µL Cocoa: 5 µL + 20 µl H_2O	25 µL	5 µL + 20 µl H_2O	25 µL

2.3 Study design

As the reference and the alternative methods share the initial pre-enrichment step, the same test portion (Item) was used for the two methods, all resulting data were treated as paired data (EN ISO 16140-2:2016).

3 METHOD COMPARISON STUDY

The method comparison study is a study performed by the expert laboratory to compare the alternative method with the reference method.

3.1 Sensitivity study

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

3.1.1 Categories and sample types

A total of seven categories were included in this validation study: five food categories, one feed category and one primary production samples category (for manual extraction protocol only).

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.

The categories, the types and the number of samples analyzed are presented in **Table 4**: Manual extraction protocol (Protocol 1), **Table 5**: Manual extraction protocol + subculture (protocols 8 and PPS) and **Table 6**: Automated extraction protocol (Protocol 7).

Table 4 - Distribution of positive and negative samples per tested category and type - Manual extraction protocol (P1)

Category		Type		Positive	Negative	Total
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	10	20
		b	Raw material	8	12	20
		c	Pastries	12	8	20
			Total	30	30	60
2	Meat and meat products	a	Fresh meat	9	13	22
		b	Fermented and cured meat products	11	14	25
		c	Cooked meat products and cooked delicatessen	10	10	20
			Total	30	37	67
3	Milk and dairy products	a	Pasteurized	12	9	21
		b	Raw	9	11	20
		c	Milk powder, infant formula	10	13	23
			Total	31	33	64
4	Egg products	a	Egg powders	8	16	24
		b	Pasteurized liquid eggs	12	8	20
		c	Egg-based products	11	9	20
			Total	31	33	64
5	Fish and seafood products	a	Raw	11	9	20
		b	Ready-to-eat	9	11	20
		c	Ready-to-reheat	11	11	22
			Total	31	31	62
6	Feed samples	a	Cattle feed	13	15	28
		b	Pet food	11	9	20
		c	Raw material	7	13	20
			Total	31	37	68
All categories (food and feed)				**184**	**201**	**385**

Table 5 - Distribution of positive and negative samples per tested category and type - Manual extraction protocol + subculture (BHI or RVS for PPS) (P8 and PPS)

Category		Type		Positive	Negative	Total
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	10	20
		b	Raw material	8	12	20
		c	Pastries	12	8	20
			Total	30	30	60
2	Meat and meat products	a	Fresh meat	9	13	22
		b	Fermented and cured meat products	11	14	25
		c	Cooked meat products and cooked delicatessen	10	10	20
			Total	30	37	67
3	Milk and dairy products	a	Pasteurized	12	9	21
		b	Raw	9	11	20
		c	Milk powder, infant formula	10	13	23
			Total	31	33	64
4	Egg products	a	Egg powders	8	16	24
		b	Pasteurized liquid eggs	12	8	20
		c	Egg-based products	11	9	20
			Total	31	33	64
5	Fish and seafood products	a	Raw	11	9	20
		b	Ready-to-eat	9	11	20
		c	Ready-to-reheat	11	11	22
			Total	31	31	62
6	Feed samples	a	Cattle feed	13	15	28
		b	Pet food	11	9	20
		c	Raw material	7	13	20
			Total	31	37	68
All categories (food and feed)				184	201	385
7	PPS	a	Animal feces	16	14	30
		b	Environmental samples and non-feces	15	28	43
			Total	31	42	73
All categories (food, feed and PPS)				**215**	**243**	**458**

Table 6 - Distribution of positive and negative samples per tested category and type - Automated extraction protocol (P7)

Category		Type		Positive	Negative	Total
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	10	20
		b	Raw material	8	12	20
		c	Pastries	12	8	20
			Total	30	30	60
2	Meat and meat products	a	Fresh meat	9	13	22
		b	Fermented and cured meat products	11	14	25
		c	Cooked meat products and cooked delicatessen	10	10	20
			Total	30	37	67
3	Milk and dairy products	a	Pasteurized	12	9	21
		b	Raw	9	11	20
		c	Milk powder, infant formula	10	13	23
			Total	31	33	64
4	Egg products	a	Egg powders	8	16	24
		b	Pasteurized liquid eggs	12	8	20
		c	Egg-based products	11	9	20
			Total	31	33	64
5	Fish and seafood products	a	Raw	11	9	20
		b	Ready-to-eat	9	11	20
		c	Ready-to-reheat	11	11	22
			Total	31	31	62
6	Feed samples	a	Cattle feed	13	15	28
		b	Pet food	11	9	20
		c	Raw material	7	13	20
			Total	31	37	68
All categories (food and feed)				**184**	**201**	**385**

A total of 385 samples were tested providing 184 positive and 201 negative results for the Manual extraction protocol (Protocol 1) and automated extraction protocol (Protocol 7).

458 samples were tested providing 215 positive and 243 negative results for the manual protocol with a subculture step (Protocol 8 and PPS).

3.1.2 Test sample preparation

Artificial contaminations were done by spiking or seeding protocols:

- **Spiking** with injured cells (heat treatment); the stress level was determined by plating onto selective and non-selective agars (TSYEA and XLD plates).

- **Seeding by:**
  * Direct inoculations of high moisture matrices using liquid cell suspensions, followed by storage periods of 48 h-72 h at 4°C, or at -20°C for 2 weeks,
  * Lyophilized strains mixed with low moisture matrices, followed by a storage period of 1 to 2 week(s) at ambient temperature.

A same strain cannot be used to inoculate more than 6 samples.

For food and feed samples: 271 samples were artificially contaminated, 166 gave positive results either by the manual extraction protocol or the automated protocol. 9.8 % of the samples were naturally contaminated.

For primary production samples: 49 samples were artificially contaminated; 25 gave positive results. 19.4 % of the samples were naturally contaminated.

For the manual extraction protocol, when all the categories are taken into account, the number of naturally contaminated samples represents 11.2 %.

For the automated protocol, the number of naturally contaminated samples represents 9.8 %.

The repartition of the positive, naturally and artificially contaminated samples is given in **Table 7**.

Table 7 - Repartition of the positive, natural and artificial contamination samples

Protocol	Categories		Naturally contaminated	Artificially contaminated						Total
				Seeding protocol			Spiking protocol			
				≤3	3<x≤10	>10	≤5	5< x<10	>10	
Manual extraction protocol	Food and feed	Positive samples	18	93	33	2	38	0	0	184
		%	9,8	50,5	17,9	1,1	20,7	0,0	0,0	100,0
	PPS	Positive samples	6	14	11	0	0	0	0	31
		%	19,4	45,2	35,5	0,0	0,0	0,0	0,0	100,0
	All categories	Positive samples	24	107	44	2	38	0	0	215
		%	11,2	49,8	20,5	0,9	17,7	0,0	0,0	100,0
Automated extraction protocol	Food and feed	Positive samples	18	93	33	2	38	0	0	184
		%	9,8	50,5	17,9	1,1	20,7	0,0	0,0	100,0

3.1.3 Protocols applied during the validation study

Incubation time

The minimum incubation time was applied during the validation study as described in **Table 8**.

Table 8 - Incubation time applied for enrichment

	Category	Manual extraction protocol	Automated extraction protocol
Pre-enrichment step (BPW)	Food and feed	18 h	19 h
	Primary production samples	16 h	/
Enrichment step (BHI)	Food and feed	3 h	/
Enrichment step (RVS)	Primary production samples	16 h	/

Confirmations

During the validation study, the presumptive positive **food**proof® *Salmonella* tests were confirmed:

- Food and feed samples:

  Tests of the ISO 6579-1 method: subculture in RVS (0.1 ml + 10 ml, 24 h ± 3 h at 41.5°C± 1°C) and MKTTn (1 ml + 10 ml, 24 h ± 3 h at

37°C± 1°C) and streaking onto XLD and ASAP. Typical colonies were confirmed by biochemical and serological tests after a purification step;

- Primary production samples:

  Tests of the ISO 6579-1 method: subculture on MSRV (24 h to 48 h at 41.5°C ± 1°C) and into MKTTN (24 h at 41.5°C ± 1°C) and streaking onto XLD and ASAP. Typical colonies were confirmed by biochemical galleries and serological tests after purification step.

3.1.4 Sensitivity study results

Table 9 shows the summary of results of the reference method and the alternative methods for all Categories and both extraction protocols.

Table 9 - Summary of sensitivity study results –
All categories for both extraction protocols

			Reference method positive (R+)	Reference method negative (R-)
Manual extraction protocol without subculture	CFX96	+	Positive agreement (R+/A+) **PA = 180**	Positive deviation (R-/A+) **PD = 0**
		-	Negative deviation (R+/A-) **ND = 4 (PPND=0)**	Negative agreement (R-/A-) **NA = 201 (PPNA=18)**
	LightCycler 480	+	Positive agreement (R+/A+) **PA = 181**	Positive deviation (R-/A+) **PD = 0**
		-	Negative deviation (R+/A-) **ND = 3 (PPND=0)**	Negative agreement (R-/A-) **NA = 201 (PPNA=18)**
Manual extraction protocol with subculture	CFX96	+	Positive agreement (R+/A+) **PA = 211**	Positive deviation (R-/A+) **PD = 1**
		-	Negative deviation (R+/A-) **ND = 3 (PPND=0)**	Negative agreement (R-/A-) **NA = 243 (PPNA=10)**
	LightCycler 480	+	Positive agreement (R+/A+) **PA = 211**	Positive deviation (R-/A+) **PD = 1**
		-	Negative deviation (R+/A-) **ND = 3 (PPND=0)**	Negative agreement (R-/A-) **NA = 243 (PPNA=9)**
Automated extraction protocol	CFX96	+	Positive agreement (R+/A+) **PA = 151**	Positive deviation (R-/A+) **PD = 0**
		-	Negative deviation (R+/A-) **ND = 3 (PPND=0)**	Negative agreement (R-/A-) **NA = 171 (PPNA=8)**
	LightCycler 480	+	Positive agreement (R+/A+) **PA = 151**	Positive deviation (R-/A+) **PD = 0**
		-	Negative deviation (R+/A-) **ND = 3 (PPND=0)**	Negative agreement (R-/A-) **NA = 171 (PPNA=4)**

Table 10, **Table 11** and **Table 12** show the interpretation of sample results between the reference and alternative method per category and type (based on confirmed alternative method).

Table 10 - Interpretation of sample results between the reference and alternative method (based on the confirmed alternative):
Manual extraction protocol

				CFX96				LC480			
Category		**Type**		**PA**	**NA**	**PD**	**ND**	**PA**	**NA**	**PD**	**ND**
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	10	0	0	10	10	0	0
		b	Raw material	7	12	0	1	8	12	0	0
		c	Pastries	12	8	0	0	12	8	0	0
		Total		29	30	0	1	30	30	0	0
2	Meat and meat products	a	Fresh meat	9	13	0	0	9	13	0	0
		b	Fermented and cured meat products	11	14	0	0	11	14	0	0
		c	Cooked meat products and cooked delicatessen	10	10	0	0	10	10	0	0
		Total		30	37	0	0	30	37	0	0
3	Milk and dairy products	a	Pasteurized	12	9	0	0	12	9	0	0
		b	Raw	8	11	0	1	9	11	0	0
		c	Milk powder, infant formula	10	13	0	0	10	13	0	0
		Total		30	33	0	1	31	33	0	0
4	Egg products	a	Egg powders	8	16	0	0	8	16	0	0
		b	Pasteurized liquid eggs	12	8	0	0	12	8	0	0
		c	Egg-based products	9	9	0	2	9	9	0	2
		Total		29	33	0	2	29	33	0	2
5	Fish and seafood products	a	Raw	11	9	0	0	11	9	0	0
		b	Ready-to-eat	9	11	0	0	9	11	0	0
		c	Ready-to-reheat	11	11	0	0	11	11	0	0
		Total		31	31	0	0	31	31	0	0
6	Feed samples	a	Cattle feed	13	15	0	0	13	15	0	0
		b	Pet food	11	9	0	0	11	9	0	0
		c	Raw material	7	13	0	0	6	13	0	1
		Total		31	37	0	0	30	37	0	1
All categories (food and feed)				**180**	**201**	**0**	**4**	**181**	**201**	**0**	**3**

NA= NA + PPNA
ND= ND + PPND

Table 11 - Interpretation of sample results between the reference and alternative method (based on the confirmed alternative):
Manual extraction protocol + Subculture (BHI or RVS for PPS)

Category		Type		CFX96				LC480			
				PA	NA	PD	ND	PA	NA	PD	ND
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	10	0	0	10	10	0	0
		b	Raw material	8	12	0	0	8	12	0	0
		c	Pastries	12	8	0	0	12	8	0	0
		Total		30	30	0	0	30	30	0	0
2	Meat and meat products	a	Fresh meat	9	13	0	0	9	13	0	0
		b	Fermented and cured meat products	11	14	0	0	11	14	0	0
		c	Cooked meat products and cooked delicatessen	10	10	0	0	10	10	0	0
		Total		30	37	0	0	30	37	0	0
3	Milk and dairy products	a	Pasteurized	12	9	0	0	12	9	0	0
		b	Raw	8	11	0	1	8	11	0	1
		c	Milk powder, infant formula	10	13	0	0	10	13	0	0
		Total		30	33	0	1	30	33	0	1
4	Egg products	a	Egg powders	8	16	0	0	8	16	0	0
		b	Pasteurized liquid eggs	12	8	0	0	12	8	0	0
		c	Egg-based products	9	9	0	2	9	9	0	2
		Total		29	33	0	2	29	33	0	2
5	Fish and seafood products	a	Raw	11	9	0	0	11	9	0	0
		b	Ready-to-eat	9	11	0	0	9	11	0	0
		c	Ready-to-reheat	11	11	0	0	11	11	0	0
		Total		31	31	0	0	31	31	0	0
6	Feed samples	a	Cattle feed	13	15	0	0	13	15	0	0
		b	Pet food	11	9	0	0	11	9	0	0
		c	Raw material	7	13	0	0	7	13	0	0
		Total		31	37	0	0	31	37	0	0
All categories (food and feed)				181	201	0	3	181	201	0	3
7	PPS	a	Animal faeces	15	14	1	0	15	14	1	0
		b	Environmental samples and non-feces	15	28	0	0	15	28	0	0
		Total		30	42	1	0	30	42	1	0
All categories (food and feed and PPS)				**211**	**243**	**1**	**3**	**211**	**243**	**1**	**3**

NA= NA + PPNA
ND= ND + PPND

Table 12 - Interpretation of sample results between the reference and alternative method (based on the confirmed alternative method):

Automated extraction protocol

				CFX96				LC480			
Category		**Type**		**PA**	**NA**	**PD**	**ND**	**PA**	**NA**	**PD**	**ND**
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	10	0	0	10	10	0	0
		b	Raw material	7	12	0	1	7	12	0	1
		c	Pastries	12	8	0	0	12	8	0	0
		Total		29	30	0	1	29	30	0	1
2	Meat and meat products	a	Fresh meat	9	13	0	0	9	13	0	0
		b	Fermented and cured meat products	11	14	0	0	11	14	0	0
		c	Cooked meat products and cooked delicatessen	10	10	0	0	10	10	0	0
		Total		30	37	0	0	30	37	0	0
3	Milk and dairy products	a	Pasteurized	12	9	0	0	12	9	0	0
		b	Raw	8	11	0	1	8	11	0	1
		c	Milk powder, infant formula	10	13	0	0	10	13	0	0
		Total		30	33	0	1	30	33	0	1
4	Egg products	a	Egg powders	8	16	0	0	8	16	0	0
		b	Pasteurized liquid eggs	12	8	0	0	12	8	0	0
		c	Egg-based products	9	9	0	2	9	9	0	2
		Total		29	33	0	2	29	33	0	2
5	Fish and seafood products	a	Raw	11	9	0	0	11	9	0	0
		b	Ready-to-eat	9	11	0	0	9	11	0	0
		c	Ready-to-reheat	11	11	0	0	11	11	0	0
		Total		31	31	0	0	31	31	0	0
6	Feed samples	a	Cattle feed	13	15	0	0	13	15	0	0
		b	Pet food	11	9	0	0	11	9	0	0
		c	Raw material	7	13	0	0	7	13	0	0
		Total		31	37	0	0	31	37	0	0
All categories (food and feed)				180	201	0	4	180	201	0	4

NA= NA + PPNA
ND= ND + PPND

3.1.5 Sensitivity study calculations

The sensitivity study parameters as specified in **Table 13** were calculated for all Categories and Types, and the overview is given:

- For the CFX 96:
  - **Table 14** (manual extraction protocol),
  - **Table 15** (manual extraction protocol + subculture (BHI or RVS for PPS),
  - **Table 16** (automated extraction protocol),

- For the LightCycler 480:
  - **Table 17** (manual extraction protocol),
  - **Table 18** (manual extraction protocol + subculture (BHI or RVS for PPS),
  - **Table 19** (automated extraction protocol).

Table 13 - 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	$FPR = \frac{(FP)}{NA} \times 100\%$

with ND = ND+PPND
NA= NA+PPNA

Table 14 - CFX 96

Overview calculated sensitivity parameters per Category and Type - Manual extraction protocol

Category		Type		PA	NA	PD	ND	PPND	PPNA	SE $_{alt}$ %	SE $_{ref}$ %	RT %	FPR %
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	10	0	0	0	0	100,0	100,0	100,0	0
		b	Raw material	7	12	0	1	0	0	87,5	100,0	95,0	0,0
		c	Pastries	12	8	0	0	0	0	100,0	100,0	100,0	0,0
			Total	29	30	0	1	0	0	96,7	100,0	98,3	0,0
2	Meat and meat products	a	Fresh meat	9	13	0	0	0	0	100,0	100,0	100,0	0,0
		b	Fermented and cured meat products	11	13	0	0	0	1	100,0	100,0	100,0	7,1
		c	Cooked meat products and cooked delicatessen	10	10	0	0	0	0	100,0	100,0	100,0	0,0
			Total	30	36	0	0	0	1	100,0	100,0	100,0	2,7
3	Milk and dairy products	a	Pasteurized	12	9	0	0	0	0	100,0	100,0	100,0	0,0
		b	Raw	8	11	0	1	0	0	88,9	100,0	95,0	0,0
		c	Milk powder, infant formula	10	13	0	0	0	0	100,0	100,0	100,0	0,0
			Total	30	33	0	1	0	0	96,8	100,0	98,4	0,0
4	Egg products	a	Egg powders	8	3	0	0	0	13	100,0	100,0	100,0	81,3
		b	Pasteurized liquid eggs	12	7	0	0	0	1	100,0	100,0	100,0	12,5
		c	Egg-based products	9	9	0	2	0	0	81,8	100,0	90,0	0,0
			Total	29	19	0	2	0	14	93,5	100,0	96,9	42,4
5	Fish and seafood products	a	Raw	11	9	0	0	0	0	100,0	100,0	100,0	0,0
		b	Ready-to-eat	9	10	0	0	0	1	100,0	100,0	100,0	9,1
		c	Ready-to-reheat	11	11	0	0	0	0	100,0	100,0	100,0	0,0
			Total	31	30	0	0	0	1	100,0	100,0	100,0	3,2
6	Feed samples	a	Cattle feed	13	15	0	0	0	0	100,0	100,0	100,0	0,0
		b	Pet food	11	9	0	0	0	0	100,0	100,0	100,0	0,0
		c	Raw material	7	11	0	0	0	2	100,0	100,0	100,0	15,4
			Total	31	35	0	0	0	2	100,0	100,0	100,0	5,4
All categories (food and feed)				**180**	**183**	**0**	**4**	**0**	**18**	**97,8**	**100,0**	**99,0**	**9,0**

Table 15 - CFX 96

Overview calculated sensitivity parameters per Category and Type - Manual extraction protocol + subculture (BHI or RVS for PPS)

Category		Type		PA	NA	PD	ND	PPND	PPNA	SE alt %	SE ref %	RT %	FPR %
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	10	0	0	0	0	100,0	100,0	100,0	0
		b	Raw material	8	12	0	0	0	0	100,0	100,0	100,0	0,0
		c	Pastries	12	8	0	0	0	0	100,0	100,0	100,0	0,0
		Total		30	30	0	0	0	0	100,0	100,0	100,0	0,0
2	Meat and meat products	a	Fresh meat	9	13	0	0	0	0	100,0	100,0	100,0	0,0
		b	Fermented and cured meat products	11	13	0	0	0	1	100,0	100,0	100,0	7,1
		c	Cooked meat products and cooked delicatessen	10	10	0	0	0	0	100,0	100,0	100,0	0,0
		Total		30	36	0	0	0	1	100,0	100,0	100,0	2,7
3	Milk and dairy products	a	Pasteurized	12	9	0	0	0	0	100,0	100,0	100,0	0,0
		b	Raw	8	11	0	1	0	0	88,9	100,0	95,0	0,0
		c	Milk powder, infant formula	10	13	0	0	0	0	100,0	100,0	100,0	0,0
		Total		30	33	0	1	0	0	96,8	100,0	98,4	0,0
4	Egg products	a	Egg powders	8	9	0	0	0	7	100,0	100,0	100,0	43,8
		b	Pasteurized liquid eggs	12	8	0	0	0	0	100,0	100,0	100,0	0,0
		c	Egg-based products	9	9	0	2	0	0	81,8	100,0	90,0	0,0
		Total		29	26	0	2	0	7	93,5	100,0	96,9	21,2
5	Fish and seafood products	a	Raw	11	9	0	0	0	0	100,0	100,0	100,0	0,0
		b	Ready-to-eat	9	11	0	0	0	0	100,0	100,0	100,0	0,0
		c	Ready-to-reheat	11	11	0	0	0	0	100,0	100,0	100,0	0,0
		Total		31	31	0	0	0	0	100,0	100,0	100,0	0,0
6	Feed samples	a	Cattle feed	13	15	0	0	0	0	100,0	100,0	100,0	0,0
		b	Pet food	11	9	0	0	0	0	100,0	100,0	100,0	0,0
		c	Raw material	7	11	0	0	0	2	100,0	100,0	100,0	15,4
		Total		31	35	0	0	0	2	100,0	100,0	100,0	5,4
All categories (food and feed)				181	191	0	3	0	10	98,4	100,0	99,2	5,0
7	PPS	a	Animal faeces	15	14	1	0	0	0	100,0	93,8	96,7	0,0
		b	Environmental samples and non-faeces	15	28	0	0	0	0	100,0	100,0	100,0	0,0
		Total		30	42	1	0	0	0	100,0	96,8	98,6	0,0
All categories (food, feed and PPS)				**211**	**233**	**1**	**3**	**0**	**10**	**98,6**	**99,5**	**99,1**	**4,1**

Table 16 - CFX 96

Overview calculated sensitivity parameters per Category and Type - Automated extraction protocol

Category		Type		PA	NA	PD	ND	PPND	PPNA	SE $_{alt}$ %	SE $_{ref}$ %	RT %	FPR %
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	10	0	0	0	0	100,0	100,0	100,0	0
		b	Raw material	7	12	0	1	0	0	87,5	100,0	95,0	0,0
		c	Pastries	12	8	0	0	0	0	100,0	100,0	100,0	0,0
			Total	29	30	0	1	0	0	96,7	100,0	98,3	0,0
2	Meat and meat products	a	Fresh meat	9	13	0	0	0	0	100,0	100,0	100,0	0,0
		b	Fermented and cured meat products	11	13	0	0	0	1	100,0	100,0	100,0	7,1
		c	Cooked meat products and cooked delicatessen	10	9	0	0	0	1	100,0	100,0	100,0	10,0
			Total	30	35	0	0	0	2	100,0	100,0	100,0	5,4
3	Milk and dairy products	a	Pasteurized	12	9	0	0	0	0	100,0	100,0	100,0	0,0
		b	Raw	8	10	0	1	0	1	88,9	100,0	95,0	9,1
		c	Milk powder, infant formula	10	13	0	0	0	0	100,0	100,0	100,0	0,0
			Total	30	32	0	1	0	1	96,8	100,0	98,4	3,0
4	Egg products	a	Egg powders	8	14	0	0	0	2	100,0	100,0	100,0	12,5
		b	Pasteurized liquid eggs	12	8	0	0	0	0	100,0	100,0	100,0	0,0
		c	Egg-based products	9	8	0	2	0	1	81,8	100,0	90,0	11,1
			Total	29	30	0	2	0	3	93,5	100,0	96,9	9,1
5	Fish and seafood products	a	Raw	11	9	0	0	0	0	100,0	100,0	100,0	0,0
		b	Ready-to-eat	9	9	0	0	0	2	100,0	100,0	100,0	18,2
		c	Ready-to-reheat	11	11	0	0	0	0	100,0	100,0	100,0	0,0
			Total	31	29	0	0	0	2	100,0	100,0	100,0	6,5
6	Feed samples	a	Cattle feed	13	15	0	0	0	0	100,0	100,0	100,0	0,0
		b	Pet food	11	9	0	0	0	0	100,0	100,0	100,0	0,0
		c	Raw material	7	13	0	0	0	0	100,0	100,0	100,0	0,0
			Total	31	37	0	0	0	0	100,0	100,0	100,0	0,0
All categories (food and feed)				**180**	**193**	**0**	**4**	**0**	**8**	**97,8**	**100,0**	**99,0**	**4,0**

Table 17 - LightCycler 480

Overview calculated sensitivity parameters per Category and Type - Manual extraction protocol

Category		Type		PA	NA	PD	ND	PPND	PPNA	SE_{alt} %	SE_{ref} %	RT %	FPR %
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	10	0	0	0	0	100,0	100,0	100,0	0
		b	Raw material	8	12	0	0	0	0	100,0	100,0	100,0	0,0
		c	Pastries	12	8	0	0	0	0	100,0	100,0	100,0	0,0
		Total		30	30	0	0	0	0	100,0	100,0	100,0	0,0
2	Meat and meat products	a	Fresh meat	9	13	0	0	0	0	100,0	100,0	100,0	0,0
		b	Fermented and cured meat products	11	13	0	0	0	1	100,0	100,0	100,0	7,1
		c	Cooked meat products and cooked delicatessen	10	10	0	0	0	0	100,0	100,0	100,0	0,0
		Total		30	36	0	0	0	1	100,0	100,0	100,0	2,7
3	Milk and dairy products	a	Pasteurized	12	9	0	0	0	0	100,0	100,0	100,0	0,0
		b	Raw	9	11	0	0	0	0	100,0	100,0	100,0	0,0
		c	Milk powder, infant formula	10	13	0	0	0	0	100,0	100,0	100,0	0,0
		Total		31	33	0	0	0	0	100,0	100,0	100,0	0,0
4	Egg products	a	Egg powders	8	3	0	0	0	13	100,0	100,0	100,0	81,3
		b	Pasteurized liquid eggs	12	8	0	0	0	0	100,0	100,0	100,0	0,0
		c	Egg-based products	9	8	0	2	0	1	81,8	100,0	90,0	11,1
		Total		29	19	0	2	0	14	93,5	100,0	96,9	42,4
5	Fish and seafood products	a	Raw	11	9	0	0	0	0	100,0	100,0	100,0	0,0
		b	Ready-to-eat	9	11	0	0	0	0	100,0	100,0	100,0	0,0
		c	Ready-to-reheat	11	11	0	0	0	0	100,0	100,0	100,0	0,0
		Total		31	31	0	0	0	0	100,0	100,0	100,0	0,0
6	Feed samples	a	Cattle feed	13	15	0	0	0	0	100,0	100,0	100,0	0,0
		b	Pet food	11	9	0	0	0	0	100,0	100,0	100,0	0,0
		c	Raw material	6	10	0	1	0	3	85,7	100,0	95,0	23,1
		Total		30	34	0	1	0	3	96,8	100,0	98,5	8,1
All categories (food and feed)				**181**	**183**	**0**	**3**	**0**	**18**	**98,4**	**100,0**	**99,2**	**9,0**

Table 18 - LightCycler 480

Overview calculated sensitivity parameters per Category and Type - Manual extraction protocol + subculture (BHI or RVS for PPS)

Category		Type		PA	NA	PD	ND	PPND	PPNA	SE alt %	SE ref %	RT %	FPR %
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	10	0	0	0	0	100,0	100,0	100,0	0
		b	Raw material	8	12	0	0	0	0	100,0	100,0	100,0	0,0
		c	Pastries	12	8	0	0	0	0	100,0	100,0	100,0	0,0
			Total	30	30	0	0	0	0	100,0	100,0	100,0	0,0
2	Meat and meat products	a	Fresh meat	9	13	0	0	0	0	100,0	100,0	100,0	0,0
		b	Fermented and cured meat products	11	13	0	0	0	1	100,0	100,0	100,0	7,1
		c	Cooked meat products and cooked delicatessen	10	10	0	0	0	0	100,0	100,0	100,0	0,0
			Total	30	36	0	0	0	1	100,0	100,0	100,0	2,7
3	Milk and dairy products	a	Pasteurized	12	9	0	0	0	0	100,0	100,0	100,0	0,0
		b	Raw	8	11	0	1	0	0	88,9	100,0	95,0	0,0
		c	Milk powder, infant formula	10	12	0	0	0	1	100,0	100,0	100,0	7,7
			Total	30	32	0	1	0	1	96,8	100,0	98,4	3,0
4	Egg products	a	Egg powders	8	12	0	0	0	4	100,0	100,0	100,0	25,0
		b	Pasteurized liquid eggs	12	8	0	0	0	0	100,0	100,0	100,0	0,0
		c	Egg-based products	9	9	0	2	0	0	81,8	100,0	90,0	0,0
			Total	29	29	0	2	0	4	93,5	100,0	96,9	12,1
5	Fish and seafood products	a	Raw	11	9	0	0	0	0	100,0	100,0	100,0	0,0
		b	Ready-to-eat	9	11	0	0	0	0	100,0	100,0	100,0	0,0
		c	Ready-to-reheat	11	11	0	0	0	0	100,0	100,0	100,0	0,0
			Total	31	31	0	0	0	0	100,0	100,0	100,0	0,0
6	Feed samples	a	Cattle feed	13	14	0	0	0	1	100,0	100,0	100,0	6,7
		b	Pet food	11	8	0	0	0	1	100,0	100,0	100,0	11,1
		c	Raw material	7	12	0	0	0	1	100,0	100,0	100,0	7,7
			Total	31	34	0	0	0	3	100,0	100,0	100,0	8,1
All categories (food and feed)				**181**	**192**	**0**	**3**	**0**	**9**	**98,4**	**100,0**	**99,2**	**4,5**
7	PPS	a	Animal faeces	15	14	1	0	0	0	100,0	93,8	96,7	0,0
		b	Environmental samples and non-faeces	15	28	0	0	0	0	100,0	100,0	100,0	0,0
			Total	30	42	1	0	0	0	100,0	96,8	98,6	0,0
All categories (food, feed and PPS)				**211**	**234**	**1**	**3**	**0**	**9**	**98,6**	**99,5**	**99,1**	**3,7**

Table 19 - LightCycler 480

Overview calculated sensitivity parameters per Category and Type - Automated extraction protocol

Category		Type		PA	NA	PD	ND	PPND	PPNA	SE $_{alt}$ %	SE $_{ref}$ %	RT %	FPR %
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	10	0	0	0	0	100,0	100,0	100,0	0
		b	Raw material	7	11	0	1	0	1	87,5	100,0	95,0	8,3
		c	Pastries	12	8	0	0	0	0	100,0	100,0	100,0	0,0
		Total		29	29	0	1	0	1	96,7	100,0	98,3	3,3
2	Meat and meat products	a	Fresh meat	9	13	0	0	0	0	100,0	100,0	100,0	0,0
		b	Fermented and cured meat products	11	13	0	0	0	1	100,0	100,0	100,0	7,1
		c	Cooked meat products and cooked delicatessen	10	10	0	0	0	0	100,0	100,0	100,0	0,0
		Total		30	36	0	0	0	1	100,0	100,0	100,0	2,7
3	Milk and dairy products	a	Pasteurized	12	9	0	0	0	0	100,0	100,0	100,0	0,0
		b	Raw	8	11	0	1	0	0	88,9	100,0	95,0	0,0
		c	Milk powder, infant formula	10	13	0	0	0	0	100,0	100,0	100,0	0,0
		Total		30	33	0	1	0	0	96,8	100,0	98,4	0,0
4	Egg products	a	Egg powders	8	16	0	0	0	0	100,0	100,0	100,0	0,0
		b	Pasteurized liquid eggs	12	8	0	0	0	0	100,0	100,0	100,0	0,0
		c	Egg-based products	9	8	0	2	0	1	81,8	100,0	90,0	11,1
		Total		29	32	0	2	0	1	93,5	100,0	96,9	3,0
5	Fish and seafood products	a	Raw	11	9	0	0	0	0	100,0	100,0	100,0	0,0
		b	Ready-to-eat	9	11	0	0	0	0	100,0	100,0	100,0	0,0
		c	Ready-to-reheat	11	11	0	0	0	0	100,0	100,0	100,0	0,0
		Total		31	31	0	0	0	0	100,0	100,0	100,0	0,0
6	Feed samples	a	Cattle feed	13	15	0	0	0	0	100,0	100,0	100,0	0,0
		b	Pet food	11	9	0	0	0	0	100,0	100,0	100,0	0,0
		c	Raw material	7	11	0	0	0	2	100,0	100,0	100,0	15,4
		Total		31	35	0	0	0	2	100,0	100,0	100,0	5,4
All categories (food and feed)				**180**	**196**	**0**	**4**	**0**	**5**	**97,8**	**100,0**	**99,0**	**2,5**

A summary of the results is given in **Table 20**.

Table 20 - Summary of results observed per extraction protocol

	Manual extraction protocol				Automated extraction protocol	
	Without subculture (Food and Feed)		With subculture (Food, Feed and PPS)		(Food and feed)	
	CFX 96	LC 480	CFX 96	LC 480	CFX 96	LC 480
Sensitivity for the alternative method (SE_{alt})	97.8 %	98.4 %	98.6 %	98.6 %	97.8 %	97.8 %
Sensitivity for the reference method (SE_{ref})	100.0 %	100.0 %	99.5 %	99.5 %	100.0 %	100.0 %
Relative trueness (RT)	99.0 %	99.2 %	99.1 %	99.1 %	99.0 %	99.0 %
False positive ratio for the alternative method (FPR) * FP = PPNA + PPND	9.0 %	9.0 %	4.1 %	3.7 %	4.0 %	2.5 %

With *ND = ND + PPND*
NA = NA + PPNA

3.1.6 Discordant results

The negative deviations are listed in **Table 21**.

The positive deviation is given **Table 22**.

Table 21 - Negative deviations

	N°Sample	English name product	Artificial contaminations		ISO 6579-1♦	foodproof® *Salmonella* spp. detection method									Category	Type
						PCR result				Confir-matory tests	Final result CFX96	Final result LC 480	Agree-ment CFX96	Agree-ment LC 480		
						CFX96		LightCycler 480								
			Strain	Inoculation level (CFU/ sample)		Ct FAM	Result	Ct FAM	Result							
Automated extraction protocol	**2038**	**Cocoa beans**	S. Bareilly Ad1687	2,8	+	-/-/-	-/-/-	-/35,93/34,26	-/+/+	+	-	-	ND	ND	1	b
	158	**Raw milk cheese**	S. Dublin Ad531	1,2	+	-/-/-	-/-/-	-/-/35,38 (atypical curve)	-/-/+d	+	-	-	ND	ND	3	b
	499	**Mayonnaise**	S. Mbandaka Ad914	3,0	+	-/-/-	-/-/-	-/-/-	-/-/-	+	-	-	ND	ND	4	c
	2854	**Egg based powder product**	S. Livingstone E1	2,4	+	-/-/-	-/-/-	-/-/-	-/-/-	+	-	-	ND	ND	4	c
Manual extraction protocol, without subculture	**2038**	**Cocoa beans**	S. Bareilly Ad1687	2,8	+	-/-/-	-/-/-	35,50	+	+	-	+	ND	PA	1	b
	158	**Raw milk cheese**	S. Dublin Ad531	1,2	+	-/39,11/41,86	-/+/+	35,70/pos/34,76	+/+/+	+	-	+	ND	PA	3	b
	499	**Mayonnaise**	S. Mbandaka Ad914	3,0	+	-/-/-	-/-/-	-/-/-	-/-/-	+	-	-	ND	ND	4	c
	2854	**Egg based powder product**	S. Livingstone E1	2,4	+	-/-/-	-/-/-	-/-/-	-/-/-	+	-	-	ND	ND	4	c
Manual extraction protocol, with subculture	**158**	**Raw milk cheese**	S. Dublin Ad531	1,2	+	-/41,05/38,60	-/+/+	-/34,11/36,83	-/+/+	+	-	-	ND	ND	3	b
	499	**Mayonnaise**	S. Mbandaka Ad914	3,0	+	-/-/-	-/-/-	-/-/-	-/-/-	+	-	-	ND	ND	4	c
	2854	**Egg based powder product**	S. Livingstone E1	2,4	+	-/37,09/38,17	-/+/+	-/35,09/39,98	-/+d/+d	+	-	-	ND	ND	4	c

Table 22 - Positive deviations

	N°Sample	English name product	Artificial contaminations		ISO 6579-1♦	foodproof® *Salmonella* spp. detection method									Category	Type
						PCR result				Confirmatory tests	Final result CFX96	Final result LC 480	Agreement CFX96	Agreement LC 480		
						CFX96		LightCycler 480								
			Strain	Inoculation level (CFU/sample)		Ct FAM	Result	Ct FAM	Result							
Manual extraction protocol, without subculture	6949	Boot socks	/	/	-	26,13	+	26,96	+	+	+	+	PD	PD	7	a

♦ Analyses performed according to the COFRAC accreditation

Four negative deviations were observed for the manual protocol (without subculture) using the CFX96, two samples (2038 and 158) gave positive PCR results using the LightCycler 480.
For one sample (158), PCR replicates gave positive results with late Ct value.

For the manual protocol with subculture, 3 negative deviations were observed with both cyclers. When PCR tests were repeated, positive results were observed twice for two samples (158 and 2854).

For the automated extraction protocol, four negative deviations were observed (same samples as for the manual protocol without subculture) with the CFX96 and the LightCycler 480. PCR replicates gave all negative results when using the CFX96. Positive results were observed twice with the LightCycler 480 for sample 2038, and doubtful results for sample 158 using the LightCycler 480.

For the samples in negative deviations, the contamination level was probably just at the limit of detection of the method.

One positive deviation was observed for the PPS category and concerns a naturally contaminated sample.

The analyses of discordant results according to the EN ISO 16140-2:2016 for a paired study design is given:

- For the CFX96:
  - **Table 23** (manual extraction protocol),
  - **Table 24** (manual extraction protocol + subculture (BHI or RVS for PPS))
  - **Table 25** (automated extraction protocol),

For the LightCycler 480:
- **Table 26** (manual extraction protocol),
- **Table 27** (manual extraction protocol + subculture (BHI or RVS for PPS),
- **Table 28** (automated extraction protocol)

Table 23 - CFX 96

Interpretation of the sensitivity study results (paired study) - Manual extraction protocol (paired study design)

Category		Type		N+	PD	ND	PPND	(ND+PPND)-PD	AL	ND+PPND+PD	AL
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	0	0	0	0		0	
		b	Raw material	8	0	1	0	1		1	
		c	Pastries	12	0	0	0	0		0	
			Total	30	0	1	0	1	3	1	6
2	Meat and meat products	a	Fresh meat	9	0	0	0	0		0	
		b	Fermented and cured meat products	11	0	0	0	0		0	
		c	Cooked meat products and cooked delicatessen	10	0	0	0	0		0	
			Total	30	0	0	0	0	3	0	6
3	Milk and dairy products	a	Pasteurized	12	0	0	0	0		0	
		b	Raw	9	0	1	0	1		1	
		c	Milk powder, infant formula	10	0	0	0	0		0	
			Total	31	0	1	0	1	3	1	6
4	Egg products	a	Egg powders	8	0	0	0	0		0	
		b	Pasteurized liquid eggs	12	0	0	0	0		0	
		c	Egg-based products	11	0	2	0	2		2	
			Total	31	0	2	0	2	3	2	6
5	Fish and seafood products	a	Raw	11	0	0	0	0		0	
		b	Ready-to-eat	9	0	0	0	0		0	
		c	Ready-to-reheat	11	0	0	0	0		0	
			Total	31	0	0	0	0	3	0	6
6	Feed samples	a	Cattle feed	13	0	0	0	0		0	
		b	Pet food	11	0	0	0	0		0	
		c	Raw material	7	0	0	0	0		0	
			Total	31	0	0	0	0	3	0	6
All categories (food and feed)				**184**	**0**	**4**	**0**	**4**	**6**	**4**	**16**

Table 24 - CFX 96

Interpretation of the sensitivity study results (paired study) - Manual extraction protocol + subculture (BHI or RVS for PPS) (paired study design)

Category		Type		N+	PD	ND	PPND	(ND+PPND)-PD	AL	ND+PPND+PD	AL
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	0	0	0	0		0	
		b	Raw material	8	0	0	0	0		0	
		c	Pastries	12	0	0	0	0		0	
			Total	30	0	0	0	0	3	0	6
2	Meat and meat products	a	Fresh meat	9	0	0	0	0		0	
		b	Fermented and cured meat products	11	0	0	0	0		0	
		c	Cooked meat products and cooked delicatessen	10	0	0	0	0		0	
			Total	30	0	0	0	0	3	0	6
3	Milk and dairy products	a	Pasteurized	12	0	0	0	0		0	
		b	Raw	9	0	1	0	1		1	
		c	Milk powder, infant formula	10	0	0	0	0		0	
			Total	31	0	1	0	1	3	1	6
4	Egg products	a	Egg powders	8	0	0	0	0		0	
		b	Pasteurized liquid eggs	12	0	0	0	0		0	
		c	Egg-based products	11	0	2	0	2		2	
			Total	31	0	2	0	2	3	2	6
5	Fish and seafood products	a	Raw	11	0	0	0	0		0	
		b	Ready-to-eat	9	0	0	0	0		0	
		c	Ready-to-reheat	11	0	0	0	0		0	
			Total	31	0	0	0	0	3	0	6
6	Feed samples	a	Cattle feed	13	0	0	0	0		0	
		b	Pet food	11	0	0	0	0		0	
		c	Raw material	7	0	0	0	0		0	
			Total	31	0	0	0	0	3	0	6
All categories (food and feed)				184	0	3	0	3	6	3	16
7	PPS ISO 6579/A1	a	Animal faeces	16	1	0	0	-1		1	
		b	Environmental samples and non-faeces	15	0	0	0	0		0	
			Total	31	1	0	0	-1	3	1	6
All categories (food, feed and PPS)				**215**	**1**	**3**	**0**	**2**	**6**	**4**	**18**

Table 25 - CFX 96

Interpretation of the sensitivity study results (paired study) - Automated extraction protocol

Category		Type		N+	PD	ND	PPND	(ND+PPND)-PD	AL	ND+PPND+PD	AL
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	0	0	0	0		0	
		b	Raw material	8	0	1	0	1		1	
		c	Pastries	12	0	0	0	0		0	
			Total	30	0	1	0	1	3	1	6
2	Meat and meat products	a	Fresh meat	9	0	0	0	0		0	
		b	Fermented and cured meat products	11	0	0	0	0		0	
		c	Cooked meat products and cooked delicatessen	10	0	0	0	0		0	
			Total	30	0	0	0	0	3	0	6
3	Milk and dairy products	a	Pasteurized	12	0	0	0	0		0	
		b	Raw	9	0	1	0	1		1	
		c	Milk powder, infant formula	10	0	0	0	0		0	
			Total	31	0	1	0	1	3	1	6
4	Egg products	a	Egg powders	8	0	0	0	0		0	
		b	Pasteurized liquid eggs	12	0	0	0	0		0	
		c	Egg-based products	11	0	2	0	2		2	
			Total	31	0	2	0	2	3	2	6
5	Fish and seafood products	a	Raw	11	0	0	0	0		0	
		b	Ready-to-eat	9	0	0	0	0		0	
		c	Ready-to-reheat	11	0	0	0	0		0	
			Total	31	0	0	0	0	3	0	6
6	Feed samples	a	Cattle feed	13	0	0	0	0		0	
		b	Pet food	11	0	0	0	0		0	
		c	Raw material	7	0	0	0	0		0	
			Total	31	0	0	0	0	3	0	6
All categories (food and feed)				**184**	**0**	**4**	**0**	**4**	**6**	**4**	**16**

Table 26 - LightCycler 480

Interpretation of the sensitivity study results (paired study) - Manual extraction protocol (paired study design)

Category		Type		N+	PD	ND	PPND	(ND+PPND)-PD	AL	ND+PPND+PD	AL
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	0	0	0	0		0	
		b	Raw material	8	0	0	0	0		0	
		c	Pastries	12	0	0	0	0		0	
			Total	30	0	0	0	0	3	0	6
2	Meat and meat products	a	Fresh meat	9	0	0	0	0		0	
		b	Fermented and cured meat products	11	0	0	0	0		0	
		c	Cooked meat products and cooked delicatessen	10	0	0	0	0		0	
			Total	30	0	0	0	0	3	0	6
3	Milk and dairy products	a	Pasteurized	12	0	0	0	0		0	
		b	Raw	9	0	0	0	0		0	
		c	Milk powder, infant formula	10	0	0	0	0		0	
			Total	31	0	0	0	0	3	0	6
4	Egg products	a	Egg powders	8	0	0	0	0		0	
		b	Pasteurized liquid eggs	12	0	0	0	0		0	
		c	Egg-based products	11	0	2	0	2		2	
			Total	31	0	2	0	2	3	2	6
5	Fish and seafood products	a	Raw	11	0	0	0	0		0	
		b	Ready-to-eat	9	0	0	0	0		0	
		c	Ready-to-reheat	11	0	0	0	0		0	
			Total	31	0	0	0	0	3	0	6
6	Feed samples	a	Cattle feed	13	0	0	0	0		0	
		b	Pet food	11	0	0	0	0		0	
		c	Raw material	7	0	1	0	1		1	
			Total	31	0	1	0	1	3	1	6
All categories (food and feed)				**184**	**0**	**3**	**0**	**3**	**6**	**3**	**16**

Table 27 - LightCycler 480

Interpretation of the sensitivity study results (paired study) - Manual extraction protocol + subculture (BHI or RVS for PPS) (paired study design)

Category		Type		N+	PD	ND	PPND	(ND+PPND)-PD	AL	ND+PPND+PD	AL
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	0	0	0	0		0	
		b	Raw material	8	0	0	0	0		0	
		c	Pastries	12	0	0	0	0		0	
			Total	30	0	0	0	0	3	0	6
2	Meat and meat products	a	Fresh meat	9	0	0	0	0		0	
		b	Fermented and cured meat products	11	0	0	0	0		0	
		c	Cooked meat products and cooked delicatessen	10	0	0	0	0		0	
			Total	30	0	0	0	0	3	0	6
3	Milk and dairy products	a	Pasteurized	12	0	0	0	0		0	
		b	Raw	9	0	1	0	1		1	
		c	Milk powder, infant formula	10	0	0	0	0		0	
			Total	31	0	1	0	1	3	1	6
4	Egg products	a	Egg powders	8	0	0	0	0		0	
		b	Pasteurized liquid eggs	12	0	0	0	0		0	
		c	Egg-based products	11	0	2	0	2		2	
			Total	31	0	2	0	2	3	2	6
5	Fish and seafood products	a	Raw	11	0	0	0	0		0	
		b	Ready-to-eat	9	0	0	0	0		0	
		c	Ready-to-reheat	11	0	0	0	0		0	
			Total	31	0	0	0	0	3	0	6
6	Feed samples	a	Cattle feed	13	0	0	0	0		0	
		b	Pet food	11	0	0	0	0		0	
		c	Raw material	7	0	0	0	0		0	
			Total	31	0	0	0	0	3	0	6
All categories (food and feed)				184	0	3	0	3	6	3	16
7	PPS ISO 6579/A1	a	Animal faeces	16	1	0	0	-1		1	
		b	Environmental samples and non-faeces	15	0	0	0	0		0	
			Total	31	1	0	0	-1	3	1	6
All categories (food, feed and PPS)				**215**	**1**	**3**	**0**	**2**	**6**	**4**	**18**

Table 28 - LightCycler 480

Interpretation of the sensitivity study results (paired study) - Automated extraction protocol

Category		Type		N+	PD	ND	PPND	(ND+PPND)-PD	AL	ND+PPND+PD	AL
1	Chocolate and bakery products	a	Cocoa powders and finished products	10	0	0	0	0		0	
		b	Raw material	8	0	1	0	1		1	
		c	Pastries	12	0	0	0	0		0	
			Total	30	0	1	0	1	3	1	6
2	Meat and meat products	a	Fresh meat	9	0	0	0	0		0	
		b	Fermented and cured meat products	11	0	0	0	0		0	
		c	Cooked meat products and cooked delicatessen	10	0	0	0	0		0	
			Total	30	0	0	0	0	3	0	6
3	Milk and dairy products	a	Pasteurized	12	0	0	0	0		0	
		b	Raw	9	0	1	0	1		1	
		c	Milk powder, infant formula	10	0	0	0	0		0	
			Total	31	0	1	0	1	3	1	6
4	Egg products	a	Egg powders	8	0	0	0	0		0	
		b	Pasteurized liquid eggs	12	0	0	0	0		0	
		c	Egg-based products	11	0	2	0	2		2	
			Total	31	0	2	0	2	3	2	6
5	Fish and seafood products	a	Raw	11	0	0	0	0		0	
		b	Ready-to-eat	9	0	0	0	0		0	
		c	Ready-to-reheat	11	0	0	0	0		0	
			Total	31	0	0	0	0	3	0	6
6	Feed samples	a	Cattle feed	13	0	0	0	0		0	
		b	Pet food	11	0	0	0	0		0	
		c	Raw material	7	0	0	0	0		0	
			Total	31	0	0	0	0	3	0	6
All categories (food and feed)				**184**	**0**	**4**	**0**	**4**	**6**	**4**	**16**

The observed values for (ND - PD) and (ND+PD) meet the acceptability limit (AL) for the seven categories (food, feed and primary production samples) whatever the protocol and the thermocycler used.

3.1.7 PCR inhibition

1228 PCR tests were performed with the CFX96 and the LC 480 for the sensitivity study, six inhibitions were observed for the manual protocol using the LC 480 and two using the CFX96 .The percentage of inhibition is 0.5% The samples concerned are listed in **Table 29**.

Table 29 – Samples with PCR inhibition

Sample n°	Product	Manual Protocol	
		CFX96	LC480
1629	Cocoa Powder	i/1/10:-	i/1/20:-
1637	Cocoa Powder	-	i/1/20:-
279	Frozen raw beef meat	+	i/1/5:+
508	Liquid egg product	i/1/5:-	i/1/5:-
510	Liquid egg yolk	i/1/5:i/1/10:i/1/20:-	i/1/5:i/1/10:i/1/20:-
515	Liquid egg product	i/1/5:i/1/10:-	i/1/5:i/1/10:-

3.1.8 Conclusion sensitivity study

The observed values for ND-PD and ND+PD for the individual categories and for all the categories combined meet the acceptability limits (observed values ≤ AL) for all the thermocyclers tested and protocols tested.

3.1.9 Confirmation

Two confirmation procedures were tested during this study for the manual protocol with subculture:

- Streaking from BHI or RVS (PPS) on selective agar plate
- Following the protocol described in the ISO 6579-1

The number of samples confirmed for each protocol is provided per category and protocol in **Table 30**.

Table 30 - Number of samples confirmed per category and protocol

Category	Confirmation protocol	PA	PD	Total confirmed
1	BHI	28	0	28
	ISO 6579-1	30	0	30
2	BHI	22	0	22
	ISO 6579-1	30	0	30
3	BHI	26	0	26
	ISO 6579-1	30	0	30
4	BHI	29	0	29
	ISO 6579-1	29	0	29
5	BHI	30	0	30
	ISO 6579-1	31	0	31
6	BHI	29	0	29
	ISO 6579-1	31	0	31
7 (PPS)	RVS	27	0	27
	ISO 6579-1	30	1	31
Total subculture in BHI or RVS		191	0	191
Total ISO 6579-1		211	1	212

For all categories combined, the presence of *Salmonella* was confirmed for 211 samples using the protocol of the ISO 6579-1 while only 191 samples were confirmed positive by streaking the BHI or the RVS on selective agar plates.

Several positive presumptive non-confirmed samples were observed for this study especially for egg powders using the manual extraction protocol (13 samples concerned).
When matrix was still available for these samples, a sample suspension was prepared in BPW (1/10) without applying any incubation and a PCR test was performed.

The results observed are given in **Table 31**.

Table 31 - PCR tests applied on non-enriched samples

N°Sample	English name product	Automated Extraction			Manual Extraction		
		Results **after matrix inoculation and enrichment**		Results **without matrix inoculation and without enrichment**	Results **after matrix inoculation and enrichment**		Results **without matrix inoculation and without enrichment**
		Cq FAM	Agreement	Cq FAM	Cq FAM	Agreement	Cq FAM
316	**Whole egg powder**	37,37	PPNA	-	36,24	PPNA	35,31
317	**Whole egg powder**	-	NA		45,64	PPNA	36,73
318	**Whole egg powder**	-	NA		37,41	PPNA	34,48
319	**Whole egg powder**	-	NA		34,86	PPNA	34,73
321	**White egg powder**	-	NA		38,1	PPNA	35,56
322	**White egg powder**	-	NA		45,1	PPNA	36,98
323	**White egg powder**	-	NA		35,37	PPNA	33,51
324	**Yolk egg powder**	-	NA		34,73	PPNA	36,22
325	**Yolk egg powder**	-	NA		35,25	PPNA	36,22
326	**Yolk egg powder**	36,45	PPNA	-	47,72	PPNA	41,2
547	White egg powder	-	NA		36,1/39,31/-	PPNA	30,45
548	Whole egg powder	-	NA		40,60/39,29	PPNA	34,48
2849	**Whole egg powder**	-	NA		36,37/36,28/44,47	PPNA	

For the twelve samples tested without any enrichment step, a positive PCR result was observed using the manual extraction protocol indicating that free DNA from dead cells was probably present in the matrices from dead cells.
The manual extraction protocol seems to be slightly more sensitive that the automated extraction protocol to detect DNA from the dead cells.

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 (proprietary) 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 were chosen for each of the categories in this validation study, as shown in **Table 32**.

Table 32 - List of selected types and strains per category, as tested within the relative level of detection study

Category	Type - Matrix	Strain	Origin	Inoculation (bulk) and storage condition	Sample size	Protocol
1 Chocolate and bakery products	a - Cocoa powder	S. Bareilly Ad1687	Chocolate industry	Seeding Lyophilized strain 2 weeks at ambient temperature	25 g	① and ⑦
2 Meat and meat products	b - Sausage	S. Enteritidis Ad926	Ready to reheat veal	Seeding 48 h at 5°C± 3°C	25 g	⑧ and ⑦
3 Milk and dairy products	b - Raw milk	S. Anatum Ad1166	Raw milk	Seeding 48 h at 5°C± 3°C	25 g	⑧ and ⑦
4 Egg products	b - Pasteurized liquid egg	S. Typhimurium Ad1484	Whole liquid egg	Seeding 48 h at 5°C± 3°C	25 g	① and ⑦
5 Fish and seafood products	b – Fish terrine	S. Brandenburg Ad 351	Seafood	Seeding 48 h at 5°C± 3°C	25 g	① and ⑦
6 Feed samples	b - Sausage for dog	S. Cerro Ad689	Raw material for pet food	Seeding 48 h at 5°C± 3°C	25 g	⑧ and ⑦
7 Primary production samples	a - Bootsocks	S. Agona Ad1306	Poultry breeding	Seeding 24 h at ambient temperature	25 g	PPS

3.2.2 Test sample preparation

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 in order to get 20 test portions providing fractional recovery,

- Higher level: One inoculated in order to get 5 test portions contaminated at a higher level.

The DNA extracts were analyzed with the CFX 96 by the expert lab, and with the LC 480 by BIOTECON DIAGNOSTICS.

3.2.3 RLOD study results

The RLOD calculations were performed using the 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 RLOD are given in **Table 33** for the manual protocol (CFX96 and LC480) in **Table 34** for the automated protocol (CFX96) and **Table 35** for the automated protocol for (LightCycler 480

Table 33 - Presentation of RLOD before and after confirmation of the automated alternative method results - Manual extraction protocol

Matrix /Strain	RLOD	RLODL	RLODU	b=ln(RLOD)	sd(b)	z-Test statistic	p-value
Cocoa powder - S. Bareilly Ad1687	1,000	0,434	2,304	0,000	0,417	0,000	1,000
Sausage - S. Enteritidis Ad926	1,000	0,437	2,287	0,000	0,414	0,000	1,000
Raw milk - S. Anatum Ad1166	1,000	0,434	2,304	0,000	0,417	0,000	1,000
Pasteurized liquid egg - S. Typhimurium Ad1484	1,000	0,485	2,063	0,000	0,362	0,000	1,000
Fish terrine - S. Brandenburg Ad351	1,119	0,550	2,276	0,112	0,355	0,316	0,752
Sausage for dog - S. Cerro Ad689	1,000	0,430	2,327	0,000	0,422	0,000	1,000
Combined	1,018	0,743	1,395	0,018	0,157	0,113	0,910

Table 34 - Presentation of RLOD before and after confirmation of the automated alternative method results - Automated extraction protocol - CFX96

Matrix /Strain	RLOD	RLODL	RLODU	b=ln(RLOD)	sd(b)	z-Test statistic	p-value
Cocoa powder - S. Bareilly Ad1687	1,000	0,434	2,304	0,000	0,417	0,000	1,000
Sausage - S. Enteritidis Ad926	1,000	0,437	2,287	0,000	0,414	0,000	1,000
Raw milk - S. Anatum Ad1166	1,000	0,434	2,304	0,000	0,417	0,000	1,000
Pasteurized liquid egg - S. Typhimurium Ad1484	1,000	0,485	2,063	0,000	0,362	0,000	1,000
Fish terrine - S. Brandenburg Ad351	1,119	0,550	2,276	0,112	0,355	0,316	0,752
Sausage for dog - S. Cerro Ad689	1,000	0,430	2,327	0,000	0,422	0,000	1,000
Boot socks - S.Agona Ad1306	0,888	0,439	1,795	-0,119	0,352	0,339	1,265
Combined	1,000	0,752	1,331	0,000	0,143	0,000	1,000

Table 35 - Presentation of RLOD before and after confirmation of the automated alternative method results - Automated extraction protocol - LC480

Matrix /Strain	RLOD	RLODL	RLODU	b=ln(RLOD)	sd(b)	z-Test statistic	p-value
Cocoa powder - S. Bareilly Ad1687	1,000	0,434	2,304	0,000	0,417	0,000	1,000
Sausage - S. Enteritidis Ad926	1,000	0,437	2,287	0,000	0,414	0,000	1,000
Raw milk - S. Anatum Ad1166	1,000	0,434	2,304	0,000	0,417	0,000	1,000
Pasteurized liquid egg - S. Typhimurium Ad1484	1,000	0,485	2,063	0,000	0,362	0,000	1,000
Fish terrine - S. Brandenburg Ad351	1,119	0,550	2,276	0,112	0,355	0,316	0,752
Sausage for dog - S. Cerro Ad689	1,000	0,430	2,327	0,000	0,422	0,000	1,000
Boot socks - S.Agona Ad1306	1,000	0,495	2,019	0,000	0,351	0,000	1,000
Combined	1,015	0,762	1,352	0,015	0,143	0,106	0,916

3.2.4 LOD50

The $LOD_{50\ \%}$ calculations according to Wilrich & Wilrich POD-LOD calculation program - version 9, 2017-09-23 test are given **Table 36, Table 37** and **Table 38**.

Table 36 - LOD_{50} results - Reference method ISO 6579-1

CFX96/LC 480			
Name	**LOD 50**	**Lower conf. Limit**	**Upper conf. Limit**
Cocoa powder - S. Bareilly Ad1687	1,354	0,776	2,362
Sausages - S. Enteritidis Ad926	0,817	0,447	1,493
Raw milk - S. Anatum Ad1166	0,787	0,456	1,359
Egg - S. Typhimurium Ad1484	0,766	0,448	1,310
Fish terrine - S. Brandenburg Ad351	0,810	0,478	1,374
Sausages for dog - S. cerro Ad689	0,936	0,514	1,705
Bootsocks (PPS) - S. Agona Ad1306	0,511	0,308	0,849

Table 37 - LOD_{50} results - Manual extraction protocol

CFX96			
Name	**LOD 50**	**Lower conf. Limit**	**Upper conf. Limit**
Cocoa powder - S. Bareilly Ad1687	1,354	0,776	2,362
Sausages - S. Enteritidis Ad926	0,817	0,447	1,493
Raw milk - S. Anatum Ad1166	0,787	0,456	1,359
Egg - S. Typhimurium Ad1484	0,766	0,448	1,310
Fish terrine - S. Brandenburg Ad351	0,901	0,529	1,534
Sausages for dog - S. cerro Ad689	0,936	0,514	1,705
Bootsocks (PPS) - S. Agona Ad1306	0,448	0,268	0,748

LC 480			
Name	**LOD 50**	**Lower conf. Limit**	**Upper conf. Limit**
Cocoa powder - S. Bareilly Ad1687	1,354	0,776	2,362
Sausages - S. Enteritidis Ad926	0,817	0,447	1,493
Raw milk - S. Anatum Ad1166	0,787	0,456	1,359
Egg - S. Typhimurium Ad1484	0,766	0,448	1,310
Fish terrine - S. Brandenburg Ad351	0,901	0,529	1,534
Sausages for dog - S. cerro Ad689	0,936	0,514	1,705
Bootsocks (PPS) - S. Agona Ad1306	0,511	0,308	0,849

Table 38 - LOD_{50} results - Automated extraction protocol

CFX96			
Name	**LOD 50**	**Lower conf. Limit**	**Upper conf. Limit**
Cocoa powder - S. Bareilly Ad1687	1,354	0,776	2,362
Sausages - S. Enteritidis Ad926	0,817	0,447	1,493
Raw milk - S. Anatum Ad1166	0,787	0,456	1,359
Egg - S. Typhimurium Ad1484	0,766	0,448	1,310
Fish terrine - S. Brandenburg Ad351	0,901	0,529	1,534
Sausages for dog - S. cerro Ad689	0,936	0,514	1,705

LC 480			
Name	**LOD 50**	**Lower conf. Limit**	**Upper conf. Limit**
Cocoa powder - S. Bareilly Ad1687	1,354	0,776	2,362
Sausages - S. Enteritidis Ad926	0,817	0,447	1,493
Raw milk - S. Anatum Ad1166	0,787	0,456	1,359
Egg - S. Typhimurium Ad1484	0,766	0,448	1,310
Fish terrine - S. Brandenburg Ad351	0,901	0,529	1,534
Sausages for dog - S. cerro Ad689	0,936	0,514	1,705

3.2.5 Conclusion RLOD study

The RLOD values meet the Acceptability limit (AL) fixed at 1.5 for a paired study design for all the tested matrix/strain pairs, for the three tested protocols using the CFX96 and the Roche LightCycler 480.

3.3 Inclusivity / exclusivity

In agreement with Microval it was decided to not run again this part with the **food**proof® *Salmonella* Detection LyoKit.

4 CONCLUSION

The **food**proof® *Salmonella* Detection LyoKit - 5' Nuclease was evaluated in association with two extractions protocols for the broad range of foods:

- the **food**proof® StarPrep One Kit (manual protocol) for:
    - Chocolate and bakery products
    - Meat and meat products
    - Milk and dairy products
    - Egg products
    - Fish and seafood products
    - Feed samples
    - Primary production samples

- the **food**proof® Magnetic Preparation Kit I (automated protocol) for:
    - Chocolate and bakery products
    - Meat and meat products
    - Milk and dairy products
    - Egg products
    - Fish and seafood products
    - Feed samples

For the manual protocol, depending on the categories tested the following enrichment procedures were evaluated:

- Food and feed
    - BPW for 18-20h at 37°C ± 1 °C
    - BPW for 18-20h at 37°C and subculture in BHI for 3h at 37°C ± 1°C

Both enrichment procedures can be used alternatively.

- For primary production samples the BPW is incubated for 16-20 h at 37°C ± 1 °C and a subculture in RVS for 16-24h at 41.5°C ± 1 °C is applied.

For the automated extraction protocol; only an enrichment procedure is available:

- BPW 19-20h at 37°C ± 1 °C

The **method comparison study conclusions** are:

☒ The method comparison study scheme corresponds to a PAIRED STUDY design as the alternative and reference methods have a common enrichment procedure.

☒ In the sensitivity study, 7 categories were tested for the Manual extraction protocol: 5 food categories, one feed category and one primary production samples category.
The ND - PD and ND + PD meet the acceptability limits (AL) for each individual category and all the categories, whatever the protocol tested and thermocycler used.

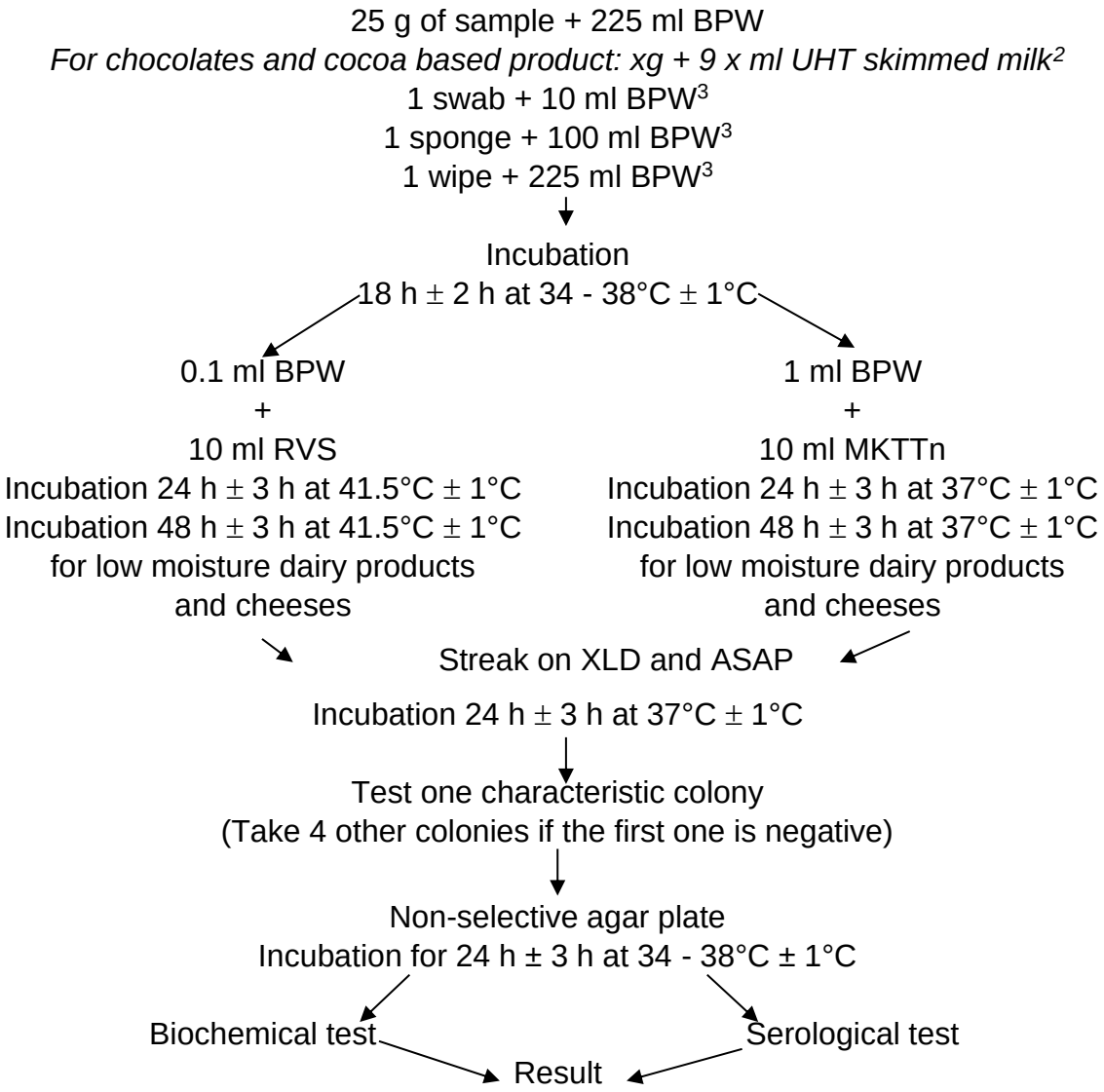
☒ The RLOD meet the AL fixed at 1.5 for a paired study design for all the tested matrix/strain pairs for the manual extraction protocols and automated extraction protocol, for the two thermocyclers tested.

Quimper, 30 October 2019

Sarah PERON	Maryse RANNOU
Technical Study Manager	Project Manager
Validation of Alternative methods	Validation of Alternative methods
Food Safety & Quality	*Food Safety & Quality*
I hereby attest to the validation of the results of the analyses carried out under the COFRAC accreditation.	I hereby attest to the validation of the verification of the conformity of the report (opinion and interpretation).

Annex A – Flow diagram of the reference method: ISO 6579-1:2017: Microbiology of the food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella* – Part 1: detection of *Salmonella* spp.

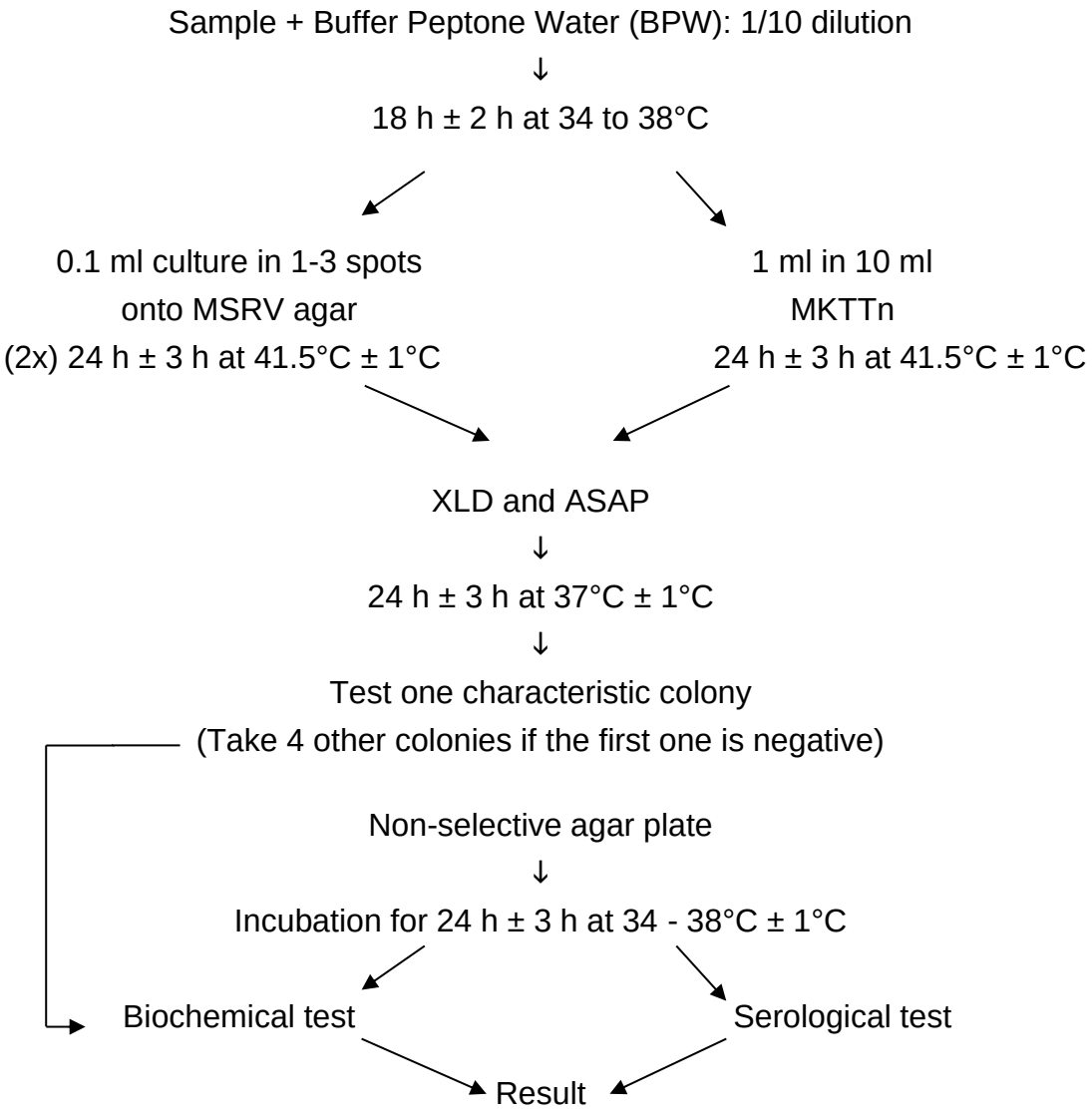
Food, feed and environmental samples: Preparation according to ISO 6887-1 to 5
Chocolate and cocoa based products: Cf. ISO 6887-4



[2] For chocolates products containing > 20 % fat, unless the products already contain sufficient emulsifier, add Tween 80. Optional: Brilliant Green 0.018 g/l

[3] For sampling after cleaning process pre-moisten
- 1 swab + 1 ml broth universal neutralizing (+ 9 ml BPW)
- 1 sponge + 10 ml broth universal neutralizing (+ 90 ml BPW)
- 1 wipe + BPW + 10 % neutralizing agent (+ 225 ml BPW)

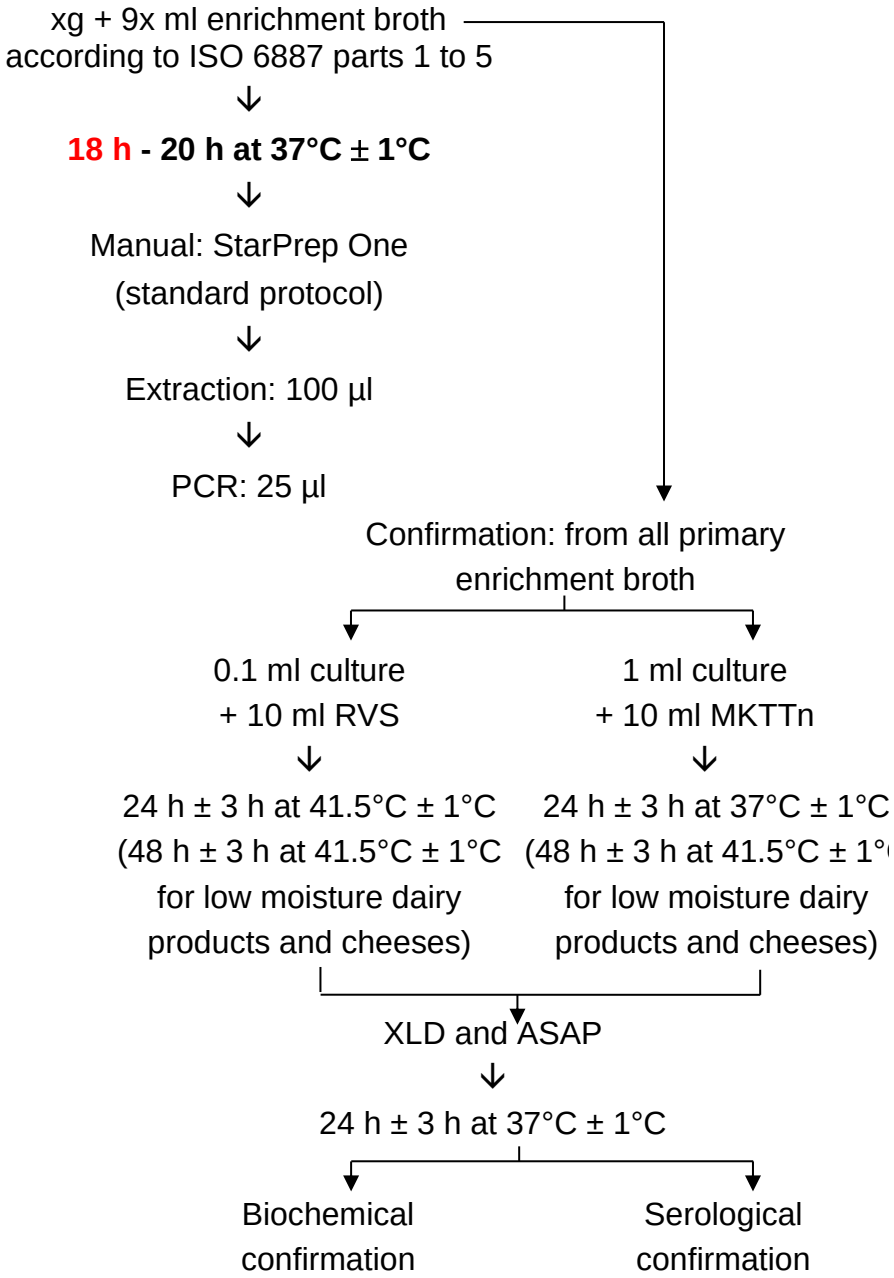
Primary production samples: faeces and environmental samples
Preparation according to ISO 6887-6



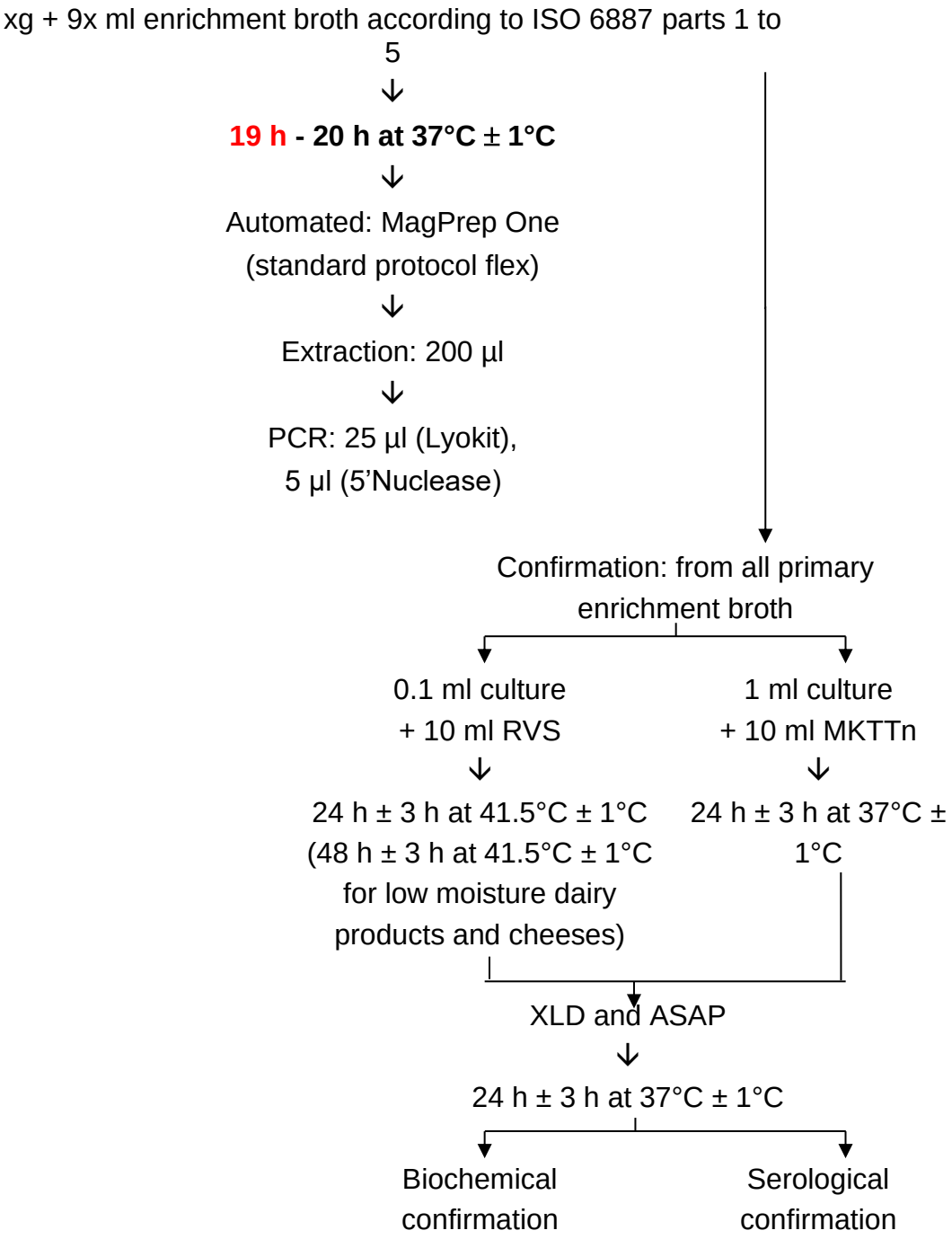
Annex B - Flow diagrams of the alternative method

Protocol ①

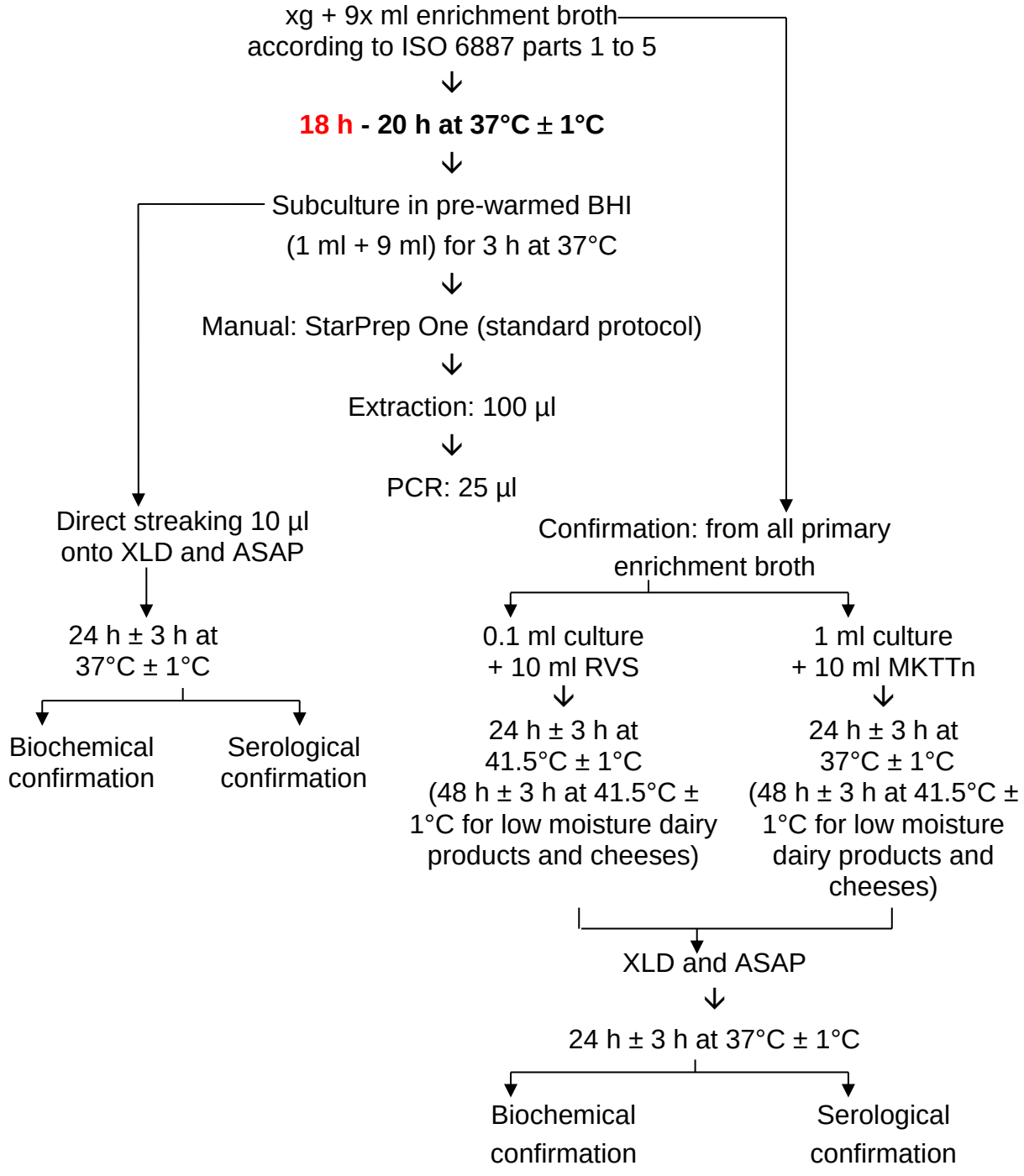
Chocolate and bakery products (25 g)
Meat and meat products (25 g)
Milk and dairy products (25 g)
Egg products (25 g)
Fish and seafood products (25 g)
Feed samples (25 g



Protocol ⑦
Chocolate and bakery products (25 g)
Meat and meat products (25 g)
Milk and dairy products (25 g)
Egg products (25 g)
Fish and seafood products (25 g)
Feed samples (25 g)



Protocol ®
Chocolate and bakery products (25 g)
Meat and meat products (25 g)
Milk and dairy products (25 g)
Egg products (25 g)
Fish and seafood products (25 g)
Feed samples (25 g



Protocol PPS
Primary production samples (25 g or sampling device)

xg + 9x ml BPW
or according to ISO 6887-6
1 wipe or 1 bootsock + 225 ml BPW
1 swab + 10 ml BPW
1 sponge + 100 ml BPW
↓
16 h - 20 h at 37°C ± 1°C
↓
Subculture in
RVS (0.1 ml + 10 ml)
16 - 24 h at 41.5°C ± 1°C
↓
Manual: StarPrep One (standard protocol)
↓
Extraction: 500 µl
↓
PCR: 5 µl + 20 µl H_2O

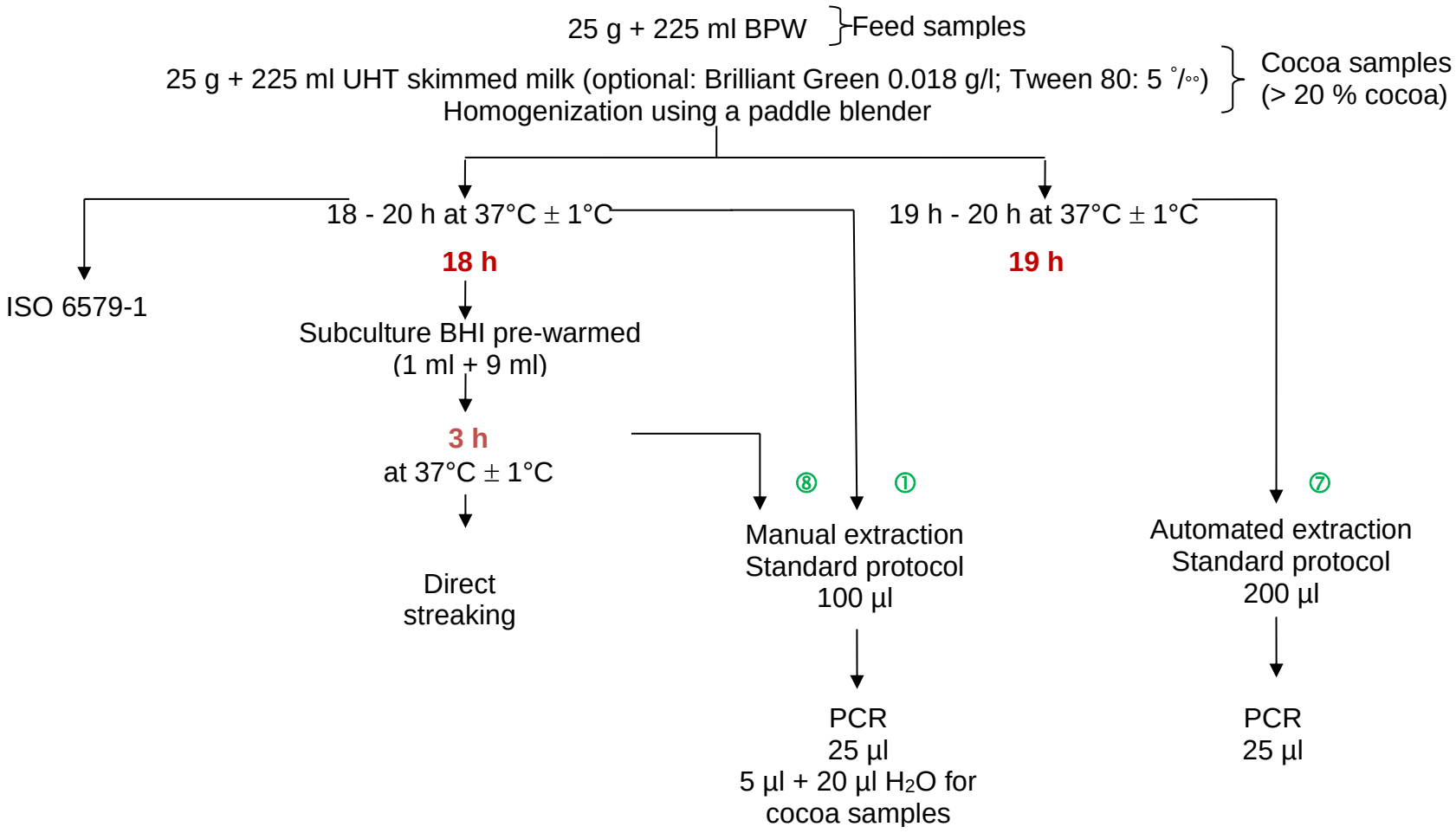
Direct streaking
10 µl onto XLD and ASAP
↓
24 h ± 3 h at
37 °C ± 1 °C

Biochemical confirmation

Serological confirmation

Kit: foodproof® *Salmonella* Detection LyoKit - 5' Nuclease - Protocols to apply per category

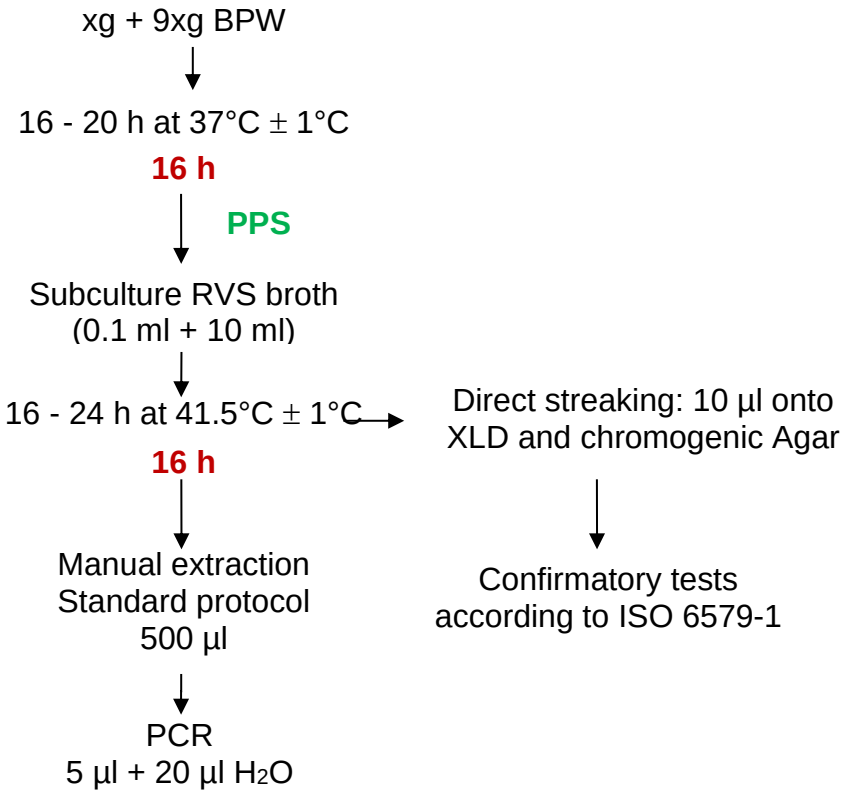
Chocolate and bakery products (Paired study design)
Feed samples (Paired study design)



Possibility to store the DNA extracts at -20°C

Kit: foodproof® *Salmonella* Detection LyoKit - 5' Nuclease - Protocols to apply per category

Primary production samples (Paired study design)



Possibility to store the DNA extracts at -20°C

Extraction protocols

foodproof® StarPrep One Kit

Procedure A: Standard Manual protocol

Shake the enrichment culture gently and let settle
↓
Transfer 100 µl (supernatant) (500 µl for PPS) in a 1.5 ml reaction tube
↓
Centrifuge for 5 min at 8000 g
↓
Remove the supernatant
↓
Add 200 µl lysis buffer
↓
Resuspend the pellet by vortex or by pipetting gently up and down
↓
Heat treatment for 10 min at 95-100°C in a heating block
↓
Keep 1 min at 15 - 25°C (room temperature)
↓
Mix by vortexing for 2 sec
↓
Centrifuge for 2 min at 13000 g
↓
PCR on 25 µl DNA extract
↓
Possibility to store the DNA extracts at -20°C

Procedure C: Ultra Rapid protocol (NOT performed during validation study)

Shake the enrichment culture gently and let settle
↓
Transfer the needed Lysis Puffer in a sterile reservoir.
↓
Transfer 200 µl StarPrep One Lysis Buffer to each tube of the 8-strip
↓
Transfer 50 µl of the sample (supernatant) to the 8-strip
Seal with sterile cap strips
↓
Incubate in the TM21 heating unit for 10 min at 100°C
↓
Carefully remove the tube strips from the heating unit and allow the tube to sit for 1 min at 15-25°C
↓
Centrifuge for 5 min at 2000 g
↓
PCR on 25 µl DNA extract from subculture or 5 µl DNA extract,
+ 20 µl H_2O from enrichment without a regrowth step
↓
Possibility to store the DNA extracts at -20°C

foodproof® Magnetic Preparation Kit I

Automated protocol

Using the foodproof Magnetic Preparation Kit in combination
with the KingFisher® Flex workstation

Preparation of kit working solutions

Binding Buffer: add 80 ml absolute isopropanol
Wash Buffer I: add 154 ml absolute isopropanol
Wash Buffer II: add 164 ml absolute isopropanol
↓
Resuspend the lysis buffer and the magnetic beads in the Binding Buffer
Place the Tip Comb 96 WH on a Tip Plate
↓
Prefill:
Lysis plate: add 320 µl lysis buffer and 25 µl Reagent P (if necessary)[4]
Washing plate I: add 750 µl Wash Buffer I
Washing plate II: add 750 µl Wash Buffer II
Washing plate II: add 750 µl Wash Buffer II
Elution plate: add 300 µl Elution Buffer
↓
Transfer 200 µl of the enriched sample into the lysis plate
↓
Choose assay file “foodproof-MPK-I” on Instrument and press Start
↓
After an elevated lysis step of 10 min, take out the plate and add 315 µl Binding Buffer. Reinsert the plate and press Start
↓
PCR on 25 µl DNA extract
↓
Possibility to store the DNA extracts at -20°C

[4] For protein rich food samples (e.g. egg, pork, chicken salmon, cheese) using the automated extraction protocol, addition of Reagent P to the Lysis Buffer is necessary.