Method Comparison and Interlaboratory Study Report for the ISO 16140-2:2016 validation of Kylt® *Salmonella* spp. 2.0, for the detection of *Salmonella* spp in 4 categories (Raw meat and ready to cook meat products, Raw poultry and ready to cook poultry products, Environmental samples and Primary production samples)

MicroVal study number: 2017LR78

Method/Kit name: Kylt® *Salmonella* spp. 2.0

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MicroVal Expert Laboratory: Campden BRI
Suzanne Jordan
Station Road,
Chipping Campden,
Gloucs,
GL55 6LD, UK
Tel: 0044 1386 842000
Email: suzanne.jordan@campdenbri.co.uk
www.campdenbri.co.uk

Foreword

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

Company:	**AniCon Labor GmbH** Muehlenstraße 13 49685 Hoeltinghausen Germany
Expert Laboratory:	Campden BRI Station Road Chipping Campden Gloucs, GL55 6LD, UK
Method/Kit name:	Kylt® *Salmonella* spp. 2.0
Validation standard:	Microbiology of the food chain— Method validation Part 1: Vocabulary (ISO 16140-1:2016) and Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method (ISO 16140-2:2016)
Reference method:	Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of *Salmonella* Part 1: Detection of Salmonella spp. (ISO 6579-1:2017)
Scope of validation:	Raw meat and ready to cook meat products Raw poultry and ready to cook poultry products Environmental sample Primary production samples

Certification orgnization: Lloyd's Register

List of abbreviations

A(lt)	Alternative method
AL	Acceptability Limit
Art. Cont.	artificial contamination
CFU	Colony Forming Units
EL	Expert Laboratory
FP	False Positive
FPR	False Positive Ratio
g	Gram
h	Hour
ILS	Interlaboratory Study
LOD	Level of Detection
MCS	Method Comparison Study
min	minute
ml	millilitre
MR	(MicroVal) Method Reviewer
MVTC	MicroVal Technical Committee
NA	Negative Agreement
na	not applicable
ND	Negative Deviation
neg (-)	negative/no growth/no reaction/target not detected
NS	Non-Suspect growth
nt	not tested
PA	Positive Agreement
PD	Positive Deviation
pos (+)	positive/growth/target detected
PPNA	Presumptive Positive Negative Argreement (belongs to the False Positive results)
PPND	Presumptive Positive Negative Deviation (belongs to the False Positive results)
R(ef)	Reference method
RLOD	Relative Level of Detection
RT	Relative Trueness
S	Suspect growth
SE	Relative Sensitivity
SP	Relative Specificity
TP	True Positive

BGA	Brilliant Green Agar
BPW	Buffered Peptone Water
LDC	l-Lysine decarboxylation medium
MKTTn	Muller-Kauffmann Tetrathionate-novobiocin broth
MSRV	Modified Semi-solid Rappaport Vassiliadis medium
RVS	Rappaport-Vassiliadis Soya broth
TSI	Triple Sugar Iron agar
UA	Urea Agar
XLD	Xylose Lysine Deoxycholate agar

Contents

1 Introduction

In this project a MicroVal validation study, based on ISO 16140-2:2016, of alternative method(s) for the detection of *Salmonella* spp in 4 different categories (Raw meat and ready to cook meat products, Raw poultry and ready to cook poultry products, Environmental samples and Primary production samples) was carried out by Campden BRI as the MicroVal Expert Laboratory.

The alternative method used was:

The Kylt® *Salmonella* spp. 2.0. This method is a polymerase chain reaction (PCR) based assay that targets *Salmonella* specific DNA split into 3 stages:

1) Sample enrichment in Buffered Peptone Water (BPW) prewarmed to 37°C and incubated for 16h at 37 ±1°C. Additional incubation conditions have been requested by the client for selected groups and these are outlined in the Table below.

Category	Additional incubation parameters
Primary production	Second selective enrichment RVS (5h at 41.5°C±1°C)

2) DNA extraction using 1 of the 2 DNA extraction methods listed in the manufacturer's protocol

- Manual reagent based lysis (Kylt® DNA Extraction-Mix II)
- Column based extraction (Kylt® RNA/ DNA Purification)

The Kylt® DNA Extraction-Mix II is available in product code 31001 (for feed and food testing) and 31299 (for veterinary samples from poultry, swine and cattle). Both of these kits are identical to each other and the study used 31001 for food and feed testing and 31299 for veterinary samples.

3) Analysis of DNA extracts by real time PCR using selected PCR platforms. The study evaluated 2 PCR programmes for *Salmonella* detection;

- Profile II: specific to bacterial pathogens
- Profile I: allows co -analysis of bacterial and viral assays in the same run.

The real-time detection kit is a single kit format that is available as part of product numbers 31001, 31302, 31299, and 31301. Product numbers 31001 and 31299 that include Kylt® DNA Extraction-Mix II will be used for the validation for food and feed testing and veterinary samples respectively.

When a sample gave a positive result from the PCR, the BPW was subcultured into RVS broth and MKTTn broth as described in ISO 6579-1 (2017) and the rest of the confirmation protocol in the reference method was followed. For PCR positive primary production samples, the BPW was subcultured onto MSRV agar and into MKTTn broth as described in ISO 6579-1 (2017) and the rest of the confirmation protocol in the reference method was followed.

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

ISO 6579-1 (2017). 25 g sample sizes were tested for all food matrices; additionally 375 gram were tested for raw and ready to cook meat and raw and ready to cook poultry.

The scope of the validation study was: 4 named categories listed within (ISO 16140-2:2016):

- Raw meat and ready to cook meat products
- Raw poultry and ready to cook poultry products
- Environmental samples
- Primary production samples.

Criteria evaluated during the study have been:

Method Comparison Study (MCS):

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

Summarized, the conclusions on the Method Comparison study are:

For 25g samples sensitivity, the observed values for ND-PD and ND+PD for the individual categories and for all categories meet the acceptability limits (observed values ≤ AL) for the following DNA extraction and PCR profiles.

- Kylt® DNA Extraction-Mix II and run on profile I
- Kylt® DNA Extraction-Mix II and run on profile II
- Kylt® RNA/ DNA Purification and run on profile I*
- Kylt® RNA/ DNA Purification and run on profile II

*Primary production samples should be run with the 5h RVS enrichment step included only.

Analysis of 375g samples revealed that the observed values for ND-PD for the individual categories and for all categories meet the acceptability limits (observed values ≤ AL) for one of the DNA extraction methods or PCR profiles. –

- Kylt® DNA Extraction-Mix II and run on profile II

The other 3 DNA extraction methods or PCR profiles did not meet the acceptability limits (observed values ≤ AL) for the individual categories and for all categories.

- Kylt® DNA Extraction-Mix II and run on profile I
- Kylt® RNA/ DNA Purification and run on profile I*
- Kylt® RNA/ DNA Purification and run on profile II

The RLOD values meet the acceptability limit for 25g environmental and primary production samples, which is 1.5 for paired studies for all DNA extraction and PCR profiles.

In addition, the RLOD values meet the acceptability limit for 375g samples of Raw poultry and ready to cook poultry products and Raw meat and ready to cook meat products, which is 2.5 for unpaired studies for DNA Extraction-Mix II profiles I and II and Kylt® RNA/ DNA Purification profiles I and II.

The alternative Kylt® *Salmonella* spp. 2.0 detection method is selective and specific for the following DNA extraction and PCR profile combinations for 25g samples only.

- Kylt® DNA Extraction-Mix II and run on profile I
- Kylt® DNA Extraction-Mix II and run on profile II
- Kylt® RNA/ DNA Purification and run on profile I*
- Kylt® RNA/ DNA Purification and run on profile II

*Primary production samples should be run with the 5h RVS enrichment step included only.

Interlaboratory study:

- Specificity
- Sensitivity
- Relative Trueness
- False positive ratio.

Summarized, the conclusions on the InterLaboratory study are: The observed values for ND-PD and ND+PD are lower than the acceptability limits.

Overall conclusions are

The Kylt® *Salmonella* spp. 2.0 is considered equivalent to the ISO standard for the following DNA extraction kit and PCR profile combinations for 25g samples.

- Kylt® DNA Extraction-Mix II profile I
- Kylt® DNA Extraction-Mix II profile II
- Kylt® RNA/ DNA Purification profile I *
- Kylt® RNA/ DNA Purification profile II

*Primary production samples should be run with the 5h RVS enrichment step included only.

The Kylt® *Salmonella* spp. 2.0 is also considered equivalent to the ISO standard for the following DNA extraction kit and PCR profile combinations for 375g samples of a raw and ready to cook meat and raw and ready to cook poultry.

- Kylt® DNA Extraction-Mix II and run on profile II

The following Table summarises the final scope of the validation for the 7500 FAST

Alternate method	Raw and ready to cook poultry	Raw and ready to cook meat	Environmental	PPS
Kylt® DNA Extraction-Mix II profile I	25g	25g	25g	25g
Kylt® DNA Extraction-Mix II profile II	25g + 375g	25g + 375g	25g	25g
Kylt® RNA/ DNA Purification profile I	25g	25g	25g	25g (after 5h RVS only)
Kylt® RNA/ DNA Purification profile II	25g	25g	25g	25g

A summary of the mutliplatform within the final scope scope of the validation are shown in the table below.

Category	DNA extraction pcr profile combination	Matrices certified						
		1	2	3	5	6	7	10
Meat 25g	Extraction mix II profile I	p	p	p	p	p	p	p
	Extraction mix II profile II	p	p	p	p	p	p	p
	RNA/DNA extraction profile I	p	p	f	p	p	p	f
	RNA/DNA extraction profile II	p	p	p	f	p	p	f
Meat 375g	Extraction mix II profile I	p	p	p	p	p	p	p
	Extraction mix II profile II	p	p	p	p	p	p	p
	RNA/DNA extraction profile I	p	p	f	p	p	p	f
	RNA/DNA extraction profile II	p	p	p	f	p	p	f
Poultry 25g	Extraction mix II profile I	p	p	p	p	p	p	p
	Extraction mix II profile II	p	p	p	p	p	p	p
	RNA/DNA extraction profile I	p	p	p	p	p	p	p

	RNA/DNA extraction profile II	p	p	p	p	p	p	p
Poultry 375g	Extraction mix II profile I	p	p	p	p	p	p	p
	Extraction mix II profile II	p	p	p	p	p	p	p
	RNA/DNA extraction profile I	p	p	p	p	p	p	p
	RNA/DNA extraction profile II	p	p	p	p	p	p	p
Environmental	Extraction mix II profile I	p	p	p	p	p	p	p
	Extraction mix II profile II	p	p	p	p	p	p	p
	RNA/DNA extraction profile I	p	p	p	p	p	p	p
	RNA/DNA extraction profile II	p	p	p	p	p	p	p
Primary production	Extraction mix II profile I	p	p	p	p	p	p	p
	Extraction mix II profile II	p	p	p	p	p	p	p
	RNA/DNA extraction profile I	p	p	p	p	p	p	p
	RNA/DNA extraction profile II	p	p	p	p	p	p	p
Primary production 5h RVS broth	Extraction mix II profile I	p	p	p	p	p	p	p
	Extraction mix II profile II	p	p	p	p	p	p	p
	RNA/DNA extraction profile I	p	p	p	p	p	p	p
	RNA/DNA extraction profile II	p	p	p	p	p	p	p

Key for platforms 1 =7500 FAST 2 = CFX96 3 = CFX 384, 5 = Rotor Gene 3000,6 = Rotor Gene 6000, 7= Aria MX Pro, 10= Light cycler 480

This report corresponds to the method comparison study and the inter-laboratory study, and gathers the observed data and interpretations according to the EN ISO 16140- 2:2016 standard and the most recent version of the MicroVal technical committee interpretations.

2 Method protocols

The two qualitative methods compared in this study are shown below

Alternative method	**Organisms covered**	**Reference method**
Kylt® Salmonella spp. 2.0 Rev004, August 2018	*Salmonella* spp.	ISO 6579-1:2017

The Method Comparison Study was carried out using 25 gram portions of sample material. Additionally, 375 gram were tested for raw and ready to cook meat and raw and ready to cook poultry for the Method Comparison Study. The 375g data was analysed separately to the 25g data. As agreed with the Microval committee, a relative limit of detection study was carried out to verify that the EL has the ability to detect *Salmonella* spp in 375g samples using ISO 6579-1:2017.

As the reference and the alternative method share the initial (pre)-enrichment step for 25g samples, the resulting data were treated as paired data (EN-ISO 16140-2). For 375g samples, the reference and the alternative method did not share the initial (pre)-enrichment step and were treated as unpaired data (EN-ISO 16140-2).

2.1 Reference method

A flow diagram outlining the stages involved in the Reference method is included in Annex A.

Sample preparations used in the reference method and the alternative method were done according to ISO 6887-series and ISO 6579-1 for all sample matrices in this study. Details of the sample preparation carried out are included in the flow diagrams in Annex A for reference.

2.2 Alternative method

Flow diagrams of the alternative method are available in Annex B.

A copy of the Kylt® *Salmonella* spp. 2.0 Real-Time PCR detection kit kit insert is displayed in Annex C and a summary of the protocol is oultined below.

The alternative method principle is based on polymerase chain reaction (PCR)

Samples were enriched in Buffered Peptone Water (BPW) pre-warmed to 37°C (dilution ratio = 1:10 for 25g samples and 1:5 for 375g samples) and incubated for 18h±2h at 37 ±1°C. During the method validation, the samples were incubated at 37 ±1°C for the shortest time stated in the incubation tolerance range of 16h. Additional incubation conditions were required for selected groups and these are outlined in the Table below.

Table 1: Additonal incubation conditions for primary production samples

Category	Additional incubation parameters	Required in
Primary production	Second selective enrichment RVS (5h at 41.5°C±1°C) at a ratio of 1:100 to reduce the impact of potential inhibitors in the samples	Sensitivity, and relative limit of detection

After incubation, the samples were prepared using 1 of the 2 DNA extraction methods listed in the manufacturer's protocol (refer to Annex C for method details)

- Manual reagent based lysis (Kylt® DNA Extraction-Mix II)
- Column based extraction (Kylt® RNA/DNA Purification)

DNA extracts were analysed by real time PCR using selected PCR platforms. The Kylt® *Salmonella* spp. 2.0 offers the capability to run the assay on multiple platforms. All method comparison study samples were run by the expert laboratory on the ABI 7500 platform (Life Technologies). In addition, DNA samples were frozen and sent blind-coded to the manufacturer to run on 9 alternative platforms listed below

- CFX 96 (BioRad)
- CFX 384 (BioRad)
- Rotor-Gene Q (Qiagen)
- Rotor-Gene 3000 (Corbett Research)
- Rotor-Gene 6000 (Corbett Research)
- AriaMx Pro (Agilent)
- LightCycler® 96 (Roche)
- LightCycler® 480 (Roche)
- Mic (BMS)

All samples were analysed for each combination of DNA extraction method, PCR platform and PCR programme. DNA samples were blind coded with a different code given for each combination of sample, DNA extraction method, PCR platform and PCR programme. All results were analysed following the guidance on how to set the threshold in the kit insert. The data generated by the manufacturer on the additional PCR instruments was sent to the EL for inclusion in the validation study.

The current study evaluated 2 PCR programmes for *Salmonella* detection;

Profile II: specific to bacterial pathogens and Profile I: allows co -analysis of bacterial and viral assays in the same run.

To address possible issues with inhibition, the strategy outlined in the manufacturer's instructions was followed. For all primary production samples, a further DNA extraction was carried out on a second enrichment in RVS broth for 5h which was analysed by Kylt® *Salmonella* spp. 2.0 PCR.

If inhibition was detected in samples from the other categories (raw meat and ready to cook meat products, raw poultry and ready to cook poultry products and environmental samples), the DNA extraction from the 5h RVS was also run as outlined manufacturer's instructions.

As the 25g samples analysed with the Reference and Alternative methods used the same enrichment broth, the 16h BPW enrichment for the Alternative and Reference methods was used to complete the *Salmonella* spp. detection protocol following ISO 6579-1:2017. For 375g samples, the reference and the alternative method did not share the initial (pre)-enrichment step and were treated as unpaired data (EN-ISO 16140-2).

Confirmation of all samples was performed as outlined in the current ISO method ISO 6579-1:2017. Presumptive positive colonies on XLD and Rambach agar were purified on NA and incubated at 37°C ±1°C aerobically for 24h ±2h. After purification, the colonies were tested with *Salmonella* Poly O and Poly H antisera, Gram stain, oxidase test and biochemical analysis using API 20E (BioMerieux).

In order to verify the stability of BPW enrichments during chilled storage (72h at 2-8°C as stated in the kit insert), the ISO detection protocol was repeated on the BPW enrichments of positive and discordant samples following storage for 72h at 2-8°C. Chilled storage for 72h at 2-8°C is representative of the storage conditions used to store samples in analytical laboratories.

For all positive samples, the ISO method only was re-run as the samples were paired and the ISO broths were also used for the isolation and confirmation of the Kylt method. This will allow a 72h storage period for enriched samples to be validated. For discordant results, both methods were run to verify the results obtained for Reference and alternative methods.

2.3 Study design

The Method Comparison Study was carried out using 25 gram test portions of the samples for most parts. For raw meat and ready to cook meat products category and raw poultry and ready to cook poultry products category, 375g samples were analysed for the RLOD and sensitivity studies. An RLOD was carried out in the EL to ensure that the sample size of 375 gram is covered by the ISO 17025 accreditation.

The samples were prepared for analysis and diluted in accordance with ISO 6887 (all parts) unless specified differently in the alternative method.

See Table 2 for specific preparations used in the validation study.

Table 2: Sample preparations required for the analysis of samples being used in the Method comparison study

Sample type	**Category**	**Appropriate part of ISO 6887 to be used**	**Preparation used**
All food products within the category	Raw meat and ready to cook meat products, Raw poultry and ready to cook poultry products	1 and 2	For 25g samples Add 225 ml BPW to 25g samples, dilution ratio = 1:10 For 375g samples Alternative method 1500ml BPW to 375g samples, dilution ratio 1:5 Reference method 3375ml to 375g samples dilution ratio = 1:10
Carcass swabs	Raw meat and ready to cook meat products	1 and 2	Add swab to 50ml tube containing 40ml BPW and make sure that the whole sample is submerged. Mix/ shake well before enrichment
Process water poultry, cattle and swine	Environmental	1 and 2	Samples <100ml added to an equal volume of double strength BPW
Dust wipe samples	Environmental	N/A	Make sure that the whole sample is submerged in BPW. Mix/ shake well before enrichment
Sponge samples	Environmental	N/A	Make sure that the whole sample is submerged in BPW. Mix/ shake well before enrichment
Dust and residue swabs	Environmental	N/A	Add swab to 50ml tube containing 40ml BPW and make sure that the whole sample is submerged. Mix/ shake well before enrichment
Swabs stainless steel, plastic surface, ceramic and rubber	Environmental	6	Add swab to 50ml tube containing 40ml BPW and make sure that the whole sample is submerged. Mix/ shake well before enrichment
Rectal swabs	Primary production	6	Add swab to 50ml tube containing 40ml BPW and make sure that the whole sample is submerged. Mix/ shake well before enrichment
Faeces pig, chicken and turkey, as well as gut contents	Primary production	6	Mix gently and add 225ml BPW to 25g samples
Bootsocks	Primary production	6	Add at least 225ml BPW and make sure that the whole sample is submerged
Hatchery samples – basket liners	Primary production	6	Samples should be at least 1m surface area. Add 1 to 2L of BPW (pre- warmed to at least room temperature, but preferably 37°C)

Sample type	Category	Appropriate part of ISO 6887 to be used	Preparation used
Transport truck sampling, waiting area swab, transport cage debris	Primary production	6	Add at least 225ml BPW and make sure that the whole sample is submerged. Mix/ shake well before enrichment
Organs lymph nodes	Primary production	6	Macerate lymph nodes by hammering a strong sterile plastic bag containing the samples. Add 9ml per g of sample

All samples were incubated at 37±1°C for the shortest time stated in the incubation tolerance range of 16h. In addition, a second selective enrichment of primary production samples was carried out in RVS broth at a dilution ratio of 1:100. This broth was incubated for 5h at 41.5°C±1°C prior to DNA extraction to reduce the impact of potential inhibitors in the samples.

As the reference and alternative methods are performed with the same test portion and have a common enrichment procedure for the 25g samples, this were analysed as a paired data study. The 375g samples had a separate enrichment procedure for the reference and alternative method and were anlaysed as an unpaired study.

3 Method comparison study

3.1 Sensitivity Study

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

3.1.1 Categories and sample types

A total of 4 Categories were included in this validation study.
A minimum of 60 Items for each Category were tested by both the reference method and the alternative method in the sensitivity study, with a minimum of 30 positive samples per Category.

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

The categories, the types and the number of samples analyzed are presented in Table 3.

Table 3 - Categories, types and number of samples analysed

Category	Type	Alternative method protocol	Test portion size	Number of samples
Raw meat and ready to cook meat products	Fresh meats (unprocessed)	Kylt® *Salmonella* spp. 2.0	25g & 375g	20
	Ready to cook (processed)	Kylt® *Salmonella* spp. 2.0		20
	Ready to eat and Ready to reheat products	Kylt® *Salmonella* spp. 2.0		20

Category	Type	Alternative method protocol	Test portion size	Number of samples
Raw poultry and ready to cook poultry products	Fresh meats (unprocessed)	Kylt® *Salmonella* spp. 2.0	25g & 375g	20
	Ready to cook (processed)	Kylt® *Salmonella* spp. 2.0		20
	Ready to eat and Ready to reheat products	Kylt® *Salmonella* spp. 2.0		20
Environmental	Dust and residues	Kylt® *Salmonella* spp. 2.0	25g	20
	Cleaning and process waters	Kylt® *Salmonella* spp. 2.0		20
	Surface samples	Kylt® *Salmonella* spp. 2.0		20
Primary production	Poultry	Kylt® *Salmonella* spp. 2.0	25g	20
	Swine/ Cattle	Kylt® *Salmonella* spp. 2.0		20
	Environmental samples	Kylt® *Salmonella* spp. 2.0		20
	Ready to cook (processed)	Kylt® *Salmonella* spp. 2.0		20
	Ready to eat and Ready to reheat products	Kylt® *Salmonella* spp. 2.0		20

300 x 25g and 120 x 375g samples were analyzed with the Kylt® *Salmonella* spp 2.0 kit with the following DNA extractions and PCR profile combinations.

- Kylt® DNA Extraction-Mix II and run on profile I
- Kylt® DNA Extraction-Mix II and run on profile II
- Kylt® RNA/ DNA Purification and run on profile I
- Kylt® RNA/ DNA Purification and run on profile II

An additional analysis was carried out on the 25g primary production, with a second DNA extraction carried out on a 5h RVS enrichment broth to reduce the impact of potential inhibitors in this sample type.

The distribution of positive and negative samples per tested category and type is given respectively in Table 4a-d.

Table 4 a- Distribution per tested category and type DNA Extraction mix II profile I

Category	**Type**		**Positive samples***	**Negative samples**	**Total**
Raw meat and ready to cook meat products 25g	a	Fresh meats (unprocessed)	10	10	20
	b	Ready to cook (processed)	12	8	20
	c	Ready to eat and Ready to reheat products	10	10	20
		Total	**32**	**28**	**60**
Raw poultry and ready to cook poultry products 25g	a	Fresh meats (unprocessed)	9	11	20
	b	Ready to cook (processed)	11	9	20
	c	Ready to eat and Ready to reheat products	10	10	20
		Total	**30**	**30**	**60**
Environmental	a	Dust and residues	10	10	20
	b	Cleaning and process waters	10	10	20
	c	Surface samples	10	10	20
		Total	**30**	**30**	**60**
Primary production	a	Poultry	7	13	20
	b	Swine/ Cattle	11	9	20
	c	Environmental samples	12	8	20
		Total	**30**	**30**	**60**
Primary	a	Poultry	7	13	20

Category	Type		Positive samples*	Negative samples	Total
Production 5h RVS	b	Swine/ Cattle	11	9	20
	c	Environmental samples	12	8	20
		Total	**30**	**30**	**60**
Total			**152**	**148**	**300**

*Positive by at least one of the methods

Table 4b - Distribution per tested category and type DNA Extraction mix II profile II

Category	Type		Positive samples*	Negative samples	Total
Raw meat and ready to cook meat products 25g	a	Fresh meats (unprocessed)	10	10	20
	b	Ready to cook (processed)	11	9	20
	c	Ready to eat and Ready to reheat products	10	10	20
		Total	**31**	**29**	**60**
Raw poultry and ready to cook poultry products 25g	a	Fresh meats (unprocessed)	10	10	20
	b	Ready to cook (processed)	11	9	20
	c	Ready to eat and Ready to reheat products	10	10	20
		Total	**31**	**39**	**60**
Environmental	a	Dust and residues	10	10	20
	b	Cleaning and process waters	10	10	20
	c	Surface samples	10	10	20
		Total	**30**	**30**	**60**
Primary production	a	Poultry	7	13	20
	b	Swine/ Cattle	11	9	20
	c	Environmental samples	12	8	20
		Total	**30**	**30**	**60**
Primary production 5h RVS	a	Poultry	7	13	20
	b	Swine/ Cattle	11	9	20
	c	Environmental samples	12	8	20
		Total	**30**	**30**	**60**
Total			**152**	**148**	**300**

Table 4c - Distribution per tested category and type RNA/DNA Purification profile I

Category	Type		Positive samples*	Negative samples	Total
Raw meat and ready to cook meat products 25g	a	Fresh meats (unprocessed)	10	10	20
	b	Ready to cook (processed)	12	8	20
	c	Ready to eat and Ready to reheat products	10	10	20
		Total	**32**	**28**	**60**
Raw poultry and ready to cook poultry products 25g	a	Fresh meats (unprocessed)	9	11	20
	b	Ready to cook (processed)	11	9	20
	c	Ready to eat and Ready to reheat products	10	10	20
		Total	**30**	**30**	**60**
Environmental	a	Dust and residues	10	10	20
	b	Cleaning and process waters	10	10	20
	c	Surface samples	10	10	20
		Total	**30**	**30**	**60**

Category	Type		Positive samples*	Negative samples	Total
Primary production 5h RVS	a	Poultry	7	13	20
	b	Swine/ Cattle	11	9	20
	c	Environmental samples	12	8	20
		Total	**30**	**30**	**60**
Total			**122**	**118**	**240**

*Positive by at least one of the methods

Note: for PPS samples , only the method with the additional 5h RVS was tested for RNA/DNA Purification profile I

Table 4d - Distribution per tested category and type RNA/DNA Purification profile II

Category	Type		Positive samples*	Negative samples	Total
Raw meat and ready to cook meat products 25g	a	Fresh meats (unprocessed)	10	10	20
	b	Ready to cook (processed)	12	8	20
	c	Ready to eat and Ready to reheat products	10	10	20
		Total	**32**	**28**	**60**
Raw poultry and ready to cook poultry products 25g	a	Fresh meats (unprocessed)	9	11	20
	b	Ready to cook (processed)	11	9	20
	c	Ready to eat and Ready to reheat products	10	10	20
		Total	**30**	**30**	**60**
Environmental	a	Dust and residues	10	10	20
	b	Cleaning and process waters	10	10	20
	c	Surface samples	10	10	20
		Total	**30**	**30**	**60**
Primary production	a	Poultry	7	13	20
	b	Swine/ Cattle	11	9	20
	c	Environmental samples	12	8	20
		Total	**30**	**30**	**60**
Primary production 5h RVS	a	Poultry	7	13	20
	b	Swine/ Cattle	11	9	20
	c	Environmental samples	12	8	20
		Total	**30**	**30**	**60**
Total			**152**	**148**	**300**

*Positive by at least one of the methods

Table 4 e- Distribution per tested category and type DNA Extraction mix II profile I

Category	Type		Positive samples*	Negative samples	Total
Raw meat and ready to cook meat products 375g	a	Fresh meats (unprocessed)	9	11	20
	b	Ready to cook (processed)	9	11	20
	c	Ready to eat and Ready to reheat products	12	8	20
		Total	**30**	**30**	**60**
Raw poultry and ready to cook poultry products 375g	a	Fresh meats (unprocessed)	7	13	20
	b	Ready to cook (processed)	10	10	20
	c	Ready to eat and Ready to reheat products	13	7	20
		Total	**30**	**30**	**60**
Total			**60**	**60**	**120**

*Positive by at least one of the methods

Table 4f- Distribution per tested category and type DNA Extraction mix II profile II

Category	**Type**		**Positive samples***	**Negative samples**	**Total**
Raw meat and ready to cook meat products 375g	a	Fresh meats (unprocessed)	9	11	20
	b	Ready to cook (processed)	9	11	20
	c	Ready to eat and Ready to reheat products	12	8	20
		Total	**30**	**30**	**60**
Raw poultry and ready to cook poultry products 375g	a	Fresh meats (unprocessed)	7	13	20
	b	Ready to cook (processed)	10	10	20
	c	Ready to eat and Ready to reheat products	13	7	20
		Total	**30**	**30**	**60**
Total			**60**	**60**	**120**

*Positive by at least one of the methods

Table 4 g- Distribution per tested category and type RNA/DNA Purification profile I

Category	**Type**		**Positive samples***	**Negative samples**	**Total**
Raw meat and ready to cook meat products 375g	a	Fresh meats (unprocessed)	9	11	20
	b	Ready to cook (processed)	9	11	20
	c	Ready to eat and Ready to reheat products	12	8	20
		Total	**30**	**30**	**60**
Raw poultry and ready to cook poultry products 375g	a	Fresh meats (unprocessed)	7	13	20
	b	Ready to cook (processed)	10	10	20
	c	Ready to eat and Ready to reheat products	13	7	20
		Total	**30**	**30**	**60**
Total			**60**	**60**	**120**

*Positive by at least one of the methods

Table 4h- Distribution per tested category and type RNA/DNA Purification profile II

Category	**Type**		**Positive samples***	**Negative samples**	**Total**
Raw meat and ready to cook meat products 375g	a	Fresh meats (unprocessed)	9	11	20
	b	Ready to cook (processed)	9	11	20
	c	Ready to eat and Ready to reheat products	12	8	20
		Total	**30**	**30**	**60**
Raw poultry and ready to cook poultry products 375g	a	Fresh meats (unprocessed)	7	13	20
	b	Ready to cook (processed)	10	10	20
	c	Ready to eat and Ready to reheat products	13	7	20
		Total	**30**	**30**	**60**
Total			**60**	**60**	**120**

*Positive by at least one of the methods

3.1.2 Test sample preparation

60% of the positive samples analysed in the study were naturally contaminated.
Artificial contaminations were done by seeding protocols.

Naturally contaminated samples were preferentially analyzed. If necessary, artificial contaminations were obtained by:

Sample type	Category	Procedure for artificial contamination
All within category	Raw meat and ready to cook meat products Raw poultry and ready to cook poultry products	Seeding and storage of samples post inoculation for 48h ±2h at 2-8°C to chill stress the cells
Environmental swabs Process waters and drinking water	Environmental	
Faeces Swine organs – gut and lymph nodes	Primary production	
Boot socks Hatchery samples Pooled dust and dust wipe samples	Primary production	Seeding and storage of samples post inoculation for 24h at 20°C to stress the cells

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

97 samples were artificially contaminated by seeding, using 24 different strains and a seeding protocol with 85-90 samples giving a positive result depending upon the DNA extraction and PCR profile combination. Most of the seeding inoculations were lower or equal to 8 CFU/sample.

3.1.3 Confirmation protocols
Reference method: ISO 6579-1 (2017)

Presumptive positive colonies on XLD and Rambach agar were purified on NA and incubated at 37°C ±1°C aerobically for 24h ±2h. After purification, the colonies were tested with *Salmonella* Poly O and Poly H antisera, Gram stain, oxidase test and biochemical analysis using API 20E (BioMerieux).

Alternative method: Kylt® *Salmonella* spp. 2.0

When a sample gave a positive result from the PCR, the BPW was subcultured into RVS broth and MKTTn broth as described in ISO 6579-1 (2017) and the rest of the confirmation protocol in the reference method was followed. For PCR positive primary production samples, the BPW was subcultured onto MSRV agar and into MKTTn broth as described in ISO 6579-1 (2017) and the rest of the confirmation protocol in the reference method was followed.

Presumptive positive colonies on XLD and Rambach agar were purified on NA and incubated at 37°C ±1°C aerobically for 24h ±2h. After purification, the colonies were tested with *Salmonella* Poly O and Poly H antisera, Gram stain, oxidase test and biochemical analysis using API 20E (BioMerieux).

3.1.4 Sensitivity study results

Table 5a-h shows the summary of results of the reference method and the alternative methods for all Categories.

Table 6 a-h shows the Interpretation of sample results between the reference and alternative method (based on the confirmed alternative method) for all Categories.

Table 5a - Summary of sensitivity study results – all categories for paired 25g samples DNA Extraction-Mix II, profile I

	Reference method positive (R+)	**Reference method negative (R-)**
Alternative method positive (A+)	Positive agreement (R+/A+) **PA =** 147	Positive déviation (R-/A+) **PD =0**
Alternative method negative (A-)	Negative deviation (R+/A-) **ND =** 5	Negative agreement (R-/A-) **NA =148**

Table 5b - Summary of sensitivity study results – all categories for paired 25g samples DNA Extraction-Mix II, profile II

	Reference method positive (R+)	**Reference method negative (R-)**
Alternative method positive (A+)	Positive agreement (R+/A+) **PA =** 149	Positive deviation (R-/A+) **PD = 0**
Alternative method negative (A-)	Negative deviation (R+/A-) **ND =** 3	Negative agreement (R-/A-) **NA = 148**

Table 5c - Summary of sensitivity study results – all categories for paired 25g samples RNA/DNA extraction profile I

	Reference method positive (R+)	**Reference method negative (R-)**
Alternative method positive (A+)	Positive agreement (R+/A+) **PA =** 117	Positive deviation (R-/A+) **PD = 0**
Alternative method negative (A-)	Negative deviation (R+/A-) **ND =** 5	Negative agreement (R-/A-) **NA = 118**

Table 5d - Summary of sensitivity study results – all categories for paired 25g samples RNA/DNA extraction profile II

	Reference method positive (R+)	**Reference method negative (R-)**
Alternative method positive (A+)	Positive agreement (R+/A+) **PA =** 146	Positive deviation (R-/A+) **PD = 0**
Alternative method negative (A-)	Negative deviation (R+/A-) **ND =** 5	Negative agreement (R-/A-) **NA = 149**

Table 5e - Summary of sensitivity study results – all categories for unpaired 375g samples DNA Extraction-Mix II, profile I

	Reference method positive (R+)	**Reference method negative (R-)**
Alternative method positive (A+)	Positive agreement (R+/A+) **PA =** 50	Positive déviation (R-/A+) **PD =1**
Alternative method negative (A-)	Negative deviation (R+/A-) **ND =8**	Negative agreement (R-/A-) **NA =61**

Table 5f - Summary of sensitivity study results – all categories for unpaired 375g samples DNA Extraction-Mix II, profile II

	Reference method positive (R+)	**Reference method negative (R-)**
Alternative method positive (A+)	Positive agreement (R+/A+) **PA =54**	Positive deviation (R-/A+) **PD = 0**
Alternative method negative (A-)	Negative deviation (R+/A-) **ND =** 4	Negative agreement (R-/A-) **NA = 62**

Table 5g - Summary of sensitivity study results – all categories for unpaired 375g samples RNA/DNA extraction profile I

	Reference method positive (R+)	**Reference method negative (R-)**
Alternative method positive (A+)	Positive agreement (R+/A+) **PA =** 51	Positive deviation (R-/A+) **PD= 2**
Alternative method negative (A-)	Negative deviation (R+/A-) **ND =** 6	Negative agreement (R-/A-) **NA =61**

Table 5h - Summary of sensitivity study results – all categories for unpaired 375g samples RNA/DNA extraction profile II

	Reference method positive (R+)	**Reference method negative (R-)**
Alternative method positive (A+)	Positive agreement (R+/A+) **PA =** 50	Positive deviation (R-/A+) **PD = 2**
Alternative method negative (A-)	Negative deviation (R+/A-) **ND =** 8	Negative agreement (R-/A-) **NA =60**

Table 6a – Interpretation of sample results between the reference and alternative method (based on the confirmed alternative method) DNA Extraction-Mix II Profile I for paired 25g samples

Category		**Type**		**PA**	**NA[1]**	**PD**	**ND[2]**	**PPNA[3]**	**PPND[3]**	**Total**
1	Raw meat and ready to cook meat products	a	Fresh meats (unprocessed)	10	10	0	0	0	0	20
		b	Ready to cook (processed)	11	7	0	1	1	0	20
		c	Ready to eat and Ready to reheat products	10	10	0	0	0	0	20
				31	**27**	**0**	**1**	**1**	**0**	**60**
2	Raw poultry and ready to cook poultry products	a	Fresh meats (unprocessed)	9	10	0	0	1	0	20
		b	Ready to cook (processed)	11	9	0	0	0	0	20
		c	Ready to eat and Ready to reheat products	10	10	0	0	0	0	20
				30	**29**	**0**	**0**	**1**	**0**	**0**
3	Environmental	a	Dust and residues	10	10	0	0	0	0	20
		b	Cleaning and process waters	10	10	0	0	0	0	20
		c	Surface samples	10	10	0	0	0	0	20
				30	**30**	**0**	**0**	**0**	**0**	**60**
4a	Primary production	a	Poultry	10	9	0	1	0	0	20
		b	Swine/ Cattle	12	8	0	0	0	0	20
		c	Environmental samples	6	13	0	1	0	0	20
				28	**30**	**0**	**2**	**0**	**0**	**60**
4b	Primary production (5h RVS)	a	Poultry	10	8	0	1	1	0	20
		b	Swine/ Cattle	12	8	0	0	0	0	20
		c	Environmental samples	6	13	0	1	0	0	20
				28	**29**	**0**	**2**	**1**	**0**	**60**
All categories				**147**	**145**	**0**	**5**	**3**	**0**	**300**

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

Table 6b – Interpretation of sample results between the reference and alternative method (based on the confirmed alternative method) DNA Extraction-Mix II Profile I for unpaired 375g samples

Category		Type		PA	NA[1]	PD	ND[2]	PPNA[3]	PPND[3]	Total
1	Raw meat and ready to cook meat products 375g	a	Fresh meats (unprocessed)	7	10	0	2	1	0	20
		b	Ready to cook (processed)	7	11	0	2	0	0	20
		c	Ready to eat and Ready to reheat products	9	9	1	1	0	0	20
				23	30	1	5	1	0	60
2	Raw poultry and ready to cook poultry products 375g	a	Fresh meats (unprocessed)	6	13	0	1	0	0	20
		b	Ready to cook (processed)	8	10	0	2	0	0	20
		c	Ready to eat and Ready to reheat products	13	6	0	0	1	0	20
				27	29	0	3	1	0	60
All categories				50	59	1	8	2	0	120

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

Table 6c – Interpretation of sample results between the reference and alternative method (based on the confirmed alternative method) DNA Extraction-Mix II Profile II for 25g paired samples

Category		Type		PA	NA[1]	PD	ND[2]	PPNA[3]	PPND[3]	Total
1	Raw meat and ready to cook meat products 25g	a	Fresh meats (unprocessed)	10	10	0	0	0	0	20
		b	Ready to cook (processed)	10	8	0	1	1	0	20
		c	Ready to eat and Ready to reheat products	10	10	0	0	0	0	20
				30	**28**	**0**	**1**	**1**	**0**	**60**
2	Raw poultry and ready to cook poultry products 25g	a	Fresh meats (unprocessed)	9	9	0	0	2	0	20
		b	Ready to cook (processed)	11	9	0	0	0	0	20
		c	Ready to eat and Ready to reheat products	10	10	0	0	0	0	20
				30	**28**	**0**	**0**	**2**	**0**	**60**
3	Environmental	a	Dust and residues	10	10	0	0	0	0	20
		b	Cleaning and process waters	10	10	0	0	0	0	20
		c	Surface samples	10	9	0	0	1	0	20
				30	**29**	**0**	**0**	**1**	**0**	**60**
4a	Primary production	a	Poultry	9	10	0	1	0	0	20
		b	Swine/ Cattle	12	7	0	0	1	0	20
		c	Environmental samples	6	13	0	1	0	0	20
				27	**30**	**0**	**2**	**1**	**0**	**60**
4b	Primary production (5h RVS)	a	Poultry	12	8	0	0	0	0	20
		b	Swine/ Cattle	12	8	0	0	0	0	20
		c	Environmental samples	8	12	0	0	0	0	20
				32	**28**	**0**	**0**	**0**	**0**	**60**
All categories				**149**	**143**	**0**	**3**	**5**	**0**	**300**

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

Table 6d – Interpretation of sample results between the reference and alternative method (based on the confirmed alternative method) DNA Extraction-Mix II II Profile II for 375g unpaired samples

Category		Type		PA	NA[1]	PD	ND[2]	PPNA[3]	PPND[3]	Total
1	Raw meat and ready to cook meat products 375g	a	Fresh meats (unprocessed)	7	12	0	1	0	0	20
		b	Ready to cook (processed)	8	11	0	1	0	0	20
		c	Ready to eat and Ready to reheat products	11	9	0	0	0	0	20
				26	**32**	**0**	**2**	**0**	**0**	**60**
2	Raw poultry and ready to cook poultry products 375g	a	Fresh meats (unprocessed)	7	11	0	0	2	0	20
		b	Ready to cook (processed)	8	10	0	2	0	0	20
		c	Ready to eat and Ready to reheat products	13	6	0	0	1	0	20
				28	**27**	**0**	**2**	**3**	**0**	**60**
All categories				**54**	**59**	**0**	**4**	***3***	**0**	**120**

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

Table 6e – Interpretation of sample results between the reference and alternative method (based on the confirmed alternative method) RNA/DNA Purification profile I for 25g paired samples

Category		Type		PA	NA[1]	PD	ND[2]	PPNA3	PPND3	Total
1	Raw meat and ready to cook meat products 25g	a	Fresh meats (unprocessed)	10	10	0	0	0	0	20
		b	Ready to cook (processed)	8	8	0	3	1	0	20
		c	Ready to eat and Ready to reheat products	10	8	0	0	2	0	20
				28	**26**	**0**	**3**	**3**	**0**	**60**
2	Raw poultry and ready to cook poultry products 25g	a	Fresh meats (unprocessed)	9	9	0	0	2	0	20
		b	Ready to cook (processed)	11	8	0	0	1	0	20
		c	Ready to eat and Ready to reheat products	10	8	0	0	2	0	20
				30	**25**	**0**	**0**	**5**	**0**	**60**
3	Environmental	a	Dust and residues	10	10	0	0	0	0	20
		b	Cleaning and process waters	10	10	0	0	0	0	20
		c	Surface samples	10	7	0	0	3	0	20
				30	**27**	**0**	**0**	**3**	**0**	**60**
4	Primary production 5h RVS	a	Poultry	10	7	0	1	2	0	20
		b	Swine/ Cattle	12	6	0	0	2	0	20
		c	Environmental samples	7	12	0	1	0	0	20
				29	**25**	**0**	**2**	**4**	**0**	**60**
All categories				**117**	**103**	**0**	**5**	**15**	**0**	**240**

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

Table 6f – Interpretation of sample results between the reference and alternative method (based on the confirmed alternative method) RNA/DNA Purification profile I for 375g unpaired samples

Category		Type		PA	NA[1]	PD	ND[2]	PPNA[3]	PPND[3]	Total
1	Raw meat and ready to cook meat products 375g	a	Fresh meats (unprocessed)	5	10	0	4	1	0	20
		b	Ready to cook (processed)	7	7	0	2	4	0	20
		c	Ready to eat and Ready to reheat products	9	9	1	0	1	0	20
				21	**26**	**1**	**6**	**6**	**0**	**60**
2	Raw poultry and ready to cook poultry products 375g	a	Fresh meats (unprocessed)	7	11	1	0	1	0	20
		b	Ready to cook (processed)	10	10	0	0	0	0	20
		c	Ready to eat and Ready to reheat products	13	4	0	0	3	0	20
				30	**25**	**1**	**0**	**4**	**0**	**60**
All categories				**51**	**51**	**2**	**6**	**10**	**0**	**120**

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

Table 6g – Interpretation of sample results between the reference and alternative method (based on the confirmed alternative method) RNA/DNA Purification profile II for 25g paired samples

Category		Type		PA	NA[1]	PD	ND[2]	PPNA[3]	PPND[3]	Total
1a	Raw meat and ready to cook meat products 25g	a	Fresh meats (unprocessed)	10	10	0	0	0	0	20
		b	Ready to cook (processed)	11	7	0	2	0	0	20
		c	Ready to eat and Ready to reheat products	10	8	0	0	2	0	20
				31	**25**	**0**	**2**	**2**	**0**	**60**
2a	Raw poultry and ready to cook poultry products 25g	a	Fresh meats (unprocessed)	9	7	0	0	4	0	20
		b	Ready to cook (processed)	11	9	0	0	0	0	20
		c	Ready to eat and Ready to reheat products	10	8	0	0	2	0	20
				30	**24**	**0**	**0**	**6**	**0**	**60**
3	Environmental	a	Dust and residues	10	10	0	0	0	0	20
		b	Cleaning and process waters	10	10	0	0	0	0	20
		c	Surface samples	10	7	0	0	3	0	20
				30	**27**	**0**	**0**	**3**	**0**	**60**
4	Primary production	a	Poultry	7	11	0	1	2	0	20
		b	Swine/ Cattle	11	7	0	0	1	0	20
		c	Environmental samples	7	12	0	1	0	0	20
				25	**30**	**0**	**2**	**3**	**0**	**60**
4b	Primaryproduction 5h RVS broth	a	Poultry	11	8	0	0	1	0	20
		b	Swine/ Cattle	12	6	0	0	2	0	20
		c	Environmental samples	7	10	0	1	2	0	20
				30	**24**	**0**	**1**	**5**	**0**	**60**
All categories				**146**	**130**	**0**	**5**	**19**	**0**	**300**

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

Table 6h- Overview calculated sensitivity parameters per Category and Type RNA/DNA extraction profile II for 375g paired

Category		Type		PA	NA[1]	PD	ND[2]	PPNA[3]	PPND[3]	Total
1	Raw meat and ready to cook meat products 375g	a	Fresh meats (unprocessed)	5	11	0	4	0	0	20
		b	Ready to cook (processed)	7	7	0	2	4	0	20
		c	Ready to eat and Ready to reheat products	9	6	1	1	3	0	20
				21	24	1	7	7	0	**60**
2	Raw poultry and ready to cook poultry products 375g	a	Fresh meats (unprocessed)	6	9	1	1	3	0	20
		b	Ready to cook (processed)	10	10	0	0	0	0	20
		c	Ready to eat and Ready to reheat products	13	5	0	0	2	0	20
				29	**24**	**1**	**1**	**5**	0	**60**
All categories				**50**	**48**	**2**	**8**	**12**	**0**	**120**

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

3.1.5 Sensitivity study calculations

The sensitivity study parameters as specified in Table 7 were calculated for all Categories and Types, and the overview is given in Table 8.

Table 7 – Formula to calculate the sensitivity parameters

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

Table 8a - Overview calculated sensitivity parameters per Category and Type DNA Extraction-Mix II profile I for 25g paired samples

Category	Type	PA	NA[1]	PD	ND[2]	FP[3]	SE alt (%)	SE ref (%)	RT (%)	FPR (%)
1a Raw meat and ready to cook meat products 25g	a	10	10	0	0	0	100.0	100.0	100.0	0.0
	b	11	7	0	1	1	91.7	100.0	90.0	10.0
	c	10	10	0	0	0	100.0	100.0	100.0	0.0
	Total	**31**	**27**	**0**	**1**	**1**	**96.9**	**100.0**	**96.7**	**3.22**
2a Raw poultry and ready to cook poultry products	a	9	10	0	0	1	100.0	100.0	95.0	10.0
	b	11	9	0	0	0	100.0	100.0	100.0	0.0
	c	10	10	0	0	0	100.0	100.0	100.0	0.0
	Total	**30**	**29**	**0**	**0**	**1**	100.0	**100.0**	**98.3**	**3.44**

Category	Type	PA	NA[1]	PD	ND[2]	FP[3]	SE alt (%)	SE ref (%)	RT (%)	FPR (%)
3 Environmental	a	10	10	0	0	0	100.0	100.0	100.0	0.0
	b	10	10	0	0	0	100.0	100.0	100.0	0.0
	c	10	10	0	0	0	100.0	100.0	100.0	0.0
	Total	**30**	**30**	**0**	**0**	**0**	**100.0**	**100.0**	**100.0**	**0.00**
4a Primary production 25g	a	10	9	0	1	0	90.9	100.0	95.0	0.0
	b	12	8	0	0	0	100.0	100.0	100.0	0.0
	c	6	13	0	1	0	85.7	100.0	95.0	0.0
	Total	**28**	**30**	**0**	**2**	**0**	**93.3**	**100.0**	**96.7**	**0.00**
4b Primary production 25g 5h RVS	a	10	8	0	1	1	100.0	100.0	90.0	12.5
	b	12	8	0	0	0	100.0	100.0	100.0	0.0
	c	6	13	0	1	0	85.7	100.0	95.0	0.0
	Total	**28**	**29**	**0**	**2**	**1**	**93.3**	**100.0**	**95.0**	**3.44**
All categories		**147**	**145**	**0**	**5**	**3**	**96.7**	**100.0**	**97.3**	**2.06**

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

Table 8b - Overview calculated sensitivity parameters per Category and Type DNA Extraction-Mix II profile I for 375g unpaired samples

Category	Type	PA	NA[1]	PD	ND[2]	FP[3]	SE alt (%)	SE ref (%)	RT (%)	FPR (%)
1 Raw meat and ready to cook meat products	a	7	10	0	2	1	77.8	100.0	85.0	10.0
	b	7	11	0	2	0	77.8	100.0	90.0	0.00
	c	9	9	1	1	0	90.9	90.9	95.0	0.00
	Total	23	30	1	5	1	**82.7**	**96.6**	**75.0**	**3.33**
2 Raw poultry and ready to cook poultry products	a	6	13	0	1	0	85.7	100.0	95.0	0.00
	b	8	10	0	2	0	80.0	100.0	90.0	0.00
	c	13	6	0	0	1	100.0	100.0	95.0	14.3
	Total	27	29	0	3	1	**90.0**	**100.0**	**93.3**	**3.44**
All categories		50	59	1	8	2	**86.4**	**98.3**	**90.8**	**3.39**

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

Table 8c - Overview calculated sensitivity parameters per Category and Type DNA Extraction-Mix II profile II for 25g paired samples

Category	Type	PA	NA[1]	PD	ND[2]	FP[3]	SE alt (%)	SE ref (%)	RT (%)	FPR (%)
1a Raw meat and ready to cook meat products 25g	a	10	10	0	0	0	100.0	100.0	100.0	0.0
	b	10	8	0	1	1	90.9	100.0	90.0	12.5
	c	10	10	0	0	0	100.0	100.0	100.0	0.0
	Total	**30**	**28**	**0**	**1**	**1**	**96.8**	**100.0**	**96.7**	**3.57**
2a Raw poultry and ready to cook poultry products 25g	a	9	9	0	0	2	100.0	100.0	90.0	22.2
	b	11	9	0	0	0	100.0	100.0	100.0	0.0
	c	10	10	0	0	0	100.0	100.0	100.0	0.0
	Total	**30**	**28**	**0**	**0**	**2**	**100.0**	**100.0**	**96.7**	**7.14**
3 Environmental	a	10	10	0	0	0	100.0	100.0	100.0	0.0
	b	10	10	0	0	0	100.0	100.0	100.0	0.0

Category	Type	PA	NA[1]	PD	ND[2]	FP[3]	SE alt (%)	SE ref (%)	RT (%)	FPR (%)
	c	10	9	0	0	1	100.0	100.0	95.0	11.1
	Total	**30**	**29**	**0**	**0**	**1**	**100.0**	**100.0**	**98.3**	**3.44**
4a Primary production 25g	a	9	10	0	1	0	90.0	100.0	95.0	0.0
	b	12	7	0	0	1	100.0	100.0	95.0	14.8
	c	6	13	0	1	0	85.7	100.0	95.0	0.0
	Total	**27**	**30**	**0**	**2**	**1**	**93.1**	**100.0**	**96.7**	**3.03**
4b Primary production 25g 5h RVS	a	12	8	0	0	0	100.0	100.0	100.0	0.0
	b	12	8	0	0	0	100.0	100.0	100.0	0.0
	c	8	12	0	0	0	100.0	100.0	100.0	0.0
	Total	**32**	**28**	**0**	**0**	**0**	**100.0**	**100.0**	**100.0**	**0.0**
All categories		149	143	0	**3**	**5**	**98.0**	**100.0**	**97.3**	**2.10**

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

Table 8d - Overview calculated sensitivity parameters per Category and Type DNA Extraction-Mix II profile II for 375g unpaired samples

Category	Type	PA	NA[1]	PD	ND[2]	FP[3]	SE alt (%)	SE ref (%)	RT (%)	FPR (%)
1b Raw meat and ready to cook meat products 375g	a	7	12	0	1	0	87.5	100.00	95.0	0.0
	b	8	11	0	1	0	88.9	100.00	95.0	0.0
	c	11	9	0	0	0	100.0	100.00	100.0	0.0
	Total	26	32	0	2	0	**92.6**	**100.00**	**96.7**	**0.0**
2b Raw poultry and ready to cook poultry products 375g	a	7	11	0	0	2	100.0	100.00	90.0	18.2
	b	8	10	0	2	0	80.0	100.00	90.0	0.0
	c	13	6	0	0	1	92.3	100.00	95.0	14.3
	Total	**28**	**27**	**0**	**2**	**3**	**93.3**	**100.00**	91.7	**16.7**
All categories		**54**	**59**	**0**	**4**	***3***	**94.7**	**100.00**	**94.2**	**5.08**

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

Table 8e - Overview calculated sensitivity parameters per Category and Type RNA/DNA Purification profile I for 25g paired samples

Category	Type	PA	NA[1]	PD	ND[2]	FP[3]	SE alt (%)	SE ref (%)	RT (%)	FPR (%)
1 Raw meat and ready to cook meat products 25g	a	10	10	0	0	0	100.0	100.0	100.0	0.0
	b	8	8	0	3	1	72.7	100.0	80.0	0.0
	c	10	8	0	0	2	100.0	100.0	90.0	25.0
	Total	**28**	**27**	**0**	**3**	**3**	**93.3**	**100.0**	**91.7**	**7.40**
2 Raw poultry and ready to cook poultry products 25g	a	9	9	0	0	2	100.0	100.0	90.0	22.2
	b	11	8	0	0	1	100.0	100.0	95.0	12.5
	c	10	8	0	0	2	100.0	100.0	90.0	22.2
	Total	**30**	**25**	**0**	**0**	**5**	100.0	**100.0**	**91.7**	**20.0**
3 Environmental	a	10	10	0	0	0	100.0	100.0	100.0	0.0
	b	10	10	0	0	0	100.0	100.0	100.0	0.0
	c	10	7	0	0	3	100.0	100.0	80.0	42.9
	Total	**30**	**27**	**0**	**0**	**3**	**100.0**	**100.0**	**93.3**	**11.1**
4 Primary production 25g 5h RVS	a	10	7	0	1	2	90.9	100.0	85.0	28.6
	b	12	6	0	0	2	100.0	100.0	90.0	33.3
	c	8	12	0	1	0	87.5	100.0	95.0	0.0

Category	Type	PA	NA[1]	PD	ND[2]	FP[3]	SE alt (%)	SE ref (%)	RT (%)	FPR (%)
	Total	**29**	**25**	**0**	**2**	**4**	**93.5**	**100.0**	**90.0**	**16.0**
All categories		**117**	**103**	**0**	**5**	**15**	**95.9**	**100.0**	**91.7**	**14.6**

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

Table 8f - Overview calculated sensitivity parameters per Category and Type RNA/DNA Pirification profile I for 375g unpaired samples

Category	Type	PA	NA[1]	PD	ND[2]	FP[3]	SE alt (%)	SE ref (%)	RT (%)	FPR (%)
1b Raw meat and ready to cook meat products 375g	a	5	10	0	4	1	55.6	100.0	75.0	10.0
	b	7	7	0	2	4	77.8	100.0	70.0	57.1
	c	9	9	1	0	1	100.0	90.0	95.0	11.1
	Total	**21**	**26**	**1**	**6**	**6**	**75.0**	**96.4**	**78.3**	**23.1**
2b Raw poultry and ready to cook poultry products 375g	a	7	11	1	0	1	100.0	87.5	90.0	9.09
	b	10	10	0	0	0	100.0	100.0	100.0	0.00
	c	13	4	0	0	3	100.0	100.0	85.0	75.0
	Total	**30**	**25**	**1**	**0**	**4**	**100.0**	**96.8**	**91.6**	**16.0**
***All* categories**		51	51	2	6	10	**89.8**	**96.7**	**85.0**	**19.6**

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

Table 8g - Overview calculated sensitivity parameters per Category and Type RNA/DNA Purification profile II for 25g paired samples

Category	Type	PA	NA[1]	PD	ND[2]	FP[3]	SE alt (%)	SE ref (%)	RT (%)	FPR (%)
1a Raw meat and ready to cook meat products 25g	a	10	10	0	0	0	100.0	100.0	100.0	0.00
	b	11	7	0	2	0	84.6	100.0	90.0	0.00
	c	10	8	0	0	2	100.0	100.0	100.0	20.0
	Total	31	27	0	0	2	**92.6**	**100.0**	**93.3**	**6.45**
2a Raw poultry and ready to cook poultry products 25g	a	9	7	0	0	4	100.0	100.0	100.0	57.1
	b	11	9	0	0	0	100.0	100.0	95.0	0.00
	c	10	8	0	0	2	100.0	100.0	90.0	25.0
	Total	30	24	0	0	6	100.0	**100.0**	90.0	25.0
3 Environmental	a	10	10	0	0	0	100.0	100.0	100.0	0.00
	b	10	10	0	0	0	100.0	100.0	100.0	0.00
	c	10	7	0	0	3	100.0	100.0	85.0	42.9
	Total	30	27	0	0	3	**100.0**	**100.0**	**95.0**	**11.1**
4a Primary production 25g	a	7	11	0	1	2	100.0	100.0	90.0	18.2
	b	11	7	0	0	1	100.0	100.0	90.0	14.3
	c	7	12	0	1	0	87.5	100.0	95.0	0.00
	Total	25	30	0	2	3	**90.9**	**100.0**	**91.7**	**10.0**
4b Primary production 25g 5h RVS	a	11	8	0	0	1	100.0	100.0	95.0	12.5
	b	12	6	0	0	2	100.0	100.0	90.0	33.3
	c	7	10	0	1	2	87.5	100.0	85.0	10.0
	Total	30	24	0	1	5	**96.8**	**100.0**	**90.0**	**20.8**
All categories		**146**	**132**	**0**	**5**	**19**	***96.7***	**100.0**	**92.7**	**14.4**

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

Table 8h- Overview calculated sensitivity parameters per Category and Type RNA/DNA purification profile II for 375g unpaired

Category	Type	PA	NA[1]	PD	ND[2]	FP[3]	SE alt (%)	SE ref (%)	RT (%)	FPR (%)
1b Raw meat and ready to cook meat products 375g	a	5	11	0	4	0	55.6	100.0	80.0	0.00
	b	7	7	0	2	4	77.7	100.0	70.0	57.1
	c	9	6	1	1	3	81.8	90.9	75.0	50.0
	Total	21	24	1	7	7	75.8	96.5	75.0	29.2
2b Raw poultry and ready to cook poultry products 375g	a	6	9	1	1	3	87.5	87.5	75.0	33.3
	b	10	10	0	0	0	100.0	100.0	100.0	0.00
	c	13	5	0	0	2	100.0	100.0	90.0	40.0
	Total	**29**	**24**	**1**	**1**	**5**	96.8	96.8	86.7	25.0
All categories		**50**	**48**	**2**	**8**	**12**	**86.7**	**96.6**	**81.6**	**25.0**

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

3.1.6 Discordant results

Negative deviations are listed in Table 9a-d.

Table 9a - Negative deviations DNA Extraction-Mix II profile I

Category	Type	Sample nº	Alternative method results	(additional) Confirmatory test results	Inoculation (CFU/Sample)
25g paired samples					
Raw meat and ready to cook meat products	Ready to cook (processed)	1-B-12	-	n/a	n/a, natural
Primary production	Poultry	4-A-2	-	n/a	n/a, natural
	Environmental samples	3-A-21	-	n/a	n/a, natural
Primary production	Poultry	4-A-2	-	n/a	n/a, natural
	Environmental samples	3-A-21	-	n/a	n/a, natural
375g unpaired samples					
Raw meat and ready to cook meat products	Fresh meats (unprocessed	1-A-30	-	n/a	8, seeded, S. Kimberley
		1-A-43	-	n/a	n/a, natural
	Ready to cook (processed)	1-B-32	-	n/a	n/a, natural
		1-B-47	-	n/a	n/a, natural
	Ready to eat and Ready to reheat products	1-C-25	-	n/a	6, Seeded S. Norwich
Raw poultry and ready to cook poultry products	Fresh meats (unprocessed)	2-A-18	-	n/a	n/a, natural
	Ready to cook (processed)	**2-B-25**	-	n/a	n/a, natural
		2-B-28	-	n/a	n/a, natural

The majority of the negative deviations for the DNA Extraction-Mix II kit samples run with profile I were natural contamination, with all 5 of the 25g samples and 7 out of the 8 375g samples negative deviations being from naturally contaminated samples. None of the 25g samples and 2 out of the 8 375g sample negative deviations were from seeded samples. This indicates a bias towards naturally contaminated

samples for negative deviations. One possible explanation for this bias could be a combination of low levels and the uneven distribution of the contamination of *Salmonella*, particularly in the unpaired samples.

Table 9b - Negative deviations DNA Extraction mix II profile II

Category/	**Type**	**Sample**	**Alternative method results**	**(additional) Confirmatory test results**	**Inoculation (CFU/Sample)**
25g paired samples					
Raw meat and ready to cook meat products	Ready to cook (processed)	1-B-12	-	n/a	n/a, natural contamination
Primary production	Poultry	4-A-2	-	n/a	n/a, natural contamination
	Environmental samples	3-A-21	-	n/a	n/a, natural contamination
375g paired samples					
Raw meat and ready to cook meat products	Fresh meats (unprocessed)	1-A-43	-	n/a	n/a, natural contamination
	Ready to cook (processed)	1-B-47	-	n/a	n/a, natural contamination
Raw poultry and ready to cook poultry products	Ready to cook (processed)	2-B-25	-	n/a	n/a, natural contamination
		2-B-28	-	n/a	n/a, natural contamination

All of the negative deviations for the DNA Extraction-Mix II kit samples run with profile I were natural contamination, with 3 out of the 3 deviations being from naturally contaminated 25g samples and 4 out of 4 of the 375g sample deviations being from naturally contaminated samples. This indicates a bias towards naturally contaminated samples for negative deviations.

Table 9c – Negative deviations RNA/DNA Purification profile I

Category/	**Type**	**Sample nº**	**Alternative metl results**	**(additional) Confirmatory test results**	**Inoculation (CFU/Sample)**
25g paired samples					
Raw meat and ready to cook meat products	Ready to cook (processed)	1-B-11	-	n/a	n/a, natural contamination
		1-B-12	-	n/a	n/a, natural contamination
		1-B-13	-	n/a	n/a, natural contamination
Primary production 5h RVS broth	Poultry	4-A-2	-	n/a	n/a, natural contamination
	Environmental samples	3-A-21	-	n/a	n/a, natural contamination
375g paired samples					
Raw meat and ready to cook meat products	Fresh meats (unprocessed)	1-A-29	-	n/a	10.5, Seeded S. S. Norwich
		1-A-30	-	n/a	8, Seeded S. S. Kimberley
		1-A-32	-	n/a	8, Seeded S. S. Kimberley

Category/	Type	Sample nº	Alternative metl results	(additional) Confirmatory test results	Inoculation (CFU/Sample)
		1-A-43	-	n/a	n/a, natural contamination
	Ready to cook (processed	1-b-32	-	n/a	n/a, natural contamination
		1-B-47	-	n/a	n/a, natural contamination

The majority of the negative deviations for the RNA/DNA Purification profile I were from natural contamination, with all 5 25g samples and 5 out of 8 375g samples negative deviations being from naturally contaminated samples. Only 3 out of 7 375g samples out of the negative deviations were seeded samples. This indicates a bias towards naturally contaminated samples for negative deviations.

Table 9d - Negative deviations RNA/DNA Purification profile II

Category/	Type	Sample n	Alternative method results	(additional) Confirmatory test results	Inoculation (CFU/Sample)
25g paired samples					
Raw meat and ready to cook meat products	Ready to cook (processed)	1-B-11	-	n/a	n/a, natural contamination
		1-B-12	-	n/a	n/a, natural contamination
Primary production	Swine/ Cattle	4-B-8	-	n/a	n/a, natural contamination
Primary production	Environmental samples	3-A-21	-	n/a	n/a, natural contamination
375g unpaired samples					
Raw meat and ready to cook meat products	Fresh meats (unprocessed)	1-A-29	-	n/a	10.5, Seeded, S. Norwich
		1-A-30	-	n/a	8, Seeded, S. Kimberley
		1-A-32	-	n/a	8, Seeded , S. Kimberley
		1-A-43	-	n/a	n/a, natural contamination
	Ready to cook (processed)	1-b-32	-	n/a	n/a, natural contamination
		1-B-47	-	n/a	n/a, natural contamination
	Ready to eat and Ready to reheat products	1-C-25	-	n/a	6, seeded S. Norwich
Raw poultry and ready to cook poultry products	Fresh meats (unprocessed)	2-A-51	-	n/a	n/a, natural contamination

The majority of the negative deviations for RNA/DNA extraction kit 25g samples run with profile II were natural contamination, with 5 out of 5 25g samples negative deviations being from naturally contaminated samples. This indicates a bias towards naturally contaminated samples for negative deviations.

This was not the case for the 375g samples where 4 out of 8 samples were natural contamination and 4 out of 8 from seeded samples, suggesting no bias towards either type of contaminated samples for negative deviations. The 375g were unpaired samples, so are more likely to have a discrepancy in the reference and alternative methods. As the inoculated samples are contaminated at a low level it is possible that one of the samples was not contaminated with the target organism during inoculation.

Positive deviations are listed in Table 10a-d.

Table 10a - Positive deviations DNA Extraction-Mix II profile I

Category/	**Type**	**Sample nº**	**Alternative method results**	**(additional) Confirmatory test results**	**Inoculation (CFU/Sample)**
Raw meat and ready to cook meat products	Ready to eat and Ready to reheat products	1-C-35	+	n/a	n/a, natural contamination

10b - Positive deviations DNA Extraction-Mix II profile II

No positive deviations observed

Table 10c – Positive deviations RNA/DNA Purification profile I

Category	**Type**	**Sample nº**	**Alternative method results**	**(additional) Confirmatory test results**	**Inoculation (CFU/Sample)**
375g unpaired samples					
Raw meat and ready to cook meat products	Ready to eat and Ready to reheat products	1-C-35	+	n/a	n/a natural contamination
Raw meat and ready to cook meat products	Fresh meats (unprocessed)	2-A-17	+	n/a	n/a natural contamination

Table 10d – Positive deviations RNA/DNA Purification profile II

Category/	**Type**	**Sample nº**	**Alternative method results**	**(additional) Confirmatory test results**	**Inoculation (CFU/Sample)**
375g unpaired samples					
Raw meat and ready to cook meat products	Ready to eat and Ready to reheat products	1-C-35	+	n/a	n/a natural contamination
Fresh meats (unprocessed)	Fresh meats (unprocessed)	2-A-17	+	n/a	n/a natural contamination

The positive deviations were all observed in the 375g samples, which was to be expected for unpaired samples. All the positive deviation samples were naturally contaminated which may result from pockets of contamination in the samples that are not homogenous across the reference and alternative samples analyzed.

No inhibitions were noted in the study on the 7500 FAST platform, resulting in a 0% inhibition rate in this part of the study.

The analysis of discordant results according to ISO 16140-2:2016 for the study is given in Tables 11a to h. The 25g paired samples and 375g unpaired samples are displayed in separate tables.

Table 11a - Interpretation of the sensitivity study results (paired study) DNA Extraction Mix-II profile I 25g samples

Category	Negative Deviations (ND[1])	Positive deviations (PD)	ND-PD	Acceptability Limit (AL)	ND+PD	Acceptability Limit (AL)
Raw meat and ready to cook meat products	1	0	1	3	1	6
Raw poultry and ready to cook poultry products	0	0	0	3	0	6
Environmental	0	0	0	3	0	6
Primary production	2	0	2	3	2	6
Primary production 5h RVS	2	0	2	3	2	6
Total	5	0	5	5	5	14

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

Table 11b - Interpretation of the sensitivity study results (paired study) DNA Extraction Mix-II profile II 25g samples

Category	Negative Deviations (ND[1])	Positive deviations (PD)	ND-PD	Acceptability Limit (AL)	ND+PD	Acceptability Limit (AL)
Raw meat and ready to cook meat products	1	0	1	3	1	6
Raw poultry and ready to cook poultry products	0	0	0	3	0	6
Environmental	0	0	0	3	0	6
Primary production	2	0	2	3	2	6
Primary production 5h RVS 25g	0	0	0			
Total	**3**	**0**	**3**	**5**	**3**	**14**

[1] ND: including PPND

Table 11c - Interpretation of the sensitivity study results (paired study) RNA/DNA Purification profile I 25g samples

Category	Negative Deviations (ND[1])	Positive deviations (PD)	ND-PD	Acceptability Limit (AL)	ND+PD	Acceptability Limit (AL)
Raw meat and ready to cook meat products	3	0	3	3	3	6
Raw poultry and ready to cook poultry products	0	0	0	3	0	6
Environmental	0	0	0	3	0	6
Primary production 5h RVS 25g	2	0	2	2	2	6

Total	**8**	**0**	**8**	**5**	**5**	**14**

[1] ND: including PPND

Table 11d - Interpretation of the sensitivity study results (paired study) RNA/DNA Purification profile II 25g samples

Category	Negative Deviations (ND[1])	Positive deviations (PD)	ND-PD	Acceptability Limit (AL)	ND+PD	Acceptability Limit (AL)
Raw meat and ready to cook meat products	2	0	2	3	2	6
Raw poultry and ready to cook poultry products	0	0	0	3	0	6
Environmental	0	0	0	3	0	6
Primary production	2	0	2	3	2	6
Primary production 5h RVS 25g	1	0	1	3	1	6
Total	**5**	**0**	**5**	**5**	**5**	**14**

[1] ND: including PPND

Table 11e – Interpretation of the sensitivity study results (unpaired study) DNA Extraction-Mix II profile I 375g samples

Category	Negative Deviations (ND[1])	Positive Deviations (PD)	ND-PD	Acceptability Limit (AL)	comments
Raw meat and ready to cook meat products	5	1	4	3	fail
Raw poultry and ready to cook poultry products	3	0	3	3	pass
Total	8	1	7	4	fail

Table 11f – Interpretation of the sensitivity study results (unpaired study) DNA Extraction-Mix II profile II 375g samples

Category	Negative Deviations (ND[1])	Positive Deviations (PD)	ND-PD	Acceptability Limit (AL)	comments
Raw meat and ready to cook meat products	2	0	2	3	pass
Raw poultry and ready to cook poultry products	2	0	2	3	pass
Total	4	0	4	4	pass

[1] ND: including PPND

Table 11g – Interpretation of the sensitivity study results (unpaired study) RNA/DNA Purification profile II 375g samples

Category	Negative Deviations (ND[1])	Positive Deviations (PD)	ND-PD	Acceptability Limit (AL)	comments
Raw meat and ready to cook meat products	6	1	5	3	fail
Raw poultry and ready to cook poultry products	0	1	-1	3	pass
Total	6	2	4	4	fail

[1] ND: including PPND

Table 11h – Interpretation of the sensitivity study results (unpaired study) RNA/DNA Purification profile II 375g samples

Category	Negative Deviations (ND[1])	Positive Deviations (PD)	ND-PD	Acceptability Limit (AL)	comments
Raw meat and ready to cook meat products	7	1	6	3	fail
Raw poultry and ready to cook poultry products	1	1	0	3	pass
Total	8	2	6	4	fail

[1] ND: including PPND

3.1.7 Conclusion sensitivity study

25g paired samples

The observed values for ND-PD and ND+PD for the individual categories and for all categories meet the acceptability limits (observed values ≤ AL) for the following DNA Extraction-Mix II and PCR profiles for 25g samples.

- Kylt® DNA Extraction-Mix II and run on profile I
- Kylt® DNA Extraction-Mix II and run on profile II
- Kylt® RNA/ DNA Purification and run on profile I*
- Kylt® RNA/ DNA Purification and run on profile II^

*Primary production samples should be run with the 5h RVS enrichment step included only.

375g unpaired samples

The observed values for ND-PD for the individual categories and for all categories did meet the acceptability limits (observed values ≤ AL) for the following DNA extraction and PCR profiles for 375g samples.

- Kylt® DNA Extraction-Mix II and run on profile II

The observed values for ND-PD for the individual categories and for all categories did not meet the acceptability limits (observed values ≤ AL) for the following DNA extraction and PCR profiles for 375g samples.

- Kylt® DNA Extraction-Mix II and run on profile I
- Kylt® RNA/ DNA Purification and run on profile I
- Kylt® RNA/ DNA Purification and run on profile II

3.1.8 Enrichment broth storage

Enrichment broth BPW storage was done at 2-8 °C for 72 hrs as detailed in the alternative method kit insert and reference method ISO6579-1 (2017).

All samples giving a cultural positive result were tested again after enrichment broth storage. Six changes were observed (listed in Table 12). As all DNA extractions and PCR profile combinations for the alternative methods shared the sample enrichment, the results have been noted in a single table. The results shown for the primary production samples will apply to the additional 5h enrichment in RVS prior to DNA extraction included in this study as the samples also shared the same confirmation.

Table 12 Observed changes in results before and after storage of the enrichment broth

Sample nr.	Result before storage			Results after storage		
	Alt. method	Confirmation	Interpretation	Alt. method	Confirmation	Interpretation
Meat 25g paired						
1-A-7	Pos	Pos	PA	Neg	Neg	NA
Meat 375g unpaired						
1-A-38	Pos	Pos	PA	Pos	Neg	PD
1-B-47	Neg	Pos	ND	Neg	Neg	NA
1-C-35	Pos	Pos	PA	Pos	Neg	PD
poultry						
2-C-47	Pos	Pos	PA	Neg	Neg	NA
Environmental						
No deviations observed						
Primary production and Primary production 5h RVS broth						
4-A-2	Neg	Pos	ND	Neg	Neg	NA

Table 13 - Interpretation of the sensitivity study results after storage of the enrichment broth (25g paired study)

Category	Negative Deviations (ND[1])	Positive deviations (PD)	ND-PD	Acceptability Limit (AL)	ND+PD	Acceptability Limit (AL)
meat	0	0	0	3	0	6
poultry	0	0	0	3	0	6
Environmental	0	0	0	3	0	6
Primary production	0	0	0	3	0	6
Primary production 5h RVS broth	0	0	0	3	0	6
Total	0	0	0	5	0	14

[1] ND: including PPND

Table 14 – Interpretation of the sensitivity study results after storage of the enrichment broth (375g unpaired study)

Category	Negative Deviations (ND[1])	Positive Deviations (PD)	ND-PD	Acceptability Limit (AL)
meat	0	2	-2	3
poultry	0	0	0	3
Total	0	2	-2	4

[1] ND: including PPND

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

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

3.2 Relative level of detection study

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

3.2.1 Categories, sample types and strains

One sample type and one relevant target micro-organism for this sample type was chosen for each of the Categories in this validation study, as shown in Table 15.

Table 15 - List of selected types and strains per category, as tested within the relative level of detection study.

Category	Sample size (g)	Type	Strain	Reference number	Strain origin	Pre-test storage of samples
Raw poultry and ready to cook poultry products	375	Fresh poultry meats (unprocessed)	S. Enteritidis	1004	Chicken	48h ±2h at 2-8°C
Raw meat and ready to cook meat products	375	Ground beef	S. Dublin	1356	Bovine	48h ±2h at 2-8°C
Environmental	25	Cleaning water cattle abattoir	S. Alachua	1274	Soil, abattoir	48h ±2h at 2-8°C
Primary production	25	faeces	S. Inverness	1377	Faeces	48h ±2h at 2-8°C

3.2.2 Test sample preparations

Three levels of artificial contamination were prepared for each type:

- Negative control level: One non-inoculated in order to get 5 test portions,
- Low level (L1): One inoculated between 2 and 5 CFU/sample in order to get 20 test portions providing fractional recovery,
- Higher level (L2): One inoculated between 9.6 and 18 CFU/sample in order to get 5 test portions contaminated at a higher level.

The level of cells used for the RLOD study is given in the table below

Category	**Level of *Salmonella* used cfu per portion**	
	low (L1)	**High (L2)**
1 Raw poultry and ready to cook poultry products	2	10
2 Raw meat and ready to cook meat products	2	18
3 Environmental	4	13
4 Primary production	5	9.6

After inoculation, the matrices were stored as described in Table 15.

3.2.3 RLOD study results

The RLOD calculations were performed using the Excel spread sheet (version 3, 15-08-15) of the international standard as described in ISO 16140-2: 2016.

The RLOD per Category is given in Table 16

Table 16a – Presentation of RLOD before and after confirmation of the alternative method results DNA Extraction-Mix II profile I

Type (Category)	RLOD using the alternative method results	RLOD using the confirmed alternative method results	AL	Pass/fail
Raw poultry and ready to cook poultry products 375g	1.368	1.368	2.5	Pass
Raw meat and ready to cook meat products 375g	1.00	1.00	2.5	Pass
Environmental 25g	1.00	1.00	1.5	Pass
Primary production 25g	1.00	1.00	1.5	Pass
Primary production 5h RVS 25g	1.00	1.00	1.5	Pass
Combined	1.056	1.056	1.5/2.5	Pass

Table 16b – Presentation of RLOD before and after confirmation of the alternative method results DNA Extraction-Mix II profile II

Type (Category)	RLOD using the alternative method results	RLOD using the confirmed alternative method results	AL	Pass/fail
Raw poultry and ready to cook poultry products 375g	1.368	1.368	2.5	Pass
Raw meat and ready to cook meat products 375g	1.00	1.00	2.5	Pass
Environmental 25g	1.00	1.00	1.5	Pass
Primary production 25g	1.00	1.00	1.5	Pass
Primary production 5h RVS 25g	1.00	1.00	1.5	Pass
Combined	1.056	1.056	1.5/2.5	Pass

Table 16c – Presentation of RLOD before and after confirmation of the alternative method results RNA/DNA Purification profile I

Type (Category)	RLOD using the alternative method results	RLOD using the confirmed alternative method results	AL	Pass/fail
Raw poultry and ready to cook poultry products 375g	1.368	1.368	2.5	Pass
Raw meat and ready to cook meat products 375g	1.497	1.497	2.5	Pass
Environmental 25g	1.00	1.00	1.5	Pass
Primary production 25g	1.00	1.00	1.5	Pass
Primary production 5h RVS 25g	1.00	1.00	1.5	Pass
Combined	1.115	1.115	1.5/2.5	Pass

Table 16d – Presentation of RLOD before and after confirmation of the alternative method results RNA/DNA Purification profile II

Type (Category)	RLOD using the alternative method results	RLOD using the confirmed alternative method results	AL	Pass/fail
Raw poultry and ready to cook poultry products 375g	1.368	1.368	2.5	Pass
Raw meat and ready to cook meat products 375g	1.931	1.931	2.5	Pass
Environmental 25g	1.00	1.00	1.5	Pass
Primary production 25g	1.00	1.00	1.5	Pass
Primary production 5h RVS 25g	1.00	1.00	1.5	Pass
Combined	1.177	1.177	1.5/2.5	Pass

In addition, LOD50 values were calculated using the equations quoted in Wilrich and Wilrich (2009) Journal of AOAC International 92 (6) 1763-1772 downloaded from

www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/wilrich.index.htm

The LOD50 per Category is given in Table 17 a – d

Table 17a – Presentation of LOD50 after confirmation of the alternative method results DNA extraction mix profile I

Type (Category)	LOD50 cfu per portion	Lower confidence limit cfu per portion	Upper confidence limit cfu per portion
Raw meat and ready to cook meat products	3.062	1.548	6.056
Raw poultry and ready to cook poultry	0.997	0.997	0.1735
Environmental	1.964	1.149	3.356
Primary production	2.642	1.589	4.393
Primary production 5h RVS	2.642	1.589	4.393
Combined	2.169	1.694	2.777

Table 17b – Presentation of LOD50 after confirmation of the alternative method results DNA extraction mix profile II

Type (Category)	LOD50 cfu per portion	Lower confidence limit cfu per portion	Upper confidence limit cfu per portion
Raw meat and ready to cook meat products	3.062	1.548	6.056
Raw poultry and ready to cook poultry	0.997	0.573	1.735
Environmental	1.964	1.149	3.356
Primary production	2.642	1.589	4.393
Primary production 5h RVS	2.642	1.589	4.393
Combined	2.169	1.694	2.777

Table 17c – Presentation of LOD50 after confirmation of the alternative method results RNA/DNA extraction profile I

Type (Category)	LOD50 cfu per portion	Lower confidence limit cfu per portion	Upper confidence limit cfu per portion
Raw meat and ready to cook meat products	4.160	2.049	8.446
Raw poultry and ready to cook poultry	0.997	0.573	1.736
Environmental	1.964	1.149	3.356
Primary production	2.642	4.393	4.393
Primary production 5h RVS	2.642	4.393	4.393
Combined	2.260	2.894	2.894

Table 17d – Presentation of LOD50 after confirmation of the alternative method results RNA/DNA extraction profile II

Type (Category)	LOD50 cfu per portion	Lower confidence limit cfu per portion	Upper confidence limit cfu per portion
Raw meat and ready to cook meat products	4.888	2.373	10.070
Raw poultry and ready to cook poultry	0.997	0.573	1.735
Environmental	1.964	3.356	3.356
Primary production	2.642	1.589	4.393
Primary production 5h RVS	2.642	1.589	4.393
Combined	2.307	1.801	2.954

The LOD50 was also calculated for the reference method and the results are given below in Table 17e.

Table 17e Presentation of LOD50 after confirmation of the reference method

Type (Category)	LOD50 cfu per portion	Lower confidence limit cfu per portion	Upper confidence limit cfu per portion
Raw meat and ready to cook meat products	3.062	1.548	6.056
Raw poultry and ready to cook poultry	0.731	0.417	1.279
Environmental	1.964	1.149	3.356
Primary production	2.642	1.589	4.393
Primary production 5h RVS	2.642	1.589	4.393
Combined	2.082	1.626	2.666

Analysis of the data showed that the LOD50 in the meat category for the reference method was similar to the LOD50 for the alternative method. This was a result of the low number of positive samples for both methods obtained during the study.

3.2.4 Conclusion RLOD study

The RLOD values (using the confirmed alternative method results) meet the acceptability limit for 25g environmental and primary production samples, which is 1.5 for paired studies for all DNA extraction and PCR profiles.

In addition, (using the confirmed alternative method results) the RLOD values meet the acceptability limit for 375g samples of Raw poultry and ready to cook poultry products and Raw meat and ready to cook meat products, which is 2.5 for unpaired studies for DNA Extraction-Mix II profiles I and II and RNA/DNA Purification profiles I and II.

3.3 Inclusivity/exclusivity study

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

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

3.3.1 Protocols

Inclusivity: 100 pure strains of the target microorganisms i.e. *Salmonella* spp were freshly cultured in BPW for 18-24h at 37°C. Dilutions were made in order to inoculate approximately 10 -100 CFU/225 ml BPW broth. The alternative method protocol was then performed (no sample material was added). After inoculation, the inclusivity samples were incubated for 16h at 37 ±1°C prior to analysis using the Alternative method

Exclusivity: Each test was performed once with the Alternative method. The 30 exclusivity isolates were be grown overnight in an appropriate broth and inoculated into BPW to achieve a concentration of 10^5 cfu per ml. Following inoculation, the exclusivity samples will be incubated for 16h at 37 ±1°C before testing using the Alternative method.

3.3.2 Results inclusivity and exclusivity study
DNA was extracted from the inclusivity and exclusivity panels with both DNA extraction kits being assessed in the study (Kylt® DNA Extraction-Mix II and Kylt® RNA/ DNA Purification) and each extract was run on both PCR profiles (I and II).

A total of 100 strains were tested for **inclusivity** using the 4 combinations of DNA extraction and PCR profile.

For samples extracted with the Kylt® DNA Extraction-Mix II and run on profile 1, 100 of these strains showed the expected positive result.

For samples extracted with the Kylt® DNA Extraction-Mix II and run on profile 2, 99 of these strains showed the expected positive result.

For samples extracted with the Kylt® RNA/ DNA Purification and run on profile I, 100 of these strains showed the expected positive result.

For samples extracted with the Kylt® RNA/ DNA Purification and run on profile II, 100 of these strains showed the expected positive result.

A total of 30 strains were tested for **exclusivity** using the 4 combinations of DNA extraction and PCR profile.

For samples extracted with the Kylt® DNA Extraction-Mix II and run on profile I, 30 of these strains showed the expected negative result. No strains showed a positive result at the presumptive positive stage.

For samples extracted with the Kylt® DNA Extraction-Mix II and run on profile II, 30 of these strains showed the expected negative result. No strains showed a positive result at the presumptive positive stage.

For samples extracted with the Kylt® RNA/ DNA Purification and run on profile I, 30 of these strains showed the expected negative result. No strains showed a positive result at the presumptive positive stage.

For samples extracted with the Kylt® RNA/ DNA Purification and run on profile II, 30 of these strains showed the expected negative result. No strains showed a positive result at the presumptive positive stage.

Conclusion inclusivity and exclusivity study

The alternative Kylt® *Salmonella* spp. 2.0 detection method is selective and specific for the following DNA extraction and PCR profile combinations.

- Kylt® DNA Extraction-Mix II and run on profile I
- Kylt® DNA Extraction-Mix II and run on profile II
- Kylt® RNA/ DNA Purification and run on profile I
- Kylt® RNA/ DNA Purification and run on profile II

3.4 Conclusions Method Comparison Study

Overall, the conclusions for the Method Comparison Study are:

For 25g samples, the observed values for ND-PD and ND+PD for the individual categories and for all categories meet the acceptability limits (observed values ≤ AL) for the following DNA extraction and PCR profiles.

- Kylt® DNA Extraction-Mix II and run on profile I
- Kylt® DNA Extraction-Mix II and run on profile II
- Kylt® RNA/ DNA Purification and run on profile I*
- Kylt® RNA/ DNA Purification and run on profile II

*Primary production samples should be run with the 5h RVS enrichment step included only.

Analysis of 375g samples revealed that the observed values for ND-PD for the individual categories and for all categories did meet the acceptability limits (observed values ≤ AL) for the following DNA extraction and PCR profiles for 375g samples.

- Kylt® DNA Extraction-Mix II and run on profile II

The observed values for ND-PD for the individual categories and for all categories did not meet the acceptability limits (observed values ≤ AL) for the following DNA extraction and PCR profiles for 375g samples.

- Kylt® DNA Extraction-Mix II and run on profile I
- Kylt® RNA/ DNA Purification and run on profile I
- Kylt® RNA/ DNA Purification and run on profile II

The RLOD values meet the acceptability limit for 25g environmental and primary production samples, which is 1.5 for paired studies for all DNA extraction and PCR profiles.

In addition, the RLOD values meet the acceptability limit for 375g samples of Raw poultry and ready to cook poultry products and Raw meat and ready to cook meat products, which is 2.5 for unpaired studies for extraction mix II profiles I and II and RNA/ DNA Purification profile I. RNA/ DNA Purification II met the acceptability criteria for Raw poultry and ready to cook poultry products but not for Raw meat and ready to cook meat products meat 375g samples.

The alternative Kylt® *Salmonella* spp. 2.0 detection method is selective and specific for the following DNA extraction and PCR profile combinations.

- Kylt® DNA Extraction-Mix II and run on profile I
- Kylt® DNA Extraction-Mix II and run on profile II
- Kylt® RNA/ DNA Purification and run on profile I
- Kylt® RNA/ DNA Purification and run on profile II

Analysis of multiplatform data

As part of the study, the DNA extracts run at the EL on the 7500 FAST were also run on 6 additional platforms. Each platform was given a code during the analysis as listed in the table below.

Code	Platform
1	7500 FAST run at the expert lab
2	CFX96
3	CFX 384
5	Rotor Gene 3000
6	Rotor Gene 6000
7	Aria MX Pro
10	Light cycler 480

The data from the additional platforms was analysed for three sections of the study.

1. Inclusivity and exclusivity study
2. Sensitivity study
3. RLOD study

Inclusivity and exclusivity study

A summary of the results for the multiple platforms is given in Table 18.

Table 18a: Summary of the inclusivity data on the 7 platforms involved in the ISO16140-2:2016

DNA extraction	PCR profile	No of isolates detected out of the 100 *Salmonella* spp isolates analysed						
		1	2	3	5	6	7	10
Extraction mix II	I	100	100	99[#]	100	100	100	10
	II	100	100	99[#]	100	100	100	100
RNA/DNA extraction	I	100	98/98	97/97	97/98[g]	99/99	99/99	93[d]/94
	II	100	98/98	97/97	97/98[g]	99/99	99/99	99/100

Key
[#] S. Bareilly isolate not detected
[d] S. Dublin isolate not detected
[g] S. Agona isolate not detected

The conclusions of the inclusivity study are:

The alternative Kylt® *Salmonella* spp. 2.0 detection method is selective and specific for the following DNA extraction and PCR profile combinations listed in the table below.

DNA extraction	PCR profile	Pass/fail decision for inclusivity study						
		1	2	3	5	6	7	10
Extraction mix II	I	pass	pass	pass	pass	pass	pass	pass
	II	pass	pass	pass	pass	pass	pass	pass
RNA/DNA extraction	I	pass	pass	pass	pass	pass	pass	pass
	II	pass	pass	pass	pass	pass	pass	pass

Exclusivity

A summary of the results for the multiple platforms in the exclusivity is given in Table 19.

Table 19 Summary of the exclusivity data on the 10 platforms involved in the ISO16140-2:2016

DNA extraction	PCR profile	No of isolates detected out of the 100 *Salmonella* spp isolates analysed						
		1	2	3	5	6	7	10
Extraction mix II	I	0	0	0	0	4[d]	1[e]	1
	II	0	0/29	0/29	3[h]	8[f]	0	0/29
RNA/DNA extraction	I	0	0/29	0	0	0	0/29	0
	II	0	0/29	0/29	0	2[b]	0/29	0/28

Key

[b] 2 presumptive positives *Morganella morganii* and *Pseudomonas fragi*

[d] 4 presumptive positives *Enterobacter aerogenes*, *Enterobacter cloacae*, *Pseudomonas fluorescens* and *Serratia proteamaculans subsp. quinovora*

[e] 1 presumptive positive 125 *Shigella boydii*

[f] 8 presumptive positives *Citrobacter freundii*, *Enterobacter cloacae*, *Escherichia coli*, *Morganella morganii*, *Citrobacter braakii*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*,*Serratia liquifaciens*

[h] 3 presumptive positives *Pseudomonas fluorescens, Pseudomonas fragi, Serratia liquifaciens*

The conclusions of the exclusivity study are:

The alternative Kylt® *Salmonella* spp. 2.0 detection method is selective and specific for the following DNA extraction and PCR profile combinations listed in the table below.

DNA extraction	PCR profile	Pass/fail decision for exclusivity study						
		1	2	3	5	6	7	10
Extraction mix II	I	pass	pass	pass	pass	pass	pass	pass
	II	pass	pass	pass	pass	pass	pass	pass
	I	pass	pass	pass	pass	pass	pass	pass

RNA/DNA extraction	II	pass	pass	pass	pass	pass	pass	pass

Sensitivity study
Due to the large number of samples processed, a few of the samples were missing from the sensitivity study and a strategy was developed to analyse the sensitivity data set. The analysis plan was agreed with the selected reviewers and is outlined below.

It was agreed that the most important point to consider in the analysis were the samples that gave a positive result when run on the platform at the EL.

Positive samples with a Ct score of 20 or less with the platform at the EL were considered to be likely to give a good signal on the platforms not tested.

Analysis of the missing data was focused on positive samples which gave a Ct score of >30 with the platform at the EL. The proportion of missing samples across the 6 platforms analyzed for the DNA extraction and PCR Profile combinations are given in the Table 20 below.

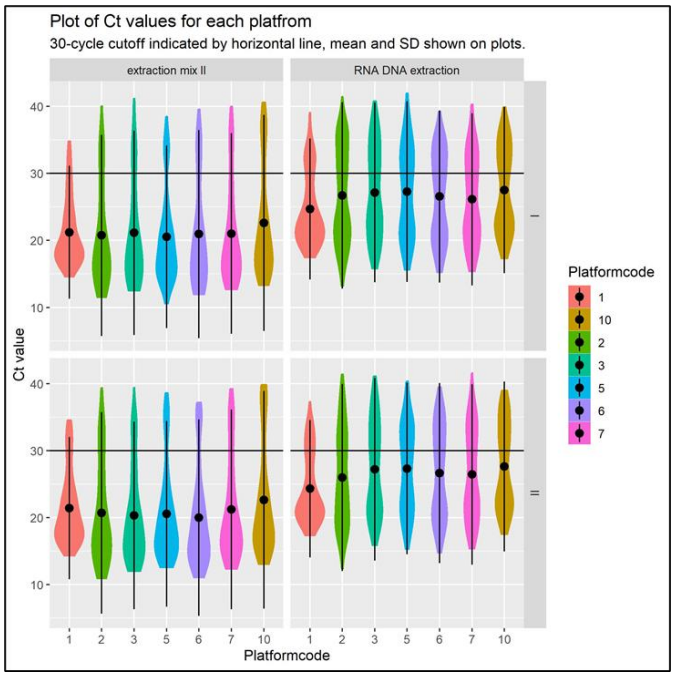
Table 20 Summary of % missing positive samples for each platform run by the manufacturer

Analysis type	% missing samples PCR platform used					
	2	**3**	**5**	**6**	**7**	**10**
Extraction mix I profile I	1.93	1.93	2.58	1.93	1.93	2.58
Extraction mix I profile II	0.92	2.31	1.39	1.85	1.39	0.92
RNA DNA extraction profile I	0.73	0.00	0.00	0.00	0.00	0.73
RNA DNA extraction profile I	0.58	1.17	1.17	1.75	1.17	2.34

The level of missing samples was no more than 2.58% of the total positive samples which was less than the 3% criteria.

In addition, the variability of the Ct scores obtained for the positive samples was analyzed as a violin plot as shown in Figure 1 below. Each DNA extraction method and PCR profile combination is plotted in a separate quarter of the plot for ease of reference and the PCR platforms are colour coded, with the key included on the right of the Figure.

Figure 1: Violin plot of Ct scores for the analysis of the sensitivity study samples run on the multiple platforms by the expert lab and manufacturer



In addition to the violin plot to visualize the variability in Ct scores between samples, statistical analysis was also carried out to determine if the differences between the Ct scores obtained. As the data did not follow a normal distribution, a non-parametric test was carried out to assess the similarity in CT scores across the PCR platforms used including the 7500 FAST run at the expert lab. The Similarity between Ct scores for each sample compared by using the Wilcoxon Signed Rank Test using a Null hypothesis that (no difference between platforms). Analysis of the data revealed that this null hypothesis is true with a probability of >0.05 or 99.5%

The data and additional was presented to the MicroVal Technical committee 16-17 September 2021 and it was agreed that the results and analysis presented gave a satisfactory analysis of the data.

RLOD study

Analysis of the RLOD data was carried out with the same criteria used to analyse the results obtained in the sensitivity study for any missing or inhibited samples. Positive samples with a Ct score of 20 or less with the platform at the EL and other platforms at the manufacturer were considered to be likely to give a good signal on the platforms not tested. A summary of the matrices that fell within the criteria for 4 categories analysed is summarized in Table 21. .

Table 21 summary of multiplatform RLOD status for multiplatform analysis

Category	DNA extraction pcr profile combination	RLOD criteria met yes/no						
		1	2	3	5	6	7	10
Meat RLOD AL = 2.5	Extraction mix II profile I	y	y	y	y	y	y	y
	Extraction mix II profile II	y	y	y	y	y	y	y
	RNA/DNA extraction profile I	y	y	n	y	y	y	n
	RNA/DNA extraction profile II	y	y	y	n	y	y	n
Poultry RLOD AL = 2.5	Extraction mix II profile I	y	y	y	y	y	y	y
	Extraction mix II profile II	y	y	y	y	y	y	y
	RNA/DNA extraction profile I	y	y	y	y	y	y	y
	RNA/DNA extraction profile II	y	y	y	y	y	y	y
Environmental RLOD AL = 1.5	Extraction mix II profile I	y	y	y	y	y	y	y
	Extraction mix II profile II	y	y	y	y	y	y	y
	RNA/DNA extraction profile I	y	y	y	y	y	y	y
	RNA/DNA extraction profile II	y	y	y	y	y	y	y
Primary production RLOD AL = 1.5	Extraction mix II profile I	y	y	y	y	y	y	y
	Extraction mix II profile II	y	y	y	y	y	y	y
	RNA/DNA extraction profile I	y	y	y	y	y	y	y
	RNA/DNA extraction profile II	y	y	y	y	y	y	y
Primary production 5h RVS broth RLOD AL = 1.5	Extraction mix II profile I	y	y	y	y	y	y	y
	Extraction mix II profile II	y	y	y	y	y	y	y
	RNA/DNA extraction profile I	y	y	y	y	y	y	y
	RNA/DNA extraction profile II	y	y	y	y	y	y	y

Key for platforms 1 =7500 FAST 2 = CFX96 3 = CFX 384, 5 = Rotor Gene 3000,6 = Rotor Gene 6000, 7= Aria MX Pro, 10= Light cycler 480

Overall conclusions on the multiplatform study. A summary of the final scope of the different platforms is given in the table below

Category	DNA extraction pcr profile combination	Matrices certified						
		1	2	3	5	6	7	10
Meat 25g	Extraction mix II profile I	p	p	p	p	p	p	p
	Extraction mix II profile II	p	p	p	p	p	p	p
	RNA/DNA extraction profile I	p	p	f	p	p	p	f
	RNA/DNA extraction profile II	p	p	p	f	p	p	f
Meat 375g	Extraction mix II profile I	p	p	p	p	p	p	p
	Extraction mix II profile II	p	p	p	p	p	p	p
	RNA/DNA extraction profile I	p	p	f	p	p	p	f
	RNA/DNA extraction profile II	p	p	p	f	p	p	f
Poultry 25g	Extraction mix II profile I	p	p	p	p	p	p	p
	Extraction mix II profile II	p	p	p	p	p	p	p
	RNA/DNA extraction profile I	p	p	p	p	p	p	p
	RNA/DNA extraction profile II	p	p	p	p	p	p	p
Poultry 375g	Extraction mix II profile I	p	p	p	p	p	p	p
	Extraction mix II profile II	p	p	p	p	p	p	p
	RNA/DNA extraction profile I	p	p	p	p	p	p	p
	RNA/DNA extraction profile II	p	p	p	p	p	p	p
Environmental	Extraction mix II profile I	p	p	p	p	p	p	p
	Extraction mix II profile II	p	p	p	p	p	p	p

	RNA/DNA extraction profile I	p	p	p	p	p	p	p
	RNA/DNA extraction profile II	p	p	p	p	p	p	p
Primary production	Extraction mix II profile I	p	p	p	p	p	p	p
	Extraction mix II profile II	p	p	p	p	p	p	p
	RNA/DNA extraction profile I	p	p	p	p	p	p	p
	RNA/DNA extraction profile II	p	p	p	p	p	p	p
Primary production 5h RVS broth	Extraction mix II profile I	p	p	p	p	p	p	p
	Extraction mix II profile II	p	p	p	p	p	p	p
	RNA/DNA extraction profile I	p	p	p	p	p	p	p
	RNA/DNA extraction profile II	p	p	p	p	p	p	p

Key for platforms 1 =7500 FAST 2 = CFX96 3 = CFX 384, 5 = Rotor Gene 3000,6 = Rotor Gene 6000, 7= Aria MX Pro, 10= Light cycler 480

4 Interlaboratory study – Raw poultry

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 organisation

Collaborators number

Samples were sent to 14 laboratories in 2 different countries; with a single collaborator were involved in the study for all laboratories.

Matrix and strain used

Raw diced chicken was inoculated with *Salmonella* Blockley strain Campden ref 1085, isolated from fresh chicken.

Samples

Samples were inoculated on 18/03/2019, as described below:

- 24 blind coded samples were prepared for analysis by the Kylt® Salmonella spp. 2.0 method and by the reference method (ISO 6579-1:2017)
- 1 non inoculated raw diced chicken was included for aerobic mesophilic flora enumeration by ISO 4833 method,
- 1 water flask labelled “Temperature Control” which was frozen with the samples to check that the temperatire conditions during transit did not defrost the samples.

All the samples were pre-weighed in stomacher bags in 25g amounts and individually inoculated at the required level.

The samples were stored frozen at ≤-18°C and defrosted prior to analysis as recommended in ISO 6887-1. The analyses was started on Monday 25 March 2019. Stability studies had been conducted to show that the required level of target organisms would be present after 7 and 6 days frozen storage. The expert lab analysed a set of samples on Tuesday 26 March 2019.

Inoculation
The target inoculation levels were:

- Level 0: 0 CFU/25 g,
- Level 1: 0.8 CFU/25 g,
- Level 2: 2.0 CFU/25 g.

Each laboratory received 24 samples of 25 g, i.e. 8 samples per inoculation level and method plus a sample for analysis of mesophilic aerobic count.

4.2 Experimental parameters controls

4.2.1 Detection Salmonella spp in the matrix before inoculation
In order to detect the presence of *Salmonella* spp., the reference method was performed on six portions (25 g) before the inoculation. All the results were negative.

4.2.2 Strain stability during transport
Three samples inoculated at 2 cfu per 25g portion were tested for detection of *Salmonella* spp. after 7days and 10 days storage at < -18°C. The mesophilic aerobic flora enumeration was also performed (See Table 22)

Table 22 – Salmonella spp stability in the matrix

Day	Reference method (detection)		
	Sample 1	Sample 2	Sample 3
Day 0	detected	detected	detected
Day 7	detected	detected	detected
Day 10	detected	detected	detected

No evolution was observed during storage at 5°C ± 3°C.

4.2.3 Contamination levels
The samples prepared for the ILS were inoculated as follows.

A culture of *Salmonella* Blockley (Campden ref 1085) was grown overnight on 14 March in Nutrient Broth incubated at 37°C. The levels in the culture were checked by plating out on count agar and the *Salmonella* Blockley was chilled for 48h prior to use in inoculating samples on 18 March 2019.

The overnight culture was diluted such that L1 samples were inoculated at a level of 0.8 CFU/25g portion and L2 were inoculated with a level of 2 CFU/25g portion on 18 March 2019. These values were used so that the cells would follow the stabilisation pattern shown in the stability trials (Table 13).

4.2.4 Logistic conditions
The temperatures measured at reception by the collaborators, the temperatures registered by the thermo-probe, and the receipt dates are given in Table 23.

Table 23 - Sample temperatures at receipt

Collaborator	Temperature measured by the probe (°C)	Temperature measured at receipt (°C)	Receipt date and time	State of the package and samples at the receipt	Analysis date
1	n/a	Water frozen	21/03/19, 08.45	frozen	25/03/19
2	n/a	Water defrosted	21/03/19, 10.30	OK	25/03/19
3	n/a	1.5°C	21/03/19, 10.30	Water defrosted	25/03/19
4	No information received				26/03/19
5	n/a	n/a	22/03/19, 11.00	Water defrosted	25/03/19
6	n/a	n/a	21/03/19, 15.10	Water defrosted	25/03/19
7	n/a	Water frozen	22/03/19, 10.15	good	25/03/19
8	n/a	Water frozen	22/03/19, 13.05	in order	25/03/19
9	n/a	Water not found	21/03/19, 12.30	good	25/03/19
11	n/a	6.8°C, Water defrosted	22/03/19, 10.55	in order	25/03/19
12	n/a	Water frozen	21/03/19, 08.15	in order	26/03/19
13	n/a	Water frozen	25/03/19	in order	25/03/19
14	n/a	Water frozen	25/03/19, 16.00	in order	25/03/19
15	n/a	Water defrosted	23/03/19, 08.00	n/a	25/03/19

No problem was encountered during the transport or at receipt for the 14 collaborators. All the samples were delivered on time and in appropriate conditions. Temperatures during shipment and at receipt were all correct.

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 24.

Table 24 – Results obtained by the expert lab.

Level	Number of positive samples				
	Reference method	Alternative method			
		Kylt® Extraction Mix II profile I	Kylt® Extraction Mix II profile II	Kylt®DNA/RNA Purification profile I	Kylt® DNA/RNA Purification profile II
L0	0/8	0/8	0/8	0/8	0/8
L1	5/8	5/8	5/8	5/8	5/8
L2	6/8	6/8	6/8	6/8	6/8

4.3.2 Results obtained by the collaborative laboratories

- *Mesophilic aerobic flora enumeration*

Depending on the Lab results, the enumeration levels varied from 1.6 x 10^4 to 1.1x10^7*CFU/g.

* High count is estimated as number of colonies recorded was greater than 300.

- *Salmonella* spp. *detection*

14 collaborators participated to the study. The results obtained by the individual collaborators in the inter-laboratory study are summarised in Table 25 (reference method) and Table 26-29 (alternative method).

Table 25 - Positive results by the reference method (ALL the collaborators)

Collaborator	**Contamination level**		
	L0	**L1**	**L2**
1	0/8	7/8	7/8
2	0/8	6/8	6/8
3	1/8	4/8	6/8
4	0/8	6/8	6/8
5	0/8	1/8	3/8
6	0/8	6/8	7/8
7	0/8	3/8	7/8
8	0/8	6/8	6/8
9	1/8	1/8	7/8
11	0/8	3/8	6/8
12	0/8	5/8	7/8
13	0/8	4/8	8/8
14	0/8	7/8	5/8
15	0/8	6/8	7/8
TOTAL	**P_0 =2**	**P_1 =65**	**P_2= 88**

Table 26 - Positive results (before and after confirmation) by the alternative methods (ALL the collaborators) Kylt® DNA Extraction-Mix II profile I

Collaborators	**Contamination level**					
	L0		**L1**		**L2**	
	Before confirmation	After confirmation	Before confirmation	After confirmation	Before confirmation	After confirmation
1	0/8	0/8	7/8	7/8	7/8	7/8
2	0/8	0/8	6/8	6/8	6/8	6/8
3	1/8	1/8	4/8	4/8	6/8	6/8
4	0/8	0/8	6/8	6/8	6/8	6/8
5	0/8	0/8	1/8	1/8	4/8	3/8
6	0/8	0/8	7/8	7/8	7/8	7/8
7	0/8	0/8	3/8	3/8	7/8	7/8
8	0/8	0/8	6/8	6/8	6/8	6/8
9	0/8	0/8	1/8	1/8	7/8	7/8
11	0/8	0/8	3/8	3/8	8/8	7/8
12	0/8	0/8	6/8	5/8	6/8	6/8
13	0/8	0/8	4/8	4/8	8/8	8/8
14	0/8	0/8	7/8	7/8	5/8	5/8
15	0/8	0/8	6/8	6/8	7/8	7/8
TOTAL	**P_0 = 1**	**CP_0 =1**	**P_1 = 67**	**CP_1 = 66**	**P_2 = 90**	**CP_2 = 88**

Table 27 - Positive results (before and after confirmation) by the alternative methods (ALL the collaborators) Kylt® DNA Extraction-Mix II profile II

Collaborators	**Contamination level**					
	L0		**L1**		**L2**	
	Before Confirmation	After Confirmation	Before Confirmation	After Confirmation	Before Confirmation	After Confirmation
1	0/8	0/8	7/8	7/8	7/8	7/8
2	0/8	0/8	6/8	6/8	6/8	6/8
3	1/8	1/8	4/8	4/8	6/8	6/8
4	0/8	0/8	6/8	6/8	6/8	6/8
5	0/8	0/8	1/8	1/8	3/8	3/8
6	0/8	0/8	7/8	7/8	7/8	7/8
7	0/8	0/8	3/8	3/8	7/8	7/8
8	0/8	0/8	6/8	6/8	6/8	6/8
9	0/8	0/8	1/8	1/8	7/8	7/8
11	0/8	0/8	3/8	3/8	8/8	7/8
12	0/8	0/8	6/8	5/8	6/8	6/8
13	0/8	0/8	4/8	4/8	8/8	8/8
14	0/8	0/8	7/8	7/8	5/8	5/8
15	0/8	0/8	6/8	6/8	7/8	7/8
TOTAL	**P_0 =1**	**CP_0 =1**	**P_1 =67**	**CP_1 =66**	**P_2 =89**	**CP_2 =88**

Table 28 - Positive results (before and after confirmation) by the alternative methods (ALL the collaborators) Kylt® RNA/ DNA Purification profile I

Collaborators	**Contamination level**					
	L0		***L1***		***L2***	
	Before confirmation	*After confirmation*	*Before confirmation*	*After confirmation*	*Before confirmation*	*After confirmation*
1	0/8	0/8	7/8	7/8	7/8	7/8
2	0/8	0/8	6/8	6/8	6/8	6/8
3	1/8	1/8	4/8	4/8	6/8	6/8
4	0/8	0/8	6/8	6/8	6/8	6/8
5	0/8	0/8	1/8	1/8	2/8	2/8
6	0/8	0/8	7/8	7/8	7/8	7/8
7	0/8	0/8	3/8	3/8	7/8	7/8
8	0/8	0/8	6/8	6/8	6/8	6/8
9	6/8	1/8	5/8	1/8	8/8	7/8
11	0/8	0/8	3/8	4/8	7/8	7/8
12	1/8	0/8	6/8	5/8	6/8	6/8
13	0/8	0/8	4/8	4/8	8/8	8/8
14	n/a	n/a	n/a	n/a	n/a	n/a
15	0/8	0/8	6/8	6/8	7/8	7/8
TOTAL	**P_0 =8**	**CP_0 =2**	**P_1 =64**	**CP_1 =58**	**P_2 =83**	**CP_2 =82**

Table 29 - Positive results (before and after confirmation) by the alternative methods (ALL the collaborators) Kylt® RNA/ DNA Purification profile II

Collaborators	**Contamination level**					
	L0		***L1***		***L2***	
	Before confirmation	*After confirmation*	*Before confirmation*	*After confirmation*	*Before confirmation*	*After confirmation*
1	0/8	0/8	7/8	7/8	7/8	7/8
2	0/8	0/8	6/8	6/8	6/8	6/8
3	1/8	1/8	4/8	4/8	6/8	6/8
4	0/8	0/8	6/8	6/8	6/8	6/8
5	0/8	0/8	1/8	1/8	3/8	3/8
6	0/8	0/8	7/8	7/8	7/8	7/8
7	0/8	0/8	3/8	3/8	7/8	7/8
8	0/8	0/8	6/8	6/8	6/8	6/8
9	5/8	1/8	4/8	1/8	8/8	7/8
11	0/8	0/8	4/8	3/8	7/8	7/8
12	0/8	0/8	6/8	5/8	6/8	6/8
13	0/8	0/8	4/8	4/8	8/8	8/8
14	n/a	n/a	n/a	n/a	n/a	n/a
15	0/8	0/8	6/8	6/8	7/8	7/8
TOTAL	**P_0 = 6/80**	**CP_0 = 2/80**	**P_1 = 64**	**CP_1 = 59**	**P_2 = 84**	**CP_2 = 83**

Remarks: Labs 5 and 9 was removed due to the low number of positive results. In addition, Lab 9 had positive results for the blank samples due the potential for cross contamination. Laboratory 13 was not included in the study as this was the supplier laboratory. The final laboratory to be excluded was lab 14 as this collaborator did not complete the sample analysis with all the DNA extraction kits. Lab 3 also had a positive blank sample which was probably due to cross contamination as *Salmonella* was not isolated in the initial blank samples analysed in the stability study or in 11 of the labs that participated in the ILS.

4.3.3 Results of the collaborators retained for interpretation

The results obtained with the 10 collaborators kept for interpretation are presented in Table 30 (reference method) and Tables 31-34 (alternative method).

Table 30 - Positive results by the reference method (Without Labs 5, 9, 13 and 14)

Collaborators	Contamination level		
	L0	**L1**	**L2**
1	0/8	7/8	7/8
2	0/8	6/8	6/8
3	1/8	4/8	6/8
4	0/8	6/8	6/8
6	0/8	7/8	7/8
7	0/8	3/8	7/8
8	0/8	6/8	6/8
11	0/8	3/8	7/8
12	0/8	5/8	7/8
15	0/8	6/8	7/8
TOTAL	**P_0 = 1/80**	**P_1 = 53/80**	**P_2 = 66/80**

Table 31 - Positive results (before and after confirmation) by the alternative methods (ALL the collaborators) Kylt® DNA Extraction-Mix II profile I (Without Labs 5, 9, 13 and 14)

Collaborator	Contamination level					
	L0		***L1***		***L2***	
	Before confirmation	*After confirmation*	*Before confirmation*	*After confirmation*	*Before confirmation*	*After confirmation*
1	0/8	0/8	7/8	7/8	7/8	7/8
2	0/8	0/8	6/8	6/8	6/8	6/8
3	1/8	1/8	4/8	4/8	6/8	6/8
4	0/8	0/8	6/8	6/8	6/8	6/8
6	0/8	0/8	7/8	7/8	7/8	7/8
7	0/8	0/8	3/8	3/8	7/8	7/8
8	0/8	0/8	6/8	6/8	6/8	6/8
11	0/8	0/8	3/8	3/8	8/8	7/8
12	0/8	0/8	6/8	5/8	6/8	6/8
15	0/8	0/8	6/8	6/8	7/8	7/8
TOTAL	**P_0 = 1/80**	**CP_0 =1/80**	**P_1 = 54/80**	**CP_1 = 53/80**	**P_2 = 66/80**	**CP_2 = 65/80**

Table 32 - Positive results (before and after confirmation) by the alternative methods (ALL the collaborators) Kylt® DNA Extraction-Mix II profile II (Without Labs 5, 9, 13 and 14)

Collaborator	Contamination level					
	L0		***L1***		***L2***	
	Before confirmation	*After confirmation*	*Before confirmation*	*After confirmation*	*Before confirmation*	*After confirmation*
1	0/8	0/8	7/8	7/8	7/8	7/8
2	0/8	0/8	6/8	6/8	6/8	6/8
3	1/8	1/8	4/8	4/8	6/8	6/8
4	0/8	0/8	6/8	6/8	6/8	6/8
6	0/8	0/8	7/8	7/8	7/8	7/8
7	0/8	0/8	3/8	3/8	7/8	7/8
8	0/8	0/8	6/8	6/8	6/8	6/8
11	0/8	0/8	3/8	3/8	8/8	7/8
12	0/8	0/8	6/8	5/8	6/8	6/8
15	0/8	0/8	6/8	6/8	7/8	7/8
TOTAL	**P_0 = 1/80**	**CP_0 =1/80**	**P_1 = 54/80**	**CP_1 = 53/80**	**P_2 = 66/80**	**CP_2 = 65/80**

Table 33 - Positive results (before and after confirmation) by the alternative methods (ALL the collaborators) Kylt® RNA/ DNA Purification profile I (Without Labs 5, 9, 13 and 14)

Collaborator	Contamination level					
	L0		***L1***		***L2***	
	Before confirmation	*After confirmation*	*Before confirmation*	*After confirmation*	*Before confirmation*	*After confirmation*
1	0/8	0/8	7/8	7/8	7/8	7/8
2	0/8	0/8	6/8	6/8	6/8	6/8
3	1/8	1/8	4/8	4/8	6/8	6/8
4	0/8	0/8	6/8	6/8	6/8	6/8
6	0/8	0/8	7/8	7/8	7/8	7/8
7	0/8	0/8	3/8	3/8	7/8	7/8
8	0/8	0/8	6/8	6/8	6/8	6/8
11	0/8	0/8	3/8	3/8	7/8	7/8
12	1/8	0/8	6/8	5/8	6/8	6/8
0/8	0/8	6/8	6/8	7/8	7/8	7/8
TOTAL	**P_0 = 2/80**	**CP_0 =1/80**	**P_1 = 54/80**	**CP_1 = 53/80**	**P_2 = 65/80**	**CP_2 = 65/80**

Table 34 - Positive results (before and after confirmation) by the alternative methods (ALL the collaborators) Kylt® RNA/ DNA Purificationprofile II (Without Labs 5, 9, 13 and 14)

Collaborator	Contamination level					
	L0		***L1***		***L2***	
	Before confirmation	*After confirmation*	*Before confirmation*	*After confirmation*	*Before confirmation*	*After confirmation*
1	0/8	0/8	7/8	7/8	7/8	7/8
2	0/8	0/8	6/8	6/8	6/8	6/8
3	1/8	1/8	4/8	4/8	6/8	6/8
4	0/8	0/8	6/8	6/8	6/8	6/8
6	0/8	0/8	7/8	7/8	7/8	7/8
7	0/8	0/8	3/8	3/8	7/8	7/8
8	0/8	0/8	6/8	6/8	6/8	6/8
11	0/8	0/8	4/8	3/8	7/8	7/8
12	0/8	0/8	6/8	5/8	6/8	6/8
15	0/8	0/8	6/8	6/8	7/8	7/8
TOTAL	**P_0 = 1/80**	**CP_0 =1/80**	**P_1 = 55/80**	**CP_1 = 53/80**	**P_2 = 65/80**	**CP_2 = 65/80**

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 L1 +L2 are the following (See Tables 33-36).

Table 32 - Percentage specificity

Specificity for the reference method	$SP_{ref} = \left(1 - \left(\frac{P_0}{N_-}\right)\right) x\ 100\ \% =$	100 %
Specificity for the alternative method Kylt® DNA Extraction-Mix II profile I	$SP_{alt} = \left(1 - \left(\frac{CP_0}{N_-}\right)\right) x\ 100\ \% =$	100 %
Specificity for the alternative method Kylt® DNA Extraction-Mix II profile II	$SP_{alt} = \left(1 - \left(\frac{CP_0}{N_-}\right)\right) x\ 100\ \% =$	100%
Specificity for the alternative method Kylt® RNA/ DNA Purification profile I	$SP_{alt} = \left(1 - \left(\frac{CP_0}{N_-}\right)\right) x\ 100\ \% =$	98.75 %
Specificity for the alternative method Kylt® RNA/ DNA Purification profile II	$SP_{alt} = \left(1 - \left(\frac{CP_0}{N_-}\right)\right) x\ 100\ \% =$	100%

N - number of all L0 tests

P_0 - total number of false-positive results obtained with the blank samples before confirmation

CP_0 - total number of false-positive results obtained with the blank samples

4.3.5 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 and the high inoculation level(s) (L1 +L2). The two inoculation levels were retained for calculation.

A summary of the results of the collaborators retained for interpretation and obtained with the reference and the alternative methods for Level 1 and Level 2 is provided in Table 32-35.

Table 33 - Summary of the obtained results with the reference method and the alternative method for Level 1 and/or Level 2 Kylt® DNA Extraction-Mix II profile I

Level	Response	Reference method positive (R+)	Reference method negative (R-)
1	**Alternative method positive (A+)**	Positive agreement (A+/R+) **PA = 53**	Positive deviation (R-/A+) **PD = 0**
	Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 0**	Negative agreement (A-/R-) **NA = 27**
2	**Alternative method positive (A+)**	Positive agreement (A+/R+) **PA = 65**	Positive deviation (R-/A+) **PD = 0**
	Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 2**	Negative agreement (A-/R-) **NA = 13**

Table 34 - Summary of the obtained results with the reference method and the alternative method for Level 1 and/or Level 2 Kylt® DNA Extraction-Mix II profile II

Level	Response	Reference method positive (R+)	Reference method negative (R-)
1	**Alternative method positive (A+)**	Positive agreement (A+/R+) **PA = 53**	Positive deviation (R-/A+) **PD = 0**
	Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 0**	Negative agreement (A-/R-) **NA = 27**
2	**Alternative method positive (A+)**	Positive agreement (A+/R+) **PA = 65**	Positive deviation (R-/A+) **PD = 0**
	Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 2**	Negative agreement (A-/R-) **NA = 13**

Table 35 - Summary of the obtained results with the reference method and the alternative method for Level 1 and/or Level 2 Kylt® RNA/ DNA Purification profile I

Level	Response	Reference method positive (R+)	Reference method negative (R-)
1	**Alternative method positive (A+)**	Positive agreement (A+/R+) **PA = 53**	Positive deviation (R-/A+) **PD = 0**
	Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 0**	Negative agreement (A-/R-) **NA = 27**
2	**Alternative method positive (A+)**	Positive agreement (A+/R+)**PA = 65**	Positive deviation (R-/A+) **PD = 0**
	Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 1**	Negative agreement (A-/R-)**NA = 14**

Table 36 - Summary of the obtained results with the reference method and the alternative method for Level 1 and/or Level 2 Kylt® RNA/ DNA Purification profile II

Level	Response	Reference method positive (R+)	Reference method negative (R-)
1	**Alternative method positive (A+)**	Positive agreement (A+/R+) **PA = 53**	Positive deviation (R-/A+) **PD = 0**
	Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 0**	Negative agreement (A-/R-) **NA = 27**
2	**Alternative method positive (A+)**	Positive agreement (A+/R+) **PA = 65**	Positive deviation (R-/A+) **PD = 0**
	Alternative method negative (A-)	Negative deviation (A-/R+) **ND = 1**	Negative agreement (A-/R-) **NA = 14**

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 Tables 37 to 40).

Table 37 - Sensitivity, relative trueness and false positive ratio percentages Kylt® DNA Extraction-Mix II profile I

		Level 1	Level 2
Sensitivity for the alternative method:	$SE_{alt} = \frac{(PA+PD)}{(PA+PD+ND)} \; x \; 100\% =$	100.0%	97.1%
Sensitivity for the reference method:	$SE_{ref} = \frac{(PA+ND)}{(PA+PD+ND)} \; x \; 100\% =$	100.0%	100%
Relative trueness	$RT = \frac{(PA+NA)}{N} \; x \; 100\% =$	98.8%	97.5%
False positive ratio for the alternative method	$FPR = \frac{FP}{NA} \; x \; 100\% =$	3.7%	7.7%

Table 38 - Sensitivity, relative trueness and false positive ratio percentages Kylt® DNA Extraction-Mix II profile II

		Level 1	**Level 2**
Sensitivity for the alternative method:	$SE_{alt} = \frac{(PA+PD)}{(PA+PD+ND)} x\ 100\% =$	100%	100%
Sensitivity for the reference method:	$SE_{ref} = \frac{(PA+ND)}{(PA+PD+ND)} x\ 100\% =$	100%	100%
Relative trueness	$RT = \frac{(PA+NA)}{N} x\ 100\% =$	98.8%	97.5%
False positive ratio for the alternative method	$FPR = \frac{FP}{NA} x\ 100\% =$	3.7%	7.7%

Table 39 - Sensitivity, relative trueness and false positive ratio percentages Kylt® RNA/ DNA Purification profile I

		Level 1	**Level 2**
Sensitivity for the alternative method:	$SE_{alt} = \frac{(PA+PD)}{(PA+PD+ND)} x\ 100\% =$	100%	98.5%
Sensitivity for the reference method:	$SE_{ref} = \frac{(PA+ND)}{(PA+PD+ND)} x\ 100\% =$	100%	100%
Relative trueness	$RT = \frac{(PA+NA)}{N} x\ 100\% =$	98.8%	98.8%
False positive ratio for the alternative method	$FPR = \frac{FP}{NA} x\ 100\% =$	3.7%	0.0%

Table 40 - Sensitivity, relative trueness and false positive ratio percentages Kylt® RNA/ DNA Purification profile II

		Level 1	**Level 2**
Sensitivity for the alternative method:	$SE_{alt} = \frac{(PA+PD)}{(PA+PD+ND)} x\ 100\% =$	100%	98.5%
Sensitivity for the reference method:	$SE_{ref} = \frac{(PA+ND)}{(PA+PD+ND)} x\ 100\% =$	100%	100%
Relative trueness	$RT = \frac{(PA+NA)}{N} x\ 100\% =$	98.8%	98.8%
False positive ratio for the alternative method	$FPR = \frac{FP}{NA} x\ 100\% =$	3.7%	0.0%

4.3.6 Interpretation of data

The negative deviations are listed in Table 41 for Level 1 and in Table 42 for Level 2.

The positive deviations are listed in Table 43 for Levels 1 and 2.

Table 41- Negative deviations for Level 1

No negative deviations were observed in this study at Level 1

Table 42- Negative deviations for Level 2

Category/	**Type**	**Sample n**	**Alternative method results**	**(additional) Confirmatory test results**	**Inoculation (CFU/Sample)**
Extraction mix II profile I – negative deviations = 2					
poultry	Raw poultry	6C4	-ve	n/a	2.0
poultry	Raw poultry	12C4	-ve	n/a	2.0
DNA Extraction-Mix II profile II– negative deviations = 2					
poultry	Raw poultry	6C4	-ve	n/a	2.0
poultry	Raw poultry	12C4	-ve	n/a	2.0
RNA/DNA purification profile I – negative deviations = 1					
poultry	Raw poultry	12C4	-ve	n/a	2.0
RNA/DNA purification profile II – negative deviations = 1					
poultry	Raw poultry	12C4	-ve	n/a	2.0

Table 43 - Positive deviations for Level 1 and 2

No positive deviations were observed in 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 (so L_1 and possibly L_2). The observed value found for (ND – PD) and (ND + PD) shall not be higher than the AL.

For 10 collaborators, the limits are the following:

Table 44 summary for Kylt® DNA Extraction-Mix II profile I

	L1			L2		
	Calculated values	AL	Conclusion	Calculated values	AL	Conclusion
ND - PD	0	3	acceptable	2	3	acceptable
ND + PD	0	4	acceptable	2	4	acceptable

Table 45 summary for Kylt® DNA Extraction-Mix II profile II

	L1			L2		
	Calculated values	AL	Conclusion	Calculated values	AL	Conclusion
ND - PD	0	3	acceptable	2	3	acceptable
ND + PD	0	4	acceptable	2	4	acceptable

Table 46 summary for Kylt® RNA/ DNA Purification profile I

	L1			L2		
	Calculated values	AL	Conclusion	Calculated values	AL	Conclusion
ND - PD	0	3	acceptable	1	3	acceptable
ND + PD	0	4	acceptable	1	4	acceptable

Table 47 summary for Kylt® RNA/ DNA Purification profile II

	L1			L2		
	Calculated values	AL	Conclusion	Calculated values	AL	Conclusion
ND - PD	0	3	acceptable	1	3	acceptable
ND + PD	0	4	acceptable	1	4	acceptable

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

4.3.7 Evaluation of the RLOD between laboratories

The RLOD was calculated using the EN ISO 16140-2:2016 Excel spreadsheet available at http://standards.iso.org/iso/16140 - RLOD (clause 5-1-4-2 Calculation and interpretation of RLOD) version 06.07.2015. The results are used only for information (see Table 47).

Table 48 – RLOD for ILS

RLOD	RLODL	RLODV	b=ln(RLOD)	sd(b)	z Test statistic	p.value	
1.061	0.799	1.409	0.059	0.142	0.415	0.678	Kylt® DNA Extraction-Mix II profile I
1.061	0.799	1.409	0.059	0.142	0.415	0.678	Kylt® DNA Extraction-Mix II profile II
1.038	0.782	1.378	0.037	0.142	0.263	0.792	Kylt® RNA/ DNA Purification profile I
1.038	0.782	1.378	0.037	0.142	0.263	0.792	Kylt® RNA/ DNA Purification profile II

4.3.8 Conclusions on ILS data

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

The observed values for ND-PD and ND+PD are lower than the acceptability limits.

The data and interpretations comply with the EN ISO 16140-2:2016 requirements.

5. CONCLUSION

The **method comparison study conclusions** are:

For 25g samples, the observed values for ND-PD and ND+PD for the individual categories and for all categories meet the acceptability limits (observed values ≤ AL) for the following DNA extraction and PCR profiles.

- Kylt® DNA Extraction-Mix II and run on profile I
- Kylt® DNA Extraction-Mix II and run on profile II
- Kylt® RNA/ DNA Purification and run on profile I*
- Kylt® RNA/ DNA Purification and run on profile II

*Primary production samples should be run with the 5h RVS enrichment step included only.

Analysis of 375g samples revealed that the observed values for ND-PD for the individual categories and for all categories met the acceptability limits (observed values ≤ AL) for the following DNA extraction and PCR profiles for 375g samples.

- Kylt® DNA Extraction-Mix II and run on profile II

The observed values for ND-PD for the individual categories and for all categories did not meet the acceptability limits (observed values ≤ AL) for the following DNA extraction and PCR profiles for 375g samples.

- Kylt® DNA Extraction-Mix II and run on profile I
- Kylt® RNA/ DNA Purification and run on profile I
- Kylt® RNA/ DNA Purification and run on profile II

The RLOD values meet the acceptability limit for 25g environmental and primary production samples, which is 1.5 for paired studies for all DNA extraction and PCR profiles.

In addition, the RLOD values meet the acceptability limit for 375g samples of Raw poultry and ready to cook poultry products and Raw meat and ready to cook meat products, which is 2.5 for unpaired studies for DNA extraction mix II profiles I and II and RNA/ DNA Purification profile I and II.

The alternative Kylt® *Salmonella* spp. 2.0 detection method is selective and specific for the following DNA extraction and PCR profile combinations.

- Kylt® DNA Extraction-Mix II and run on profile 1
- Kylt® DNA Extraction-Mix II and run on profile 2
- Kylt® RNA/ DNA Purification and run on profile 1
- Kylt® RNA/ DNA Purification and run on profile 2

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

The observed values for ND-PD and ND+PD are lower than the acceptability limits.

There are no individual categories tested for the ILS

The data and interpretations comply with the EN ISO 16140-2:2016 requirements.

The Kylt® *Salmonella* spp. 2.0 is considered equivalent to the ISO standard for the following DNA extraction kit and PCR profile combinations for 25g samples.

- Kylt® DNA Extraction-Mix II profile I
- Kylt® DNA Extraction-Mix II profile II
- Kylt® RNA/ DNA Purification profile I
- Kylt® RNA/ DNA Purification profile II

The Kylt® *Salmonella* spp. 2.0 is also considered equivalent to the ISO standard for the following DNA extraction kit and PCR profile combinations for 375g samples of a raw and ready to cook meat and raw and ready to cook Poultry.

- Kylt® DNA Extraction-Mix II and run on profile II

The following Table summarises the final scope of the validation for 7500 FAST

Alternate method	Raw and ready to cook poultry	Raw and ready to cook meat	Environmental	PPS
Kylt® DNA Extraction-Mix II profile I	25g	25g	25g	25g
Kylt® DNA Extraction-Mix II profile II	25g + 375g	25g + 375g	25g	25g
Kylt® RNA/ DNA Purification profile I	25g	25g	25g	25g (after 5h RVS only)
Kylt® RNA/ DNA Purification profile II	25g	25g	25g	25g

The following Table summarises the final scope of the validation for the mutliplatform studies

DNA extraction PCR profile combination	Category	Platforms certified						
		A	B	C	D	E	F	G
Extraction mix II profile I	Meat 25	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Meat 375g	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Poultry 25g	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Poultry 375g	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Environmental	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Primary production	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Primary production 5h RVS broth	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Key A =7500 FAST B = CFX96 C = CFX 384, D = Rotor Gene 3000, E = Rotor Gene 6000, F= Aria MX Pro, G= Light cycler 480

DNA extraction PCR profile combination	Category	Platforms certified						
		A	B	C	D	E	F	G
Extraction mix II profile II	Meat 25g	yes	yes	yes	yes	yes	yes	yes
	Meat 375g	yes	yes	yes	yes	yes	yes	yes
	Poultry 25g	yes	yes	yes	yes	yes	yes	yes
	Poultry 375g	yes	yes	yes	yes	yes	yes	yes
	Environmental	yes	yes	yes	yes	yes	yes	yes
	Primary production	yes	yes	yes	yes	yes	yes	yes
	Primary production 5h RVS broth	yes	yes	yes	yes	yes	yes	yes

Key A =7500 FAST B = CFX96 C = CFX 384, D = Rotor Gene 3000, E = Rotor Gene 6000, F= Aria MX Pro, G= Light cycler 480

DNA extraction PCR profile combination	Category	Platforms certified						
		A	B	C	D	E	F	G
RNA/DNA extraction profile I	Meat 25g	yes	yes	no	yes	yes	yes	no
	Meat 375g	yes	yes	no	yes	yes	yes	no
	Poultry 25g	yes	yes	yes	yes	yes	yes	yes
	Poultry 375g	yes	yes	yes	yes	yes	yes	yes
	Environmental	yes	yes	yes	yes	yes	yes	yes
	Primary production	yes	yes	yes	yes	yes	yes	yes
	Primary production 5h RVS broth	yes	yes	yes	yes	yes	yes	yes

Key A =7500 FAST B = CFX96 C = CFX 384, D = Rotor Gene 3000, E = Rotor Gene 6000, F= Aria MX Pro, G= Light cycler 480

DNA extraction PCR profile combination	Category	Platforms certified						
		A	B	C	D	E	F	G
RNA/DNA extraction profile II	Meat 25g	yes	yes	yes	no	yes	yes	no
	Extraction mix II profile I	yes	yes	yes	no	yes	yes	no
	Poultry 25g	yes	yes	yes	yes	yes	yes	yes
	Poultry 375g	yes	yes	yes	yes	yes	yes	yes
	Environmental	yes	yes	yes	yes	yes	yes	yes
	Primary production	yes	yes	yes	yes	yes	yes	yes
	Primary production 5h RVS broth	yes	yes	yes	yes	yes	yes	yes

Key A =7500 FAST B = CFX96 C = CFX 384, D = Rotor Gene 3000, E = Rotor Gene 6000, F= Aria MX Pro, G= Light cycler 48

Date, 12 February 2022

Signature Suzanne Jordan

Dr. Suzanne Jordan, Campden BRI

Annexes

- ANNEX A: Flow diagram of the reference method
- ANNEX A2 Workflow for ISO 6579-1 (2017) for the analysis of primary production samples
- ANNEX B1: Flow diagram of the alternative method - Work flow for Kylt® detection kit for the analysis of meat, poultry and environmental samples
- ANNEX B2: Work flow for Kylt® detection kit for the analysis of primary production samples

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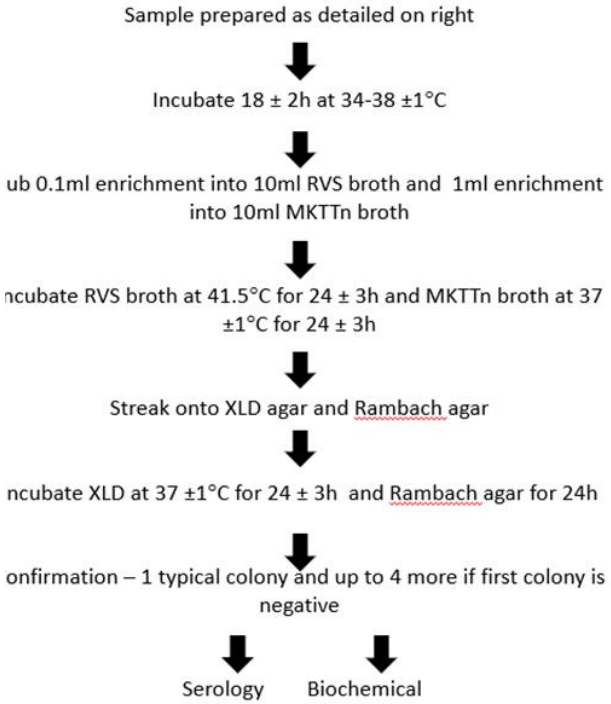
ANNEX A: Flow diagram of the reference method

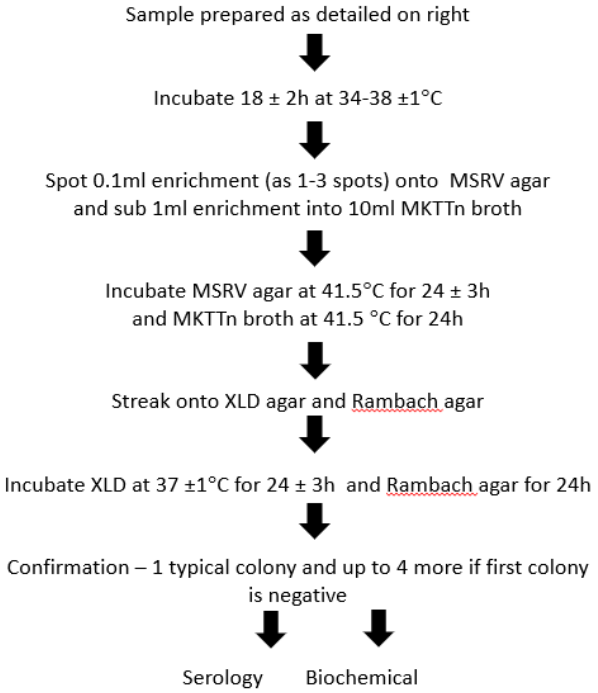
A 1 Workflow for ISO 6579-1 (2017) for the analysis of meat and poultry and environmental samples



Sample preparation

Sample type	Appropriate part of ISO 6887 to be used	Preparation needed
All food products within the category Raw meat and ready to cook meat products, Raw poultry and ready to cook poultry products	1 and 2	Add 225 ml BPW to 25g samples or 3375ml BPW to 375g samples
Carcass swabs	1 and 2 and ISO 18593	Add 100ml of diluent and make sure that the whole sample is submerged in BPW. Make sure that the whole sample is submerged. Mix/ shake well before enrichment
Process water, cleaning water cattle wash water	1	Samples <100ml added to an equal volume of double strength BPW
Dust wipe samples	1 and ISO 18593	Make sure that the whole sample is submerged in BPW. Mix/ shake well before enrichment
Sponge samples	1 and ISO 18593	Make sure that the whole sample is submerged in BPW. Mix/ shake well before enrichment
Dust and residue swabs	1	Add swab to 50ml tube containing 40ml BPW and make sure that the whole sample is submerged. Mix/ shake well before enrichment
Swabs stainless steel, plastic surface, ceramic and rubber	6	Add swab to 50ml tube containing 40ml BPW and make sure that the whole sample is submerged. Mix/ shake well before enrichment

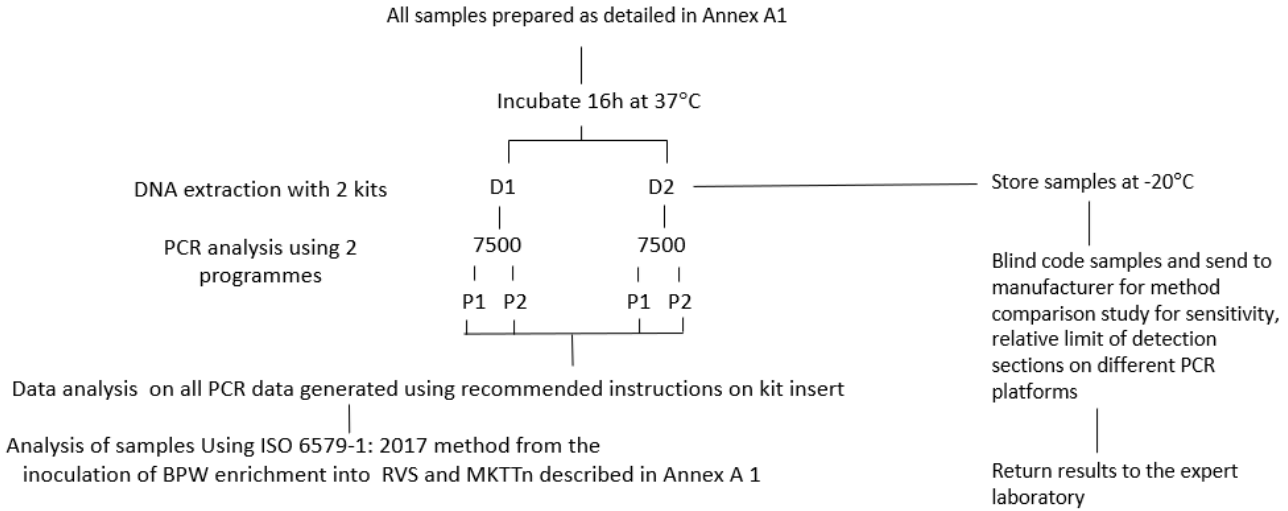
Annex A 2 Workflow for ISO 6579-1 (2017) for the analysis of primary production samples



Sample preparation

Sample type	Appropriate part of ISO 6887 to be used	Preparation needed
Rectal swabs	6	Add swab to 50ml tube containing 40ml BPW and make sure that the whole sample is submerged. Mix/ shake well before enrichment
Faeces pig, chicken and turkey, as well as gut contents	6	Mix gently and add 225ml BPW to 25g samples
Bootsocks	6	Add at least 225ml BPW and make sure that the whole sample is submerged
Hatchery samples – basket liners	6	Samples should be at least 1m surface area. Add 1 to 2L of BPW (pre- warmed to at least room temperature, but preferably 37°C)
Transport truck sampling, waiting area swab, transport cage debris	6	Add at least 225ml BPW and make sure that the whole sample is submerged. Mix/ shake well before enrichment
Organs lymph nodes	6	Macerate lymph nodes by hammering a strong sterile plastic bag containing the samples. Add 9ml per g of sample

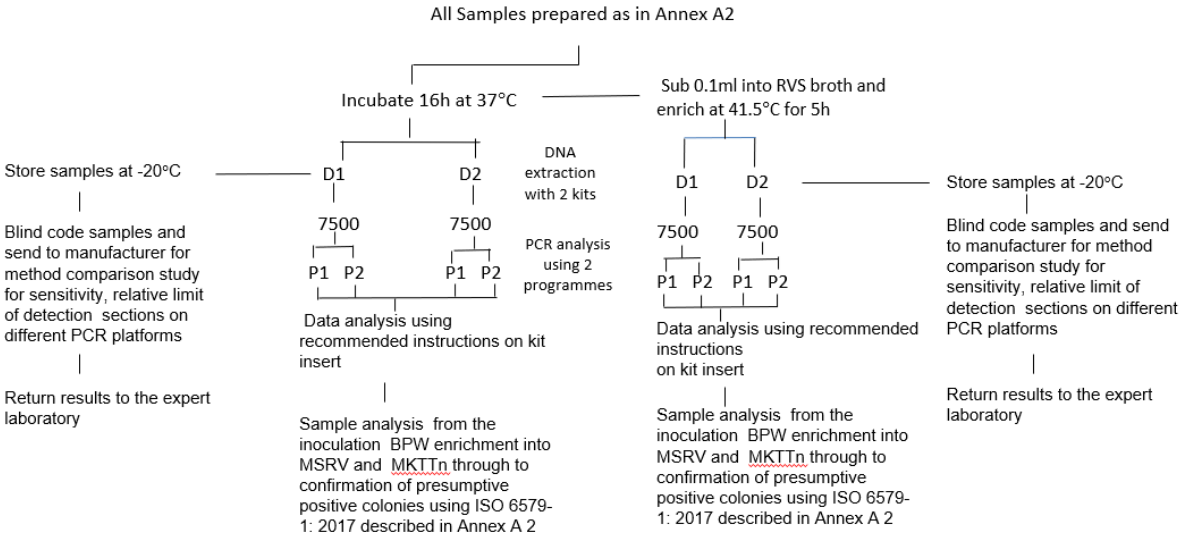
ANNEX B1: Flow diagram of the alternative method - Work flow for Kylt® detection kit for the analysis of meat, poultry and environmental samples



Key
D1 Kylt® DNA Extraction-Mix II
D2 Kylt ® RNA/DNA Purification
7500 : Applied Biosystems 7500 FAST
P1 PCR programme 1
P2 PCR programme 2

Nb. The incubation tolerance for the enrichment in BPW ISO is 18h ±2h at 37 ±1°C, however the incubation of the samples in the study was done at the shortest time of 16h

ANNEX B2: **Work flow for Kylt® detection kit for the analysis of primary production samples**



Key
D1 Kylt® DNA Extraction-Mix II
D2 Kylt ® RNA/DNA Purification
7500: Applied Biosystems 7500 FAST
P1 PCR programme 1
P2 PCR programme 2