Method Comparison Study Report for the ISO 16140-2:2016 validation of validation of Neogen One plate for *Listeria* (OP-L), for the enumeration of *L. monocytogenes* and *Listeria* spp. in a broad range of foods

MicroVal study number: 2019 LR89

Method/Kit name: One plate for *Listeria* (OP-L)

Report version:Summary report

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Foreword

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

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Method/Kit name: Neogen One plate for *Listeria* (OP-L)

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 and enumeration of *Listeria monocytogenes and of Listeria spp.*

Part 2: Enumeration method (ISO 11290-2:2017)

Scope of validation: Broad range of foods covering

- Meat and poultry products (raw and RTE)
- Dairy products
- Fresh produce and fruit
- Seafood & Fishery products
- Multicomponent foods

Certification orgnization: Lloyd's Register

List of abbreviations

-	AL	Acceptability Limit
-	AP	Accuracy Profile
-	Art. Cont.	Artificial contamination
-	CFU	Colony Forming Units
-	CL	confidence limit (usually 95%)
-	EL	Expert Laboratory
-	$\bar{D}$	Average difference
-	g	Gram
-	h	Hour
-	ILS	Interlaboratory Study
-	Inc/Ex	Inclusivity and Exclusivity
-	LOQ	Level of Quantification
-	MCS	Method Comparison Study
-	min	minute
-	ml	Millilitre
-	MR	(MicroVal) Method Reviewer
-	MVTC	MicroVal Technical Committee
-	EL	Expert Laboratory
-	n	number of samples
-	na	not applicable
-	neg	negative (target not detected)
-	NG	no growth
-	nt	not tested
-	RT	Relative Trueness
-	SD	standard deviation of differences
-	10^{-1} dilution	10-fold dilution of original food
-	10^{-2} dilution	100-fold dilution of original food

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1 Introduction

In this project a MicroVal validation study, based on ISO 16140-2:2016, of alternative method(s) for the enumeration of 2 different targets - *Listeria monocytogenes* and *Listeria* spp. in 5 different food categories was carried out by Campden BRI as the MicroVal Expert Laboratory.

The alternative method used was: Neogen One plate Listeria (OP-L). This is a chromogenic medium for the detection and enumeration of *Listeria* spp. and *L. monocytogenes*. Characteristic colonies of *L. monocytogenes* appear blue to blue-green and are surrounded by an opaque halo. Characteristic colonies of *Listeria* spp appear blue to blue-green with or without an opaque halo. This method has the option to enumerate the target organsism in different plating formats – 1ml pour plate and 0.1ml spread plate.

The reference method used is: Microbiology of the food chain — Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria spp.* Part 2: Enumeration method (ISO 11290-2:2017)

Scope of the validation study was: A broad range of foods

Categories included:

- Meat and poultry products (raw and RTE)
- Dairy products
- Fresh produce and fruit
- Seafood & Fishery products
- Multicomponent foods

Criteria evaluated during the study have been:

- Relative trueness study;
- Accuracy profiles;
- Limits of quantification (LOQ);
- Inclusivity and exclusivity
- ILS

The final conclusion on the Method Comparison study and ILS is summarized below:

The alternative method Neogen One plate Listeria (OP-L) shows comparable performance to the reference method ISO 11290-2:2017 for the enumeration of *Listeria monocytogenes* and *Listeria* spp. in a broad range of foods.

Overall, the conclusions for the Method Comparison Study and ILS are:

- The alternative method One plate OP-L (pour and spread plate format) enumeration method for *L. monocytogenes* and *Listeria* spp. shows satisfactory results for relative trueness.

- The alternative One plate OP-L (pour and spread plate format) enumeration for *L. monocytogenes* and *Listeria* spp. shows satisfactory results for accuracy profile.

- The alternative One plate OP-L enumeration method is selective and specific to *Listeria monocytogenes* and *Listeria* spp. with both the pour and spread plate formats.

2 Method protocols

The Method Comparison Study was carried out using 10 gram portions of sample material.

According to ISO 16140-2 the reference method and alternative methods were performed with, as far as possible , exactly the same sample.

2.1 Reference method

See the flow diagram in Annex A. Each sample was plated using 2 different volumes; 1ml from a single dilution was spread onto 3 plates to increase the sensitivity and 0.1ml spread plates carried out from 2 consecutive dilutions.

Sample preparations used in the reference method and the alternative method were done according to ISO 6887-series for all sample matrices in this proposal.

2.2 Alternative method

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

A summary of the protocol is outlined below.

The alternative method principle is based on chromagenic detection of *L.monocytogenes* and *Listeria* spp following the ISO 11290-1&11290-2 Ottaviani and Agosti agar formula. The agar can be used as a surface plating technique or a pour plating technique.

2.3 Study design

Samples of product containing the target organism were diluted 1 in 10 with buffered peptone water (BPW) and homogenised in a stomacher. Appropriate serial dilutions were made and all relevant dilutions were analysed using the reference method and alternative method.

3 Method comparison study

3.1 Relative trueness study

The trueness study is a comparative study between the results obtained by the reference method and the results of the alternative method. This study was conducted using naturally or artificially contaminated samples. Different categories, types and items were tested for this.

A total of 5 categories were included in this validation study. A minimum of 15 items for each category were tested by both the reference method and the alternative method in the relative trueness study, with a minimum of 15 interpretable results per category.

Each category was made up of 3 types, with at least 5 items representative for each type.

3.1.1 Number of samples

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

Table 1 – Categories, types and number of samples analysed L. monocytogenes 1ml OPL pour plate and 0.1ml OPL spread plate and Listeria spp. 1ml OPL pour plate and 0.1ml OPL spread plate

Category	**Type**		**Number of *L. monocytogenes* Samples analysed**	**Number of *Listeria* spp samples analysed**	**Preparation**	**ISO 6887 used**
Meat and poultry products (Raw and RTE)	a	Fresh meats (unprocessed)	5	5	BPW ISO @ room temperature	6887-2
	b	Ready to cook (processed)	5	5		6887-2
	c	Ready to eat and Ready to reheat products	5	5		6887-2
		Total	15	15		
Multicomponent foods or meal components	a	Composite foods with subtsantial raw ingredinets (excluding patisserie)	5	5	BPW ISO @ room temperature	6887-2
	b	Composite processed foods (cooked)	5	5		6887-2
	c	Mayonnaise based deli salads	5	5		6887-2
		Total	15	15		
Dairy products (pasteurised and raw)	a	Pasteurised dairy products	5	5	BPW ISO @ room temperature	6887-5
	b	Pasteruised milk based products	5	5		6887-5
	c	Raw milk products	5	5		6887-5
		Total	15	15		
Fresh produce and fruits	a	Ready to eat fruit	5	5	BPW ISO @ room temperature	6887-2
	b	Cut ready to eat vegetables/sprouts	5	5		6887-2
	c	Leafy greens	5	5		6887-2
		Total	15	15		
Seafood & Fishery products	a	Unprocessed	5	5	BPW ISO @ room temperature	6887-3
	b	RTE	5	5		6887-3
	c	Processed RTC	5	5		6887-3
		Total	15	15		
Total			75	75		

75 samples were analysed, leading to 75 exploitable results for both target organsims.

3.1.2 Test sample preparation

No naturally contaminated samples were found in pre-screening studies. It was therefore necessary to use artificial contamination procedures. Artificial procedures used a range of seeding protocols and strains in order to examine a wide range of different conditions.

Artificial contaminations were obtained by seeding with strains isolated from the same samples type, before storage for 48 h to 72h at 4°C or at -20°C for 72 h to 18 days, with lyophilised strains.

The same strain was not used to inoculate more than 5 samples.

Samples were inoculated with either *L. monocytogenes or Listeria spp.*strains before storage of the inoculated samples, e.g. frozen foods were stored for at least 2 weeks at -20 °C, perishable foods were stored for at least 48 h at 2 – 8 °C, and shelf stable foods were stored for at least 2 weeks at room temperature.

15 *L. monocytogenes* isolates and 15 *Listeria spp* strains were used for artificial inoculations. These cultures preferably originated from comparable sample types as the ones to be inoculated. Each particular strain was used to contaminate up to 5 different items.

Inoculation of samples was generally at the range usually associated with the test organisms and within the capabilities of the test methods, covering the range 10^2cfu/g to 10^6cfu/g.

In accordance with ISO 16140-2, a minimum of 15 items for each category were tested by both the reference method and the alternative method in the relative trueness study, made up of at least three types with at least 5 interpretable results per type.

All results were tabulated, calculated and interpreted according to ISO 16140-2.

3.1.3 Protocols applied during the validation study.

Incubation time

The incubation time for the alternative method was 48h at 37°C.

Confirmations if required for the alternative method

Confirmations were carrried out by streaking presumptive positve colonies purified on TSAYE and incubated at 37°C ±1°C aerobically for 24h ±2h. After purification, the colonies were analysed by MALDI ToF with the Maldi Biotyper complete solution (Bruker Daltonik GmbH) with the microflex LT/SH MALDI-MS system.

3.1.4 Test results

The results are split into the 2 target organisms *L. monocytogenes* and *Listeria* spp and further divided into the 2 plating formats used in the validation 1ml pour plates and 0.1ml spread plates.

The samples were analysed by the reference and the alternative methods in order to have 15 interpretable results per incubation protocol, and 5 interpretable results per tested type.

3.1.5 Calculation and interpretation of relative trueness study

The obtained data were analyzed using the scatter plot. The graphs are provided with the line of identity (y = x).

3.1.5.1 L. monocytogenes 1ml OPL pour plate

Figure 1 shows the scatter plot for *L. monocytgenes* in Meat and poultry products with OPL pour plates.
Figure 2 shows the scatter plot for *L. monocytgenes* in Multicomponent foods or meal components with OPL pour plates.
Figure 3 shows the scatter plot for *L. monocytgenes* in Dairy products with OPL pour plates.
Figure 4 shows the scatter plot for *L. monocytgenes* in Fresh produce and fruits with OPL pour plates.
Figure 5 shows the scatter plot for *L. monocytgenes* in Seafood & Fishery products with OPL pour plates.
The Figure 6 shows the scatter plot for all the categories for *L. monocytgenes* plated onto OPL pour plates.

Figure 1 - Scatter plot of the reference method versus alternative method results for L. monocytogenes in Meat and poultry products with 1ml OPL pour plates.

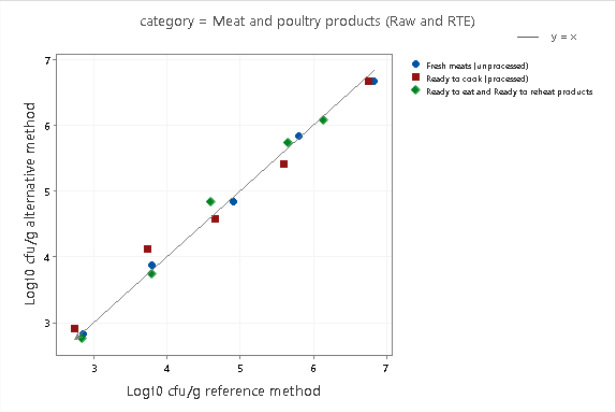


Figure 2- Scatter plot of the reference method versus alternative method results for the L. monocytogenes in Multicomponent foods or meal components with 1ml OPL pour plates.

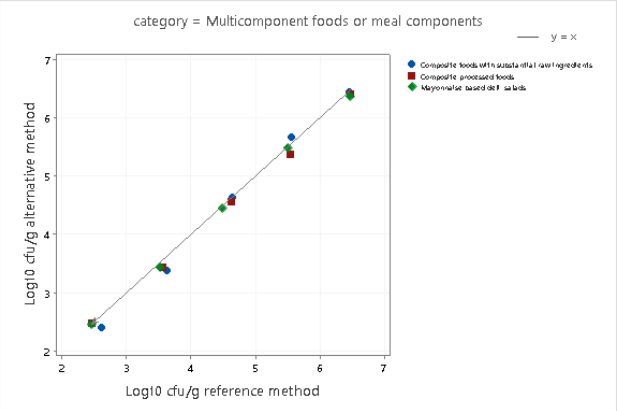


Figure 3- Scatter plot of the reference method versus alternative method results for L. monocytogenes in dairy products with 1ml OPL pour plates.

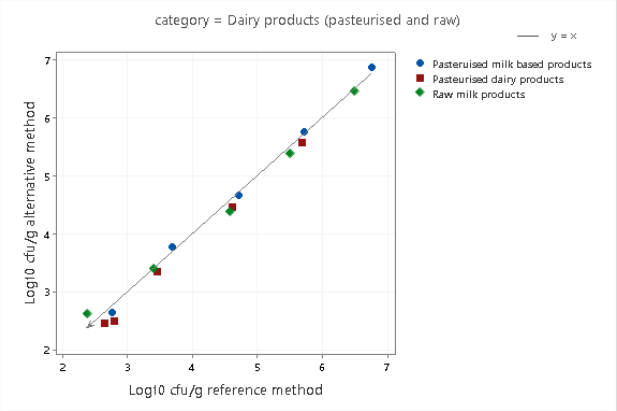


Figure 4- Scatter plot of the reference method versus alternative method results for L. monocytogenes in Fresh produce and fruits with 1ml OPL pour plates.

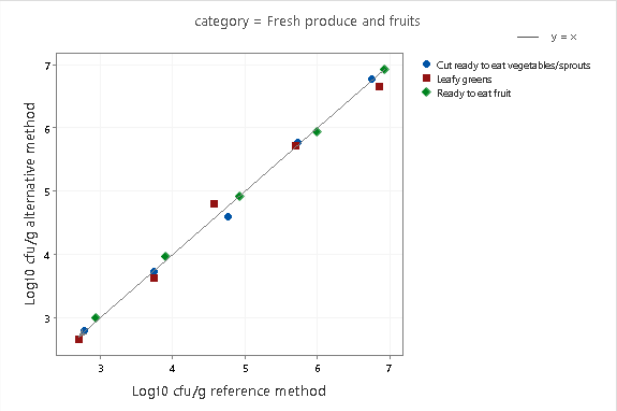


Figure 5- Scatter plot of the reference method versus alternative method results for L. monocytogenes in Seafood & Fishery products with 1ml OPL pour plates.

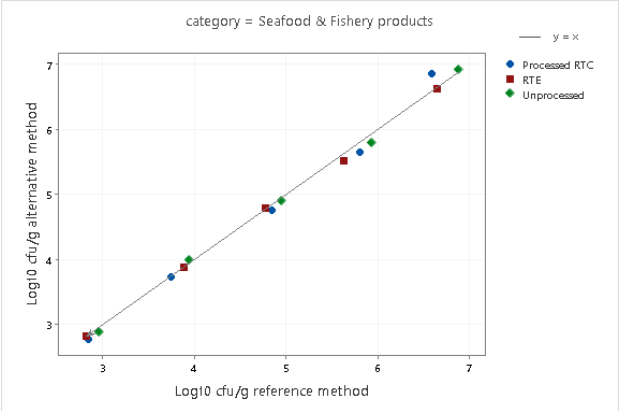
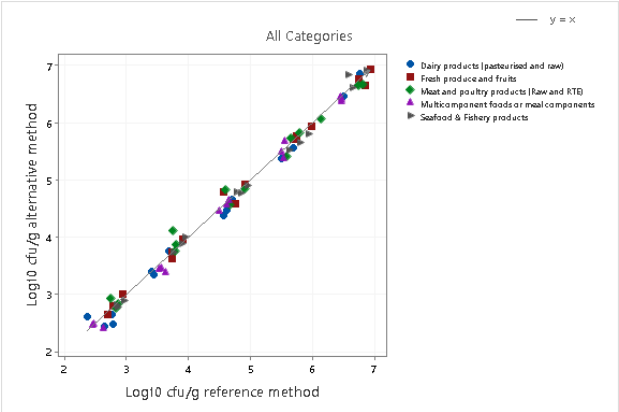


Figure 6 - Scatter plot of the reference method versus alternative method results for all the categories for L. monocytogenes plated onto 1ml OPL pour plates.



According to ISO16140-2:2016 6.1.2.3, the results of the scatter plot are interpreted on the visual observation of the amount of bias and extreme results. The scatter plots for 1ml OPL pour plates for *L. monocytogenes* show good agreement between the reference method and alternative method.

There are no obvious disagreements between the two methods and no real bias was observed. This is further described in the Bland Altman plot analysis in Figure 7.

A summary of the calculated values per category is provided in Table 2

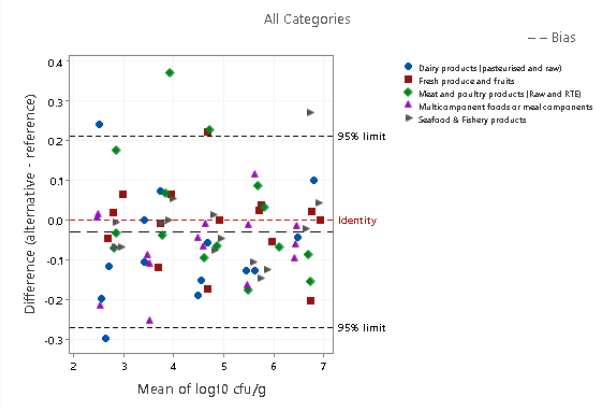
Table 2 - Summary of the calculated values per category L. monocytogenes 1ml OPL pour plates.

Category	n	$\bar{D}$	SD	95 % low limit	95 % upper limit
Dairy products (pasteurised and raw)	15	-0.063	0.136	-0.365	0.238
Fresh produce and fruits	15	-0.009	0.103	-0.238	0.220
Meat and poultry products (Raw and RTE)	15	0.013	0.152	-0.323	0.349
Multicomponent foods or meal components	15	-0.067	0.094	-0.275	0.142
Seafood & Fishery products	15	-0.018	0.100	-0.239	0.202
All products	75	-0.029	0.120	-0.270	0.212

$\bar{D}$: Average difference SD: standard deviation of differences n:number of samples

The Bland-Altman difference plot for all the samples is given Figure 7.

Figure 7 – Bland-Altman difference plot for all the samples L. monocytogenes 1ml OPL pour.



Samples for which the difference between the result observed with the reference and the alternative methods is above or lower than the limits are listed in the Table 3.

Table 3 - Data which are outside of the accepted limits L.monocytogenes 1ml OPL pour.

Category	Type	N° Sample	Reference method Log cfu/g	Alternative method Log cfu/g	Mean Log cfu/g	Difference Alt – ref	Lower / Upper limits
Dairy products (pasteurised and raw)	Pasteurised dairy products	T32	2.785	2.490	2.638	-0.295	-0.270
Dairy products (pasteurised and raw)	Raw milk products	T41	2.371	2.613	2.492	0.242	0.212
Meat and poultry products (Raw and RTE)	Ready to cook (processed)	T7	3.740	4.114	3.927	0.374	0.212
Fresh produce and fruits	Leafy greens	T58	4.568	4.792	4.680	0.224	0.212
Meat and poultry products (Raw and RTE)	Ready to eat and Ready to reheat products	T13	4.602	4.833	4.717	0.230	0.212
Seafood & Fishery products	Processed RTC	T75	6.580	6.851	6.716	0.271	0.212

The Bland Altman showed good agreement between the Reference method and the Alternative method. There were 6 data points from a total of 75 data points which

were outside of the accepted limits. However, all of these were <0.4log difference and covered 4 different categories. The overall bias between the methods was -0.029.

3.1.5.2 L. monocytogenes 0.1ml OPL spread

Figure 8 shows the scatter plot for *L. monocytgenes* in Meat and poultry products with 0.1ml OPL spread

Figure 9 shows the scatter plot for *L. monocytgenes* in Multicomponent foods or meal components with 0.1ml OPL spread plates

Figure 10 shows the scatter plot for *L. monocytgenes* in Dairy products with 0.1ml OPL spread plates

Figure 11 shows the scatter plot for *L. monocytgenes* in Fresh produce and fruits with 0.1ml OPL spread plates

Figure 12 shows the scatter plot for *L. monocytgenes* in Seafood & Fishery products with 0.1ml OPL spread plates

The Figure 13 shows the scatter plot for all the categories for *L. monocytgenes* plated onto 0.1ml OPL spread plates.

Figure 8 - Scatter plot of the reference method versus alternative method results for L. monocytogenes in Meat and poultry products with 0.1ml OPL spread plates.

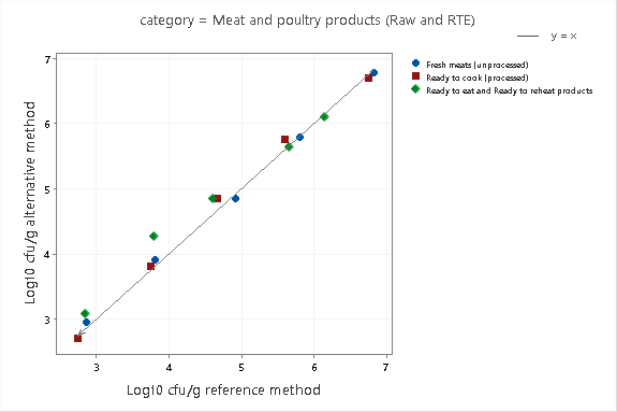


Figure 9- Scatter plot of the reference method versus alternative method results for the L. monocytgenes in Multicomponent foods or meal components with 0.1ml OPL spread plates.

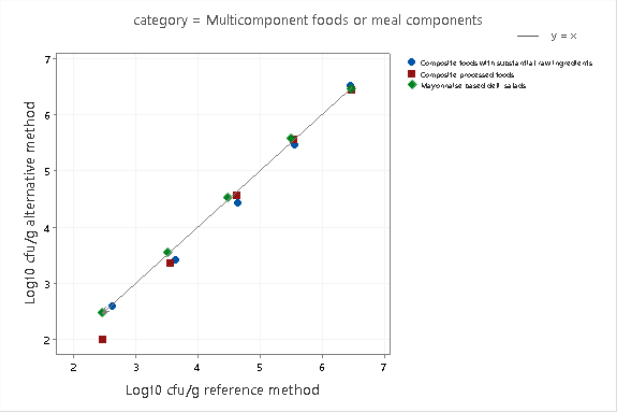


Figure 10- Scatter plot of the reference method versus alternative method results for L. monocytgenes in dairy products with 0.1ml OPL spread plates.

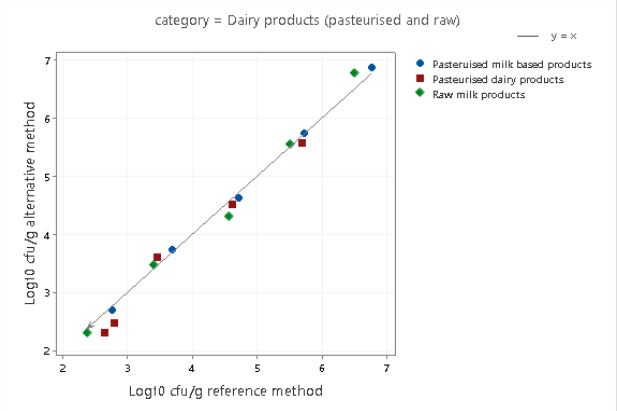


Figure 11- Scatter plot of the reference method versus alternative method results for L. monocytogenes in Fresh produce and fruits with 0.1ml OPL spread plates.

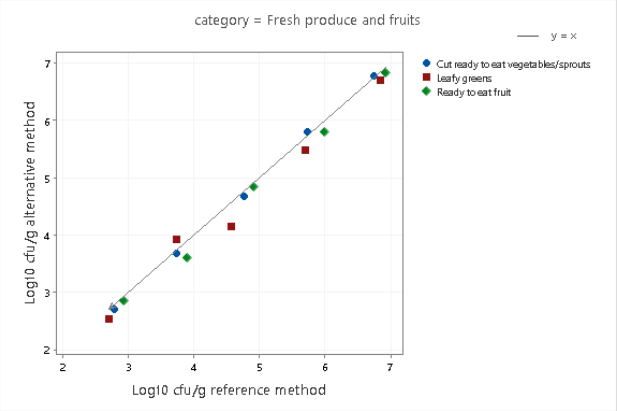


Figure 12- Scatter plot of the reference method versus alternative method results for L. monocytogenes in Seafood & Fishery products with 0.1ml OPL spread plates.

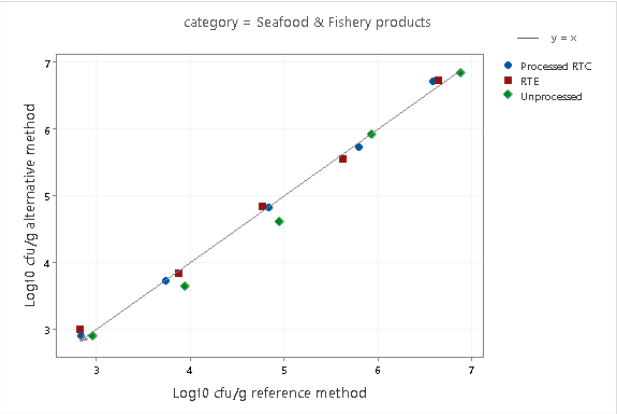
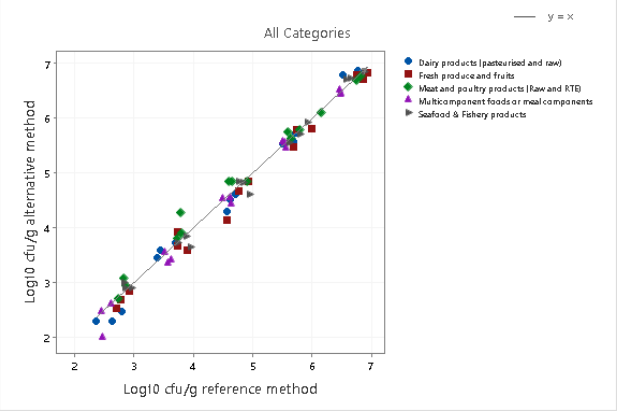


Figure 13 - Scatter plot of the reference method versus alternative method results for all the categories for L. monocytogenes plated onto 0.1ml OPL spread plates.



According to ISO16140-2:2016 6.1.2.3, the results of the scatter plot are interpreted on the visual observation of the amount of bias and extreme results. The scatter plots for 1ml OPL pour plates for *L. monocytogenes* show good agreement between the reference method and alternative method.

There are no obvious disagreements between the two methods and no real bias was obsevred. This is further described in the Bland Altman plot analysis in Figure 14.

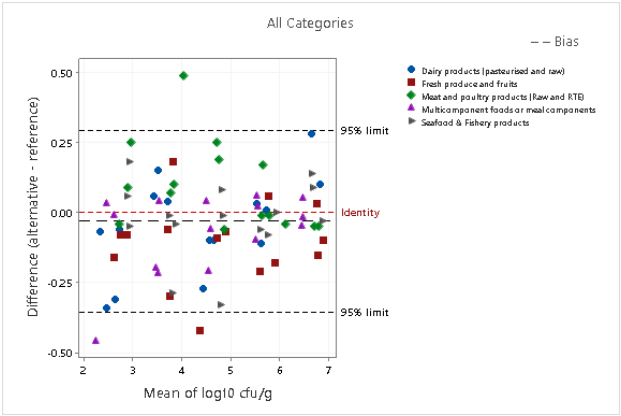
Table 4 - Summary of the calculated values per category L. monocytogenes OPL spread.

Category	n	$\overline{D}$	SD	95 % low limit	95 % upper limit
Dairy products (pasteurised and raw)	15	-0.046	0.171	-0.425	0.333
Fresh produce and fruits	15	-0.109	0.144	-0.428	0.210
Meat and poultry products (Raw and RTE)	15	0.090	0.157	-0.257	0.437
Multicomponent foods or meal components	15	-0.073	0.144	-0.392	0.247
Seafood & Fishery products	15	-0.023	0.139	-0.331	0.284
All products	75	-0.032	0.162	-0.357	0.293

$\overline{D}$: Average difference SD: standard deviation of differences n:number of samples

The Bland-Altman difference plot for all the samples is given Figure 14.

Figure 14 – Bland-Altman difference plot for all the samples L. monocytogenes 0.1ml OPL spread.



Samples for which the difference between the result observed with the reference and the alternative methods is above or lower than the limits are listed in the Table 5.

Table 5 - Data which are outside of the accepted limits L. monocytogenes 0.1ml OPL spread.

Category	Type	N° Sample	Reference method Log cfu/g	Alternative method Log cfu/g	Mean Log cfu/g	Difference Alt – ref)	Lower / Upper limits
Meat and poultry products (Raw and RTE)	Ready to eat and Ready to reheat products	T12	3.79	4.28	4.03	0.49	0.322
Multicomponent foods or meal components	Composite processed foods	T21	2.46	2.00	2.23	-0.46	-0.402
Fresh produce and fruits	Leafy greens	T58	4.57	4.15	4.36	-0.42	-0.402

The Bland Altman showed good agreement between the Reference method and the Alternative method. There were 3 data points from a total of 75 data points which were outside of the accepted limits representing 3 different categories. The overall bias between the methods was -0.032.

3.1.5.3 *Listeria* spp OPL 1mL pour.

Figure 15 shows the scatter plot for *Listeria* spp. in Meat and poultry products with 1ml OPL pour plates.

Figure 16 shows the scatter plot for *Listeria* spp. in Multicomponent foods or meal components with 1ml OPL pour plates.

Figure 157shows the scatter plot for *Listeria* spp. in Dairy products with1ml OPL pour plates.

Figure 18 shows the scatter plot for *Listeria* spp. in Fresh produce and fruits with 1ml OPL pour plates.

Figure 19 shows the scatter plot for *Listeria* spp. in Seafood & Fishery products with 1ml OPL pour plates.

The Figure 20 shows the scatter plot for all the categories for *Listeria* spp. *plated onto OPL pour plates*.

Figure 15 - Scatter plot of the reference method versus alternative method results for Listeria spp. in Meat and poultry products with 1ml OPL pour plates.

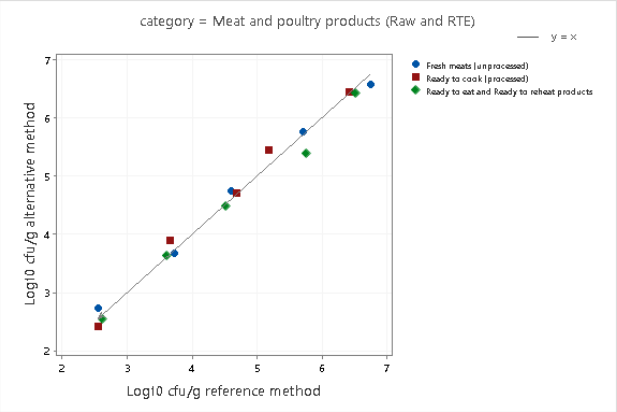


Figure 16- Scatter plot of the reference method versus alternative method results for Listeria spp. in Multicomponent foods or meal components with 1ml OPL pour plates.

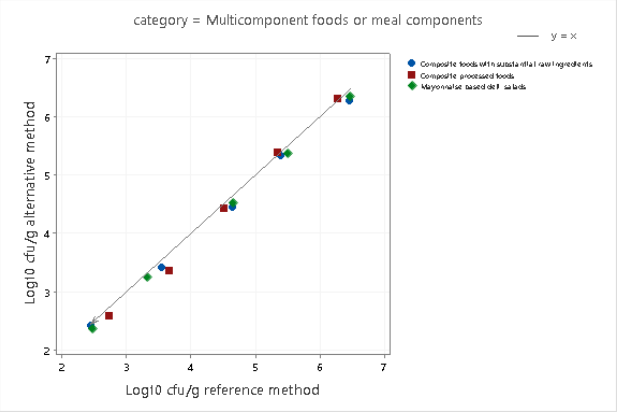


Figure 17- Scatter plot of the reference method versus alternative method results for Listeria spp. in Dairy products with 1ml OPL pour plates.

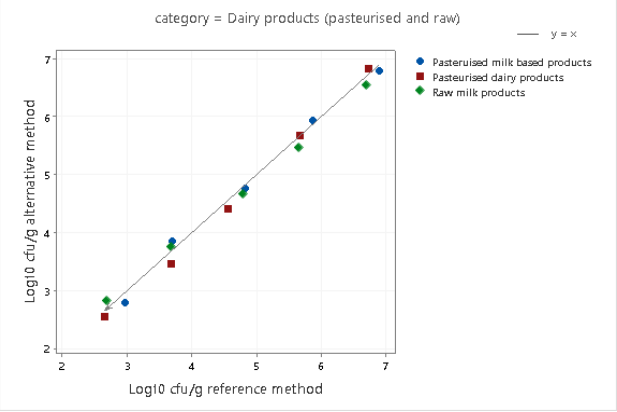


Figure 18- Scatter plot of the reference method versus alternative method results for Listeria spp. in Fresh produce and fruits with 1ml OPL pour plates.

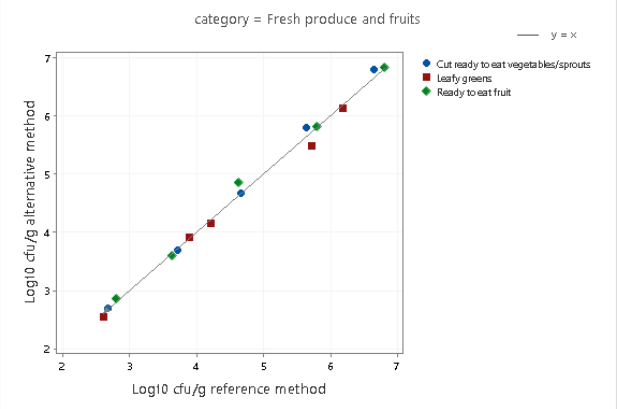


Figure 19- Scatter plot of the reference method versus alternative method results for Listeria spp. in Seafood & Fishery products with 1ml OPL pour plates.

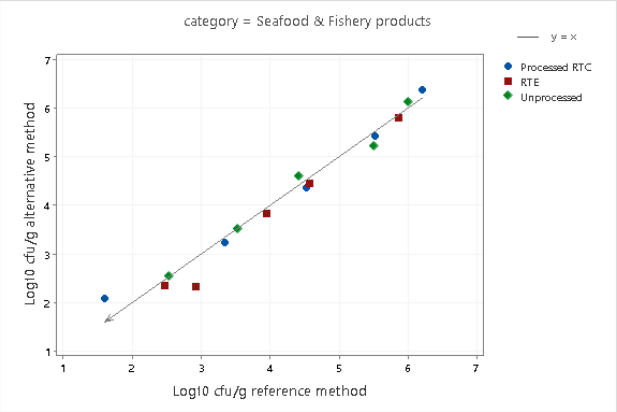
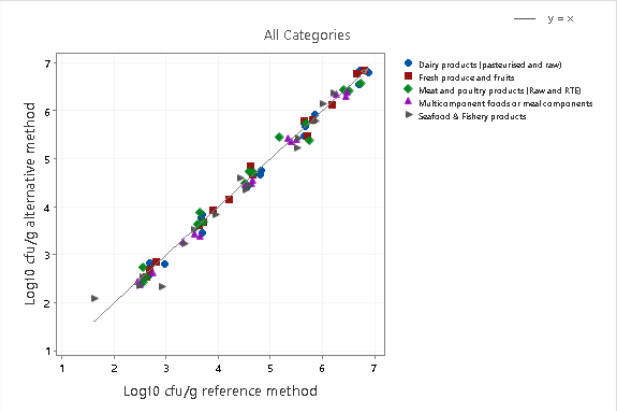


Figure 20 - Scatter plot of the reference method versus alternative method results for all the categories for Listeria spp. plated onto 1ml OPL pour plates.



According to ISO16140-2:2016 6.1.2.3, the results of the scatter plot are interpreted on the visual observation of the amount of bias and extreme results. The scatter plots for 1ml OPL pour plates for *L. monocytogenes* show good agreement between the reference method and alternative method.

There are no obvious disagreements between the two methods and no real bias was observed. This is further described in the Bland Altman plot analysis in Figure 21.

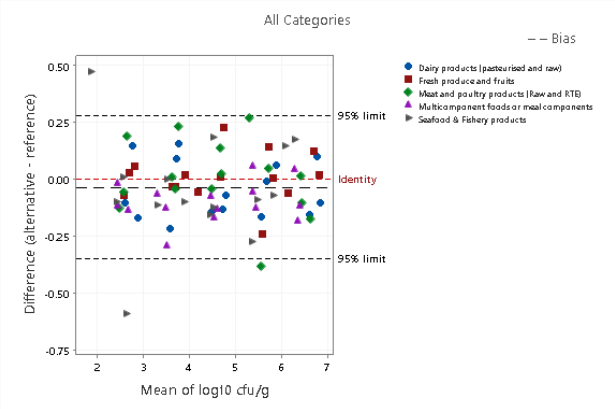
Table 6 - Summary of the calculated values per category Listeria spp 1ml OPL pour.

Category	n	$\bar{D}$	SD	95 % low limit	95 % upper limit
Dairy products (pasteurised and raw)	15	-0.047	0.127	-0.328	0.234
Fresh produce and fruits	15	0.010	0.108	-0.229	0.248
Meat and poultry products (Raw and RTE)	15	0.000	0.168	-0.373	0.373
Multicomponent foods or meal components	15	-0.101	0.088	-0.296	0.094
Seafood & Fishery products	15	-0.041	0.239	-0.570	0.488
All products	75	-0.036	0.156	-0.349	0.278

$\bar{D}$: Average difference SD: standard deviation of differences n:number of samples

The Bland-Altman difference plot for all the samples is given Figure 21.

Figure 21 – Bland-Altman difference plot for all the samples Listeria spp 1ml OPL pour.



Samples for which the difference between the result observed with the reference and the alternative methods is above or lower than the limits are listed in the Table 7.

Table 7 - Data which are outside of the accepted limits Listeria spp OPL pour plate.

Category	Type	N° Sample	Reference method Log cfu/g	Alternative method Log cfu/g	Mean Log cfu/g	Difference Alt – ref)	Lower / Upper limits
Seafood & Fishery products	Processed RTC	T146	1.60	2.08	1.84	0.48	0.278
Seafood & Fishery products	RTE	T142	2.91	2.32	2.62	-0.59	-0.349
Meat and poultry products (Raw and RTE)	Ready to eat and Ready to reheat products	T89	5.76	5.38	5.57	-0.38	-0.349

Comments

The Bland Altman showed good agreement between the Reference method and the Alternative method. There were 3 data points from a total of 75 data points which were outside of the accepted limits. However, all of these were <0.6log difference and covered 2 different categories.

3.1.5.4 *Listeria* spp. OPL 0.1mL spread

Figure 22 shows the scatter plot for *Listeria* spp. in Meat and poultry products with 0.1ml OPL spread plates.

Figure 23 shows the scatter plot for *Listeria* spp. in Multicomponent foods or meal components with 0.1ml OPL spread plates.

Figure 24 shows the scatter plot for *Listeria* spp. in Dairy products with 0.1ml OPL spread plates.

Figure 25 shows the scatter plot for *Listeria* spp. in Fresh produce and fruits with 0.1ml OPL spread plates.

Figure 26 shows the scatter plot for *Listeria* spp. in Seafood & Fishery products with 0.1ml OPL spread plates.

The Figure 27 shows the scatter plot for all the categories for *Listeria* spp. *plated onto 0.1ml OPL spread plates*.

Figure 22 - Scatter plot of the reference method versus alternative method results for Listeria spp. in Meat and poultry products with 0.1ml OPL spread plates.

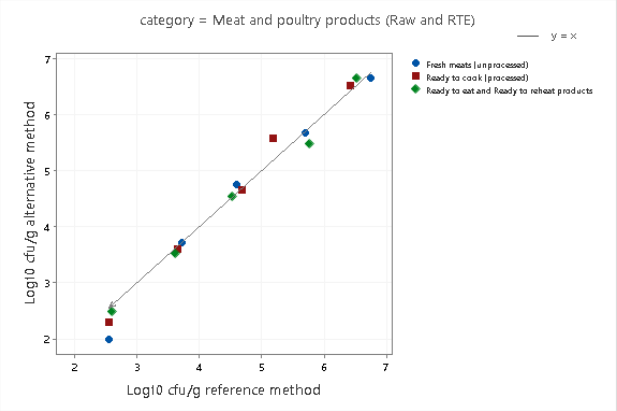


Figure 23- Scatter plot of the reference method versus alternative method results for the Listeria spp. in Dairy products with 0.1ml OPL spread plates.

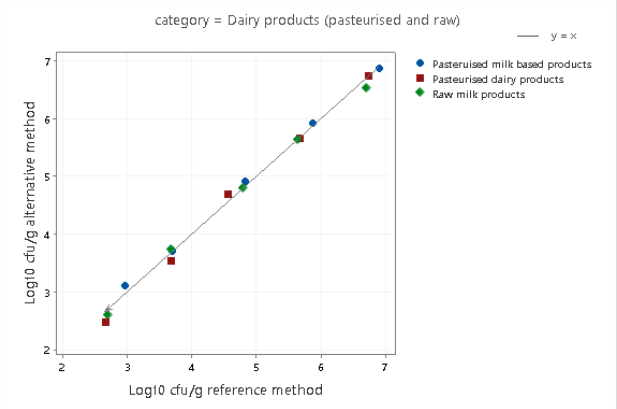


Figure 24- Scatter plot of the reference method versus alternative method results for Listeria spp. in Multicomponent foods or meal components with 0.1ml OPL spread plates.

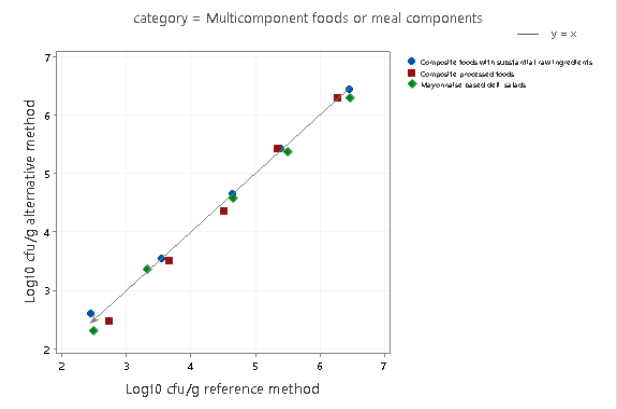


Figure 25- Scatter plot of the reference method versus alternative method results for Listeria spp in fresh produce and fruits with 0.1ml OPL spread plates.

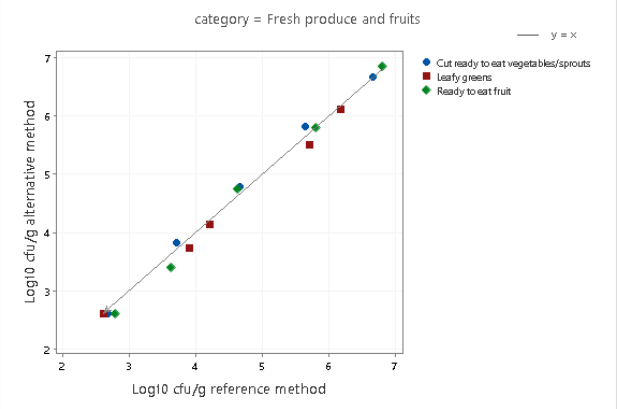


Figure 26- Scatter plot of the reference method versus alternative method results for Listeria spp. in Seafood & Fishery products with 0.1ml OPL spread plates.

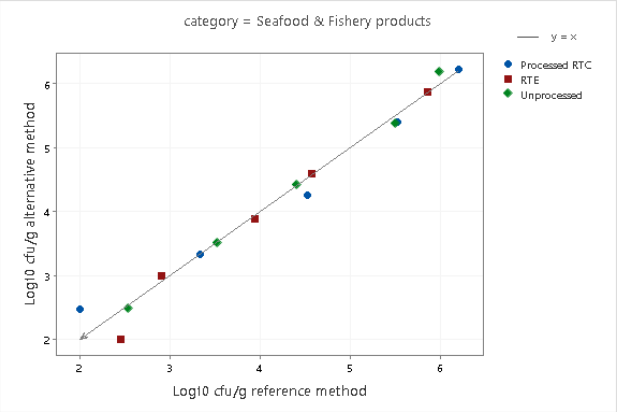
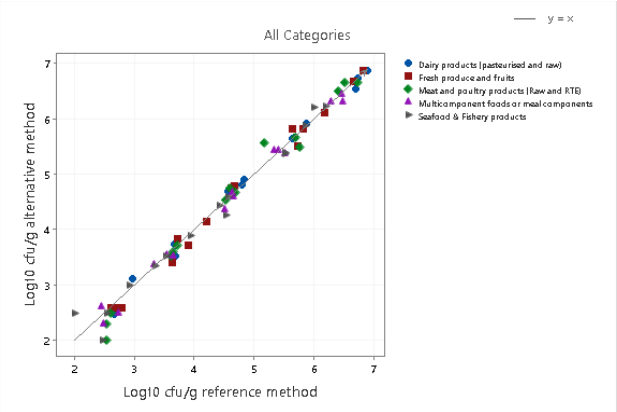


Figure 27 - Scatter plot of the reference method versus alternative method results for all the categories for Listeria spp. plated onto OPL spread plates.



According to ISO16140-2:2016 6.1.2.3, the results of the scatter plot are interpreted on the visual observation of the amount of bias and extreme results. The scatter plots for 1ml OPL pour plates for *L. monocytogenes* show good agreement between the reference method and alternative method. There are no obvious disagreements between the two methods and no real bias was obsevred. This is further described in the Bland Altman plot analysis in Figure 28.

Table 8- Summary of the calculated values per category Listeria spp 0.1ml OPL spread.

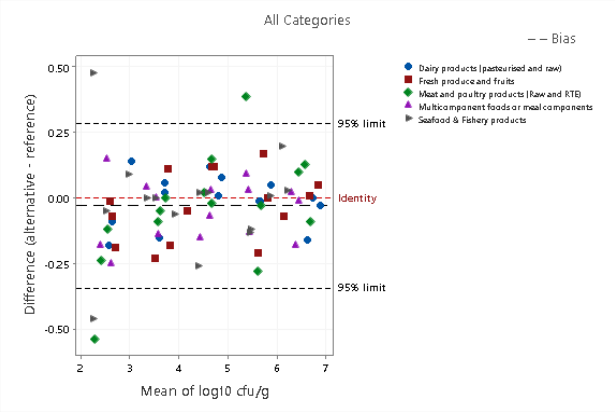
Category	n	$\bar{D}$	SD	95 % low limit	95 % upper limit
Dairy products (pasteurised and raw)	15	-0.010	0.098	-0.228	0.208
Fresh produce and fruits	15	-0.029	0.130	-0.318	0.260
Meat and poultry products (Raw and RTE)	15	-0.045	0.213	-0.516	0.427

Multicomponent foods or meal components	15	-0.050	0.116	-0.306	0.207
Seafood & Fishery products	15	-0.015	0.206	-0.471	0.441
All products	75	-0.030	0.156	-0.343	0.284

$\bar{D}$: Average difference SD: standard deviation of differences n: number of samples

The Bland-Altman difference plot for all the samples is given Figure 28.

Figure 28 – Bland-Altman difference plot for all the samples Listeria spp OPL spread.



Samples for which the difference between the result observed with the reference and the alternative methods is above or lower than the limits are listed in the Table 9.

Table 9 - Data which are outside of the accepted limits Listeria spp OPL spread

Category	**Type**	**N° Sample**	**Reference method Log cfu/g**	**Alternative method Log cfu/g**	**Mean Log cfu/g**	**Difference Alt – ref)**	**Lower / Upper limits**
Seafood & Fishery products	Processed RTC	T146	2.00	2.48	2.24	0.480	0.335
Meat and poultry products (Raw and RTE)	Ready to cook (processed)	T84	5.176	5.568	5.372	0.392	0.335
Seafood & Fishery products	RTE	T141	2.462	2.000	2.231	-0.462	-0.384
Meat and poultry products (Raw and RTE)	Fresh meats (unprocessed)	T76	2.544	2.000	2.272	-0.544	-0.384

Comments

The Bland Altman showed good agreement between the Reference method and the Alternative method. There were 4 data points from a total of 75 data points which were outside of the accepted limits. Two of these were positively biased and two were negatively biased and covered 2 different categories.

3.1.6 Conclusion (RT study)

The relative trueness of the Alternative method is satisfied as there was a good agreement between the reference method and alternative method for *Listeria* spp and *L.monocytogenes* as a pour plate or spread plate. The 95% confidence limits were less than 0.5logs for all Bland Altman plots and there was no bias between the reference method and the alternative method.

3.2 Accuracy profile study

The accuracy profile study is a comparative study between the results obtained by the reference and the results of the alternative method. This study is conducted using artificially contaminated samples, using one type per category.

3.2.1 Categories, sample types and strains

Five food categories were tested with a single batch of two different food types using 6 samples per type.

Two samples were contaminated at a low level, 2 at intermediate level, 2 at a high level. For each sample, 5 replicates (5 different test portions) were tested. A total of 30 samples were analysed per food type. The following food type/strain pairs were studied (See Table 10)

Each sample was bulk inoculated and five replicate test portions examined from the bulk sample/ individually inoculated as a separate test portion, with the exception of salad where single test portions were inoculated.

Table 10a - Categories, types, items, strains and inoculation levels for accuracy profile study L.monocytogenes

Category	Types	Strain of *L. monocytogenes*	Level cfu/g
Poultry and meat products (Raw and RTE)	Cooked sliced chicken	*L. monocytogenes* 3b CRA 1168 isolated from cooked turkey	Level 1x5: $8x10^2$- $2x10^3$
			Level 2x5: $7x10^3$- $2x10^4$
			Level 3x5: $6x10^4$- $1x10^5$
	Cooked sliced turkey		Level 1x5: $8x10^2$- $2x10^3$
			Level 2x5: $9x10^3$- $2x10^4$
			Level 3x5: $8x10^4$- $1x10^5$
Dairy products (pasteurised and raw)	Raw milk	*L. monocytogenes* 4b CRA 1177 isolated ice-cream	Level 1x5: $2x10^3$- $4x10^3$
			Level 2x5: $2x10^4$ - $3x10^4$
			Level 3x5: $1x10^5$- $2x10^5$
	Raw milk cheese (Chaource)		Level 1x5: $3x10^2$ - $6x10^2$
			Level 2x5: $9x10^3$- $2x10^4$
			Level 3x5: $5x10^5$- $1x10^6$
Fresh produce and fruits	Bagged salads	*L. monocytogenes* 1/2a CRA 1102 isolated from lettuce	Level 1x5: $6x10^1$ -$2x10^2$
			Level 2x5: $6x10^2$ -$2x10^3$
			Level 3x5: $3x10^3$ - $6x10^3$
	Bagged salads		Level 1x5: $1x10^2$ -$2x10^2$
			Level 2x5: 5 $x10^2$- $2x10^3$
			Level 3x5: $6x10^3$- $8x10^3$
Seafood & Fishery products	RTC frozen fishcakes	*L. monocytogenes* (CRA 5219) isolated from salmon fish cakes	Level 1x5: $5x10^3$-$7x10^3$
			Level 2x5: $3x10^4$- $9x10^4$
			Level 3x5: $1x10^6$- $6x10^6$
	Frozen fish		Level 1x5: $4x10^3$- $6x10^3$
			Level 2x5: $4x10^4$- $5x10^4$
			Level 3x5: $4x10^6$- $6x10^6$
Multicomponent foods	Couscous salad	*L. monocytogenes* 3c CRA 1173 iolsated from chicken and lettuce sandwich	Level 1x5: $2x10^2$-$4x10^2$
			Level 2x5: $2x10^3$- $5x10^3$
			Level 3x5: $3x10^4$- $6x10^4$
	Pasta salad		Level 1x5: $1x10^2$-$6x10^2$
			Level 2x5: $3x10^3$- $6x10^3$
			Level 3x5: $2x10^4$- $6x10^4$

Table 10b - Categories, types, items, strains and inoculation levels for accuracy profile study Listeria spp

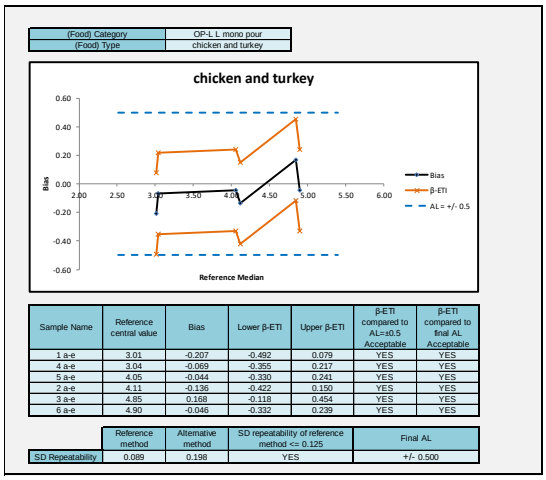
Category	Types	Strain of ***Listeria spp.***	Level cfu/g
Poultry and meat products (Raw and RTE)	Cooked sliced chicken	*Listeria seeligeri* CRA 1142 islated from pork loaf	Level 1x5: $9x10^2$- $2x10^3$
			Level 2x5: $7x10^3$- $3x10^4$
			Level 3x5: $7x10^4$- $1x10^5$
	Cooked sliced turkey		Level 1x5: $1x10^3$-$2x10^3$
			Level 2x5: $1x10^4$ - $5x10^4$
			Level 3x5: $1x10^5$- $1x10^6$
Dairy products (pasteurised and raw)	Raw milk	*L. innocua* CRA 1111 isolated from Camembert	Level 1x5: $1x10^2$- $6x10^2$
			Level 2x5: $4x10^3$- $6x10^3$
			Level 3x5: $2x10^4$ - $5x10^4$
	Raw milk cheese (Chaource)		Level 1x5: $1x10^2$ -$6x10^2$
			Level 2x5: $3x10^3$- $3x10^3$
			Level 3x5: $2x10^4$ - $6x10^4$
Fresh produce and fruits	Mixed leaf bagged salad	*Listeria grayii* CRA 5164 isolated from salmon fishcakes	Level 1x5: $1x10^2$- $1x10^3$
			Level 2x5: $2x10^3$- $3x10^3$
			Level 3x5: $8x10^3$- $2x10^4$
	Baby leaf bagged salad		Level 1x5: $2x10^3$- $6x10^3$
			Level 2x5: $5x10^3$- $1x10^4$
			Level 3x5: $1x10^4$- $4x10^4$
Seafood & Fishery products	RTC frozen fishcakes	*Listeria grayii* CRA 5164 isolated from salmon fishcakes	Level 1x5: $2x10^2$-$4x10^2$
			Level 2x5: $2x10^3$-$4x10^3$
			Level 3x5: $2x10^4$- $6x10^4$
	Frozen fish		Level 1x5: $2x10^2$-$4x10^2$
			Level 2x5: 8 $x10^2$- $4x10^3$
			Level 3x5: $2x10^4$- $4x10^4$
Multicomponent foods	Pasta salad	*L. monocytogenes* 3c CRA1173 isolated from chicken and lettuce sandwich	Level 1x5: $2x10^2$-$4x10^2$
			Level 2x5: $2x10^3$- $5x10^3$
			Level 3x5: $3x10^4$- $6x10^4$
	Cous cous salad		Level 1x5: $1x10^2$-$6x10^2$
			Level 2x5: $3x10^3$- $6x10^3$
			Level 3x5: $2x10^4$- $6x10^4$

3.2.2 Calculations and interpretation of accuracy profile study

The statistical results and the accuracy profiles are provided Figures 29-33.

The calculations were done using the AP Calculation Tool MCS (Clause 6-1-3-3 calculation and interpretation of accuracy profile study) available on http://standards.iso.org/iso/16140

Figure 29 – Accuracy profile L. monocytogenes meat and poultry on 1ml OPL pour plates

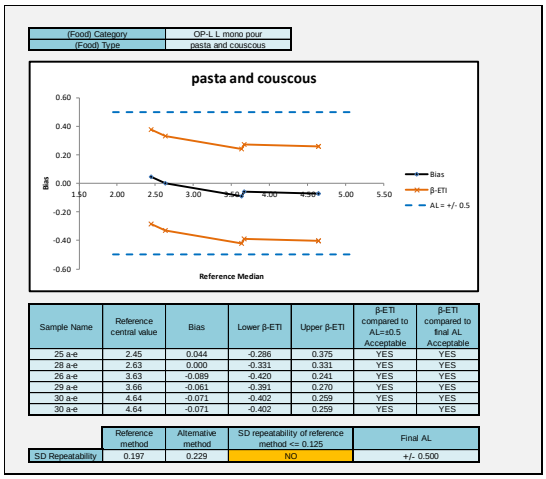


(Food) Category	OP-L L mono pour
(Food) Type	chicken and turkey

Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
1 a-e	3.01	-0.207	-0.492	0.079	YES	YES
4 a-e	3.04	-0.069	-0.355	0.217	YES	YES
5 a-e	4.05	-0.044	-0.330	0.241	YES	YES
2 a-e	4.11	-0.136	-0.422	0.150	YES	YES
3 a-e	4.85	0.168	-0.118	0.454	YES	YES
6 a-e	4.90	-0.046	-0.332	0.239	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.089	0.198	YES	+/- 0.500

Figure 30– Accuracy profile L. monocytogenes multicomponent foods on 1ml OPL pour plates

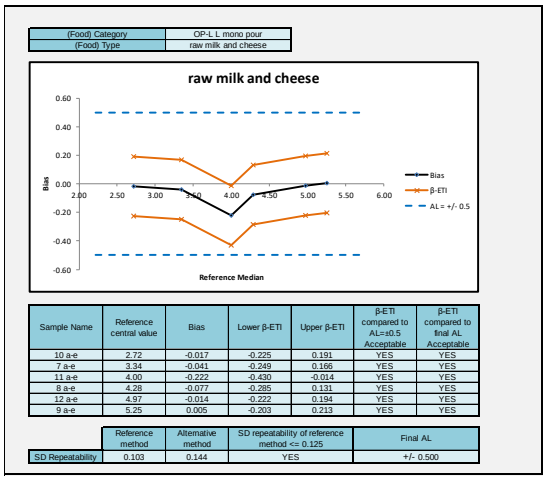


(Food) Category	OP-L L mono pour
(Food) Type	pasta and couscous

Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
25 a-e	2.45	0.044	-0.286	0.375	YES	YES
28 a-e	2.63	0.000	-0.331	0.331	YES	YES
26 a-e	3.63	-0.089	-0.420	0.241	YES	YES
29 a-e	3.66	-0.061	-0.391	0.270	YES	YES
30 a-e	4.64	-0.071	-0.402	0.259	YES	YES
30 a-e	4.64	-0.071	-0.402	0.259	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.197	0.229	NO	+/- 0.500

Figure 31– Accuracy profile L. monocytogenes dairy products on 1ml OPL pour plates

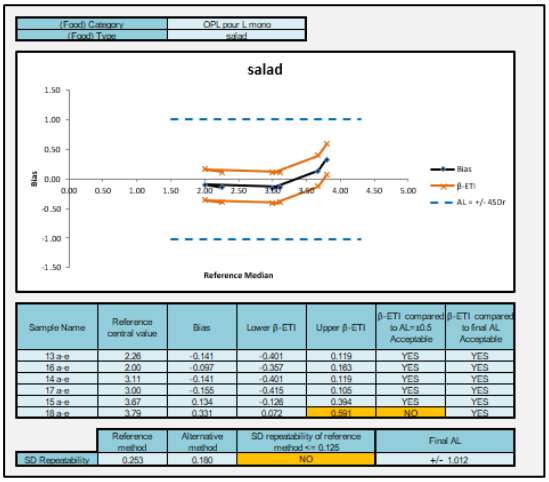


(Food) Category	OP-L L mono pour
(Food) Type	raw milk and cheese

Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
10 a-e	2.72	-0.017	-0.225	0.191	YES	YES
7 a-e	3.34	-0.041	-0.249	0.166	YES	YES
11 a-e	4.00	-0.222	-0.430	-0.014	YES	YES
8 a-e	4.28	-0.077	-0.285	0.131	YES	YES
12 a-e	4.97	-0.014	-0.222	0.194	YES	YES
9 a-e	5.25	0.005	-0.203	0.213	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.103	0.144	YES	+/- 0.500

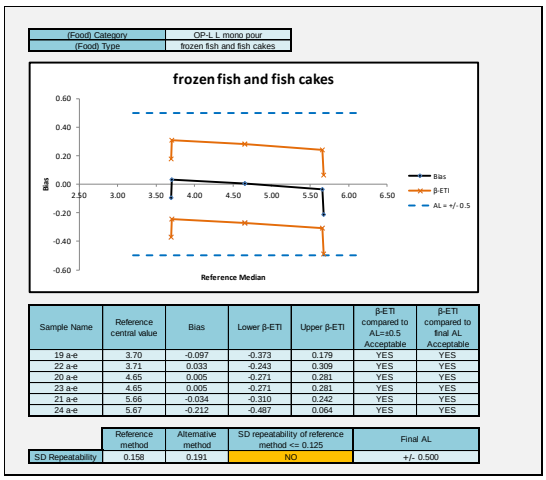
Figure 32 – Accuracy profile L. monocytogenes fresh produce and fruits on 1ml OPL pour plates



Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
13 a-e	2.26	-0.141	-0.401	0.119	YES	YES
16 a-e	2.00	-0.097	-0.357	0.163	YES	YES
14 a-e	3.11	-0.141	-0.401	0.119	YES	YES
17 a-e	3.00	-0.155	-0.415	0.105	YES	YES
15 a-e	3.67	0.134	-0.126	0.394	YES	YES
18 a-e	3.79	0.331	0.072	0.591	NO	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.253	0.180	NO	+/- 1.012

Figure 33 – Accuracy profile L. monocytogenes fish and seafood products on 1ml OPL pour plates



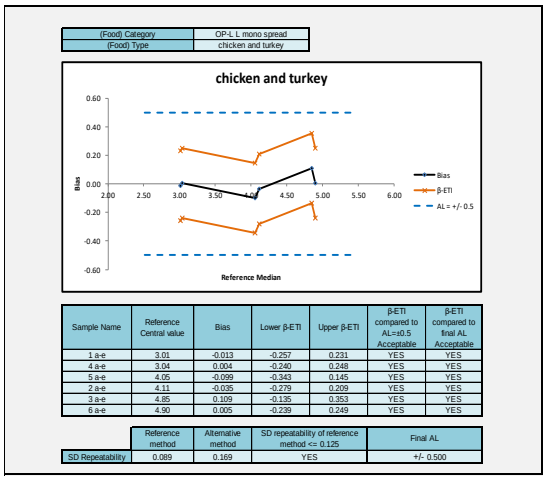
Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
19 a-e	3.70	-0.097	-0.373	0.179	YES	YES
22 a-e	3.71	0.033	-0.243	0.309	YES	YES
20 a-e	4.65	0.005	-0.271	0.281	YES	YES
23 a-e	4.65	0.005	-0.271	0.281	YES	YES
21 a-e	5.66	-0.034	-0.310	0.242	YES	YES
24 a-e	5.67	-0.212	-0.487	0.064	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.158	0.191	NO	+/- 0.500

For the enumeration of *L. monocytogenes* with 0.1ml OPL spread plates, the following categories met the AL of 0.5log meat and poultry, dairy, fish and seafood and multicomponent foods. 1 category (produce and fruit) required the new AL to be calculated. All data met the new AL value of 1.012.

Analysis of the category with a re-calculated AL (fresh produce and fruit on OP-L 1 ml pour) 1 out of the 12 ß-ETI values exceeded the 0.5log AL. This was for high level baby leaf salad. All of the categories met the re-calculated AL of 1.012. Although this is a relatively large AL, the recalculation was required due to the repeatability of the reference method which was 0.253 compared to 0.180 for the alternative method. The baby leaf and mixed leaves were difficult to achieve a homogenous inoculum

Figure 34 – Accuracy profile L. monocytogenes meat and poultry on 0.1ml OPL spread plates

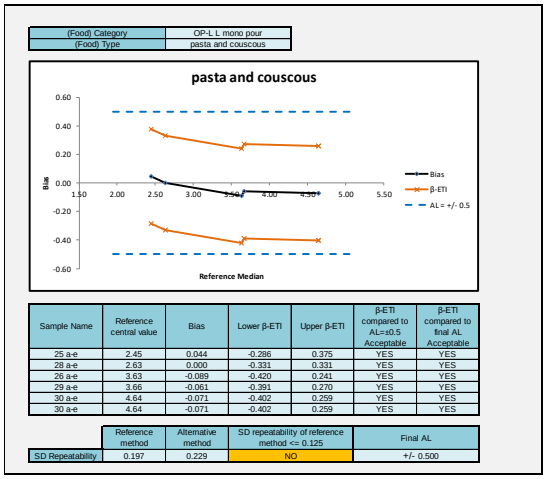


(Food) Category	OP-L L mono spread
(Food) Type	chicken and turkey

Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
1 a-e	3.01	-0.013	-0.257	0.231	YES	YES
4 a-e	3.04	0.004	-0.240	0.248	YES	YES
5 a-e	4.05	-0.099	-0.343	0.145	YES	YES
2 a-e	4.11	-0.035	-0.279	0.209	YES	YES
3 a-e	4.85	0.109	-0.135	0.353	YES	YES
6 a-e	4.90	0.005	-0.239	0.249	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.089	0.169	YES	+/- 0.500

Figure 35– Accuracy profile L. monocytogenes multicomponent foods on 0.1ml OPL spread plates

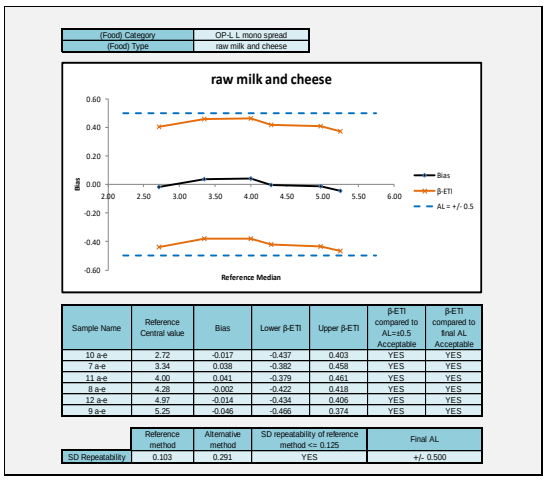


(Food) Category	OP-L L mono pour
(Food) Type	pasta and couscous

Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
25 a-e	2.45	0.044	-0.286	0.375	YES	YES
28 a-e	2.63	0.000	-0.331	0.331	YES	YES
26 a-e	3.63	-0.089	-0.420	0.241	YES	YES
29 a-e	3.66	-0.061	-0.391	0.270	YES	YES
30 a-e	4.64	-0.071	-0.402	0.259	YES	YES
30 a-e	4.64	-0.071	-0.402	0.259	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.197	0.229	NO	+/- 0.500

Figure 36– Accuracy profile L. monocytogenes dairy products on 0.1ml OPL spread plates

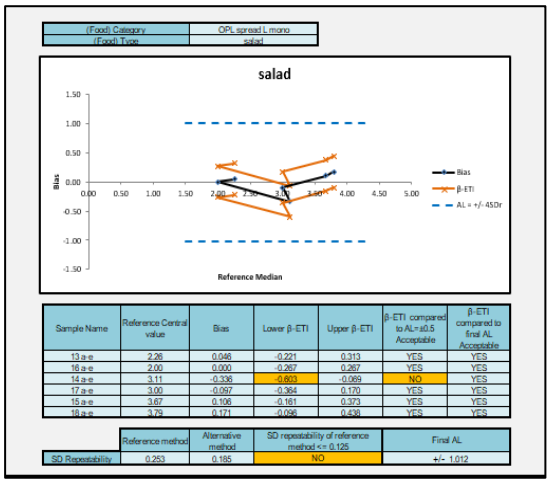


(Food) Category	OP-L L mono spread
(Food) Type	raw milk and cheese

Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
10 a-e	2.72	-0.017	-0.437	0.403	YES	YES
7 a-e	3.34	0.038	-0.382	0.458	YES	YES
11 a-e	4.00	0.041	-0.379	0.461	YES	YES
8 a-e	4.28	-0.002	-0.422	0.418	YES	YES
12 a-e	4.97	-0.014	-0.434	0.406	YES	YES
9 a-e	5.25	-0.046	-0.466	0.374	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.103	0.291	YES	+/- 0.500

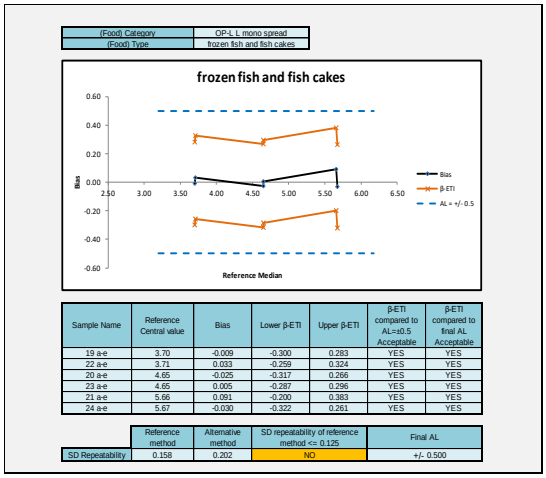
Figure 37 – Accuracy profile L. monocytogenes fresh produce and fruits on 0.1ml OPL spread plates



Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
13 a-e	2.26	0.046	-0.221	0.313	YES	YES
16 a-e	2.00	0.000	-0.267	0.267	YES	YES
14 a-e	3.11	-0.336	-0.603	-0.069	NO	YES
17 a-e	3.00	-0.097	-0.364	0.170	YES	YES
15 a-e	3.67	0.106	-0.161	0.373	YES	YES
18 a-e	3.79	0.171	-0.096	0.438	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.253	0.185	NO	+/- 1.012

Figure 38 – Accuracy profile L. monocytogenes fish and seafood products on 0.1ml OPL spread plates



Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
19 a-e	3.70	-0.009	-0.300	0.283	YES	YES
22 a-e	3.71	0.033	-0.259	0.324	YES	YES
20 a-e	4.65	-0.025	-0.317	0.266	YES	YES
23 a-e	4.65	0.005	-0.287	0.296	YES	YES
21 a-e	5.66	0.091	-0.200	0.383	YES	YES
24 a-e	5.67	-0.030	-0.322	0.261	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.158	0.202	NO	+/- 0.500

For the enumeration of *L. monocytogenes* with 0.1ml OPL spread plates, the following categories met the AL of 0.5log dairy, meat and poultry, fish and seafood, multicomponent foods. 1 category (fresh produce) required the new AL to be calculated. All data met the new AL value of 1.012.

For the fresh produce and fruit only 1 out of the 12 ß-ETI values exceeded the 0.5log AL. This was for medium level baby leaf salad where the lower ß-ETI value was higher than the 0.5log AL. All of the categories met the re-calculated value of 1.012 log. Although the AL relatively high, this was impacted by with repeatability of the reference method which was 0.253 compared to 0.185 for the alternative method.

Figure 39 – Accuracy profile Listeria spp. meat and poultry on 1ml OPL pour plates

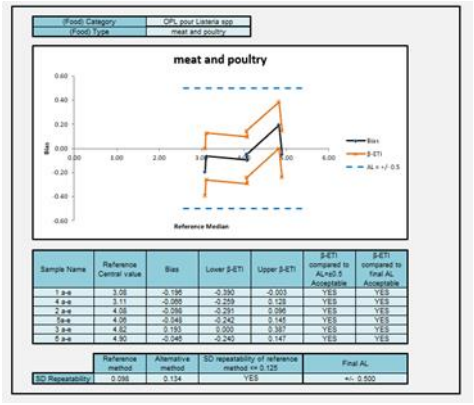


Figure 40– Accuracy profile Listeria spp. multicomponent foods on 1ml OPL pour plates

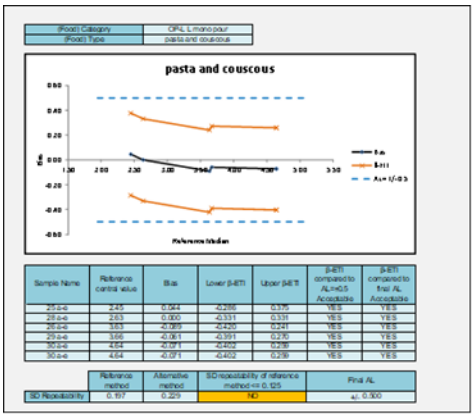


Figure 41 – Accuracy profile Listeria spp. dairy products on 1ml OPL pour plates

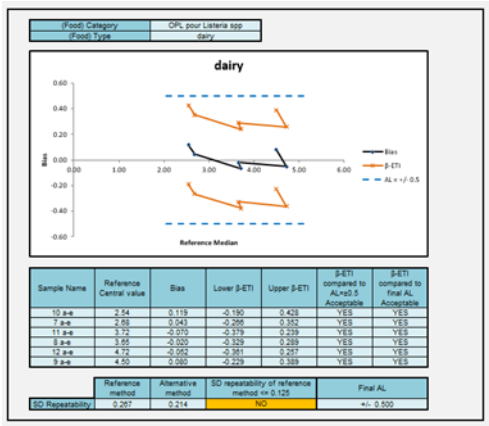


Figure 42 – Accuracy profile Listeria spp. fresh produce and fruits on 1ml OPL pour plates

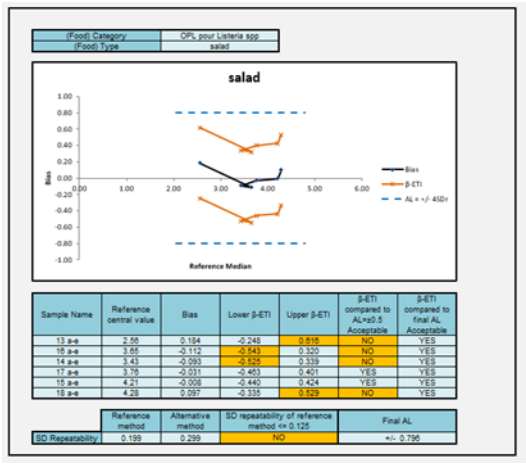
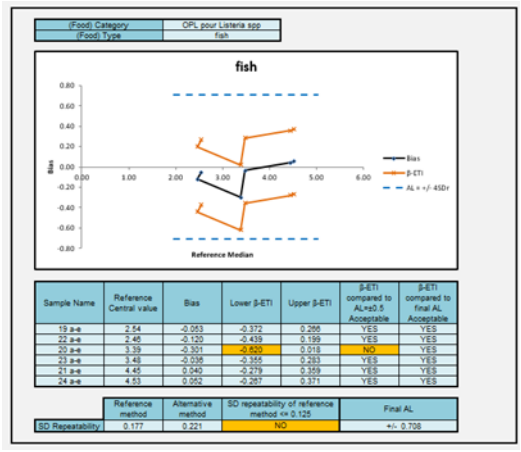


Figure 43– Accuracy profile Listeria spp. fish and seafood products on 1ml OPL pour plate



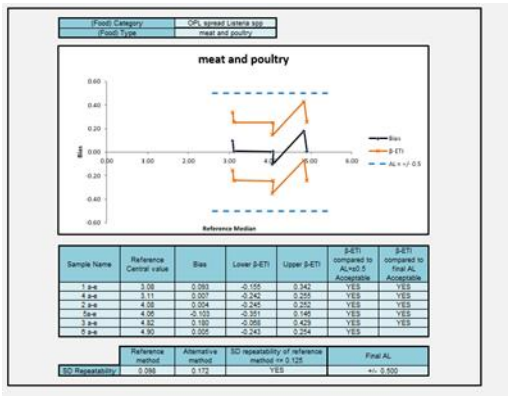
For the enumeration of *Listeria* spp. with 1ml OPL pour plates, the following categories met the AL of 0.5log meat and poultry, fish and seafood, dairy multicomponent foods. Two categories required the new AL to be calculated fish and seafood and fresh produce. All data met the new AL value of 0.708 and 0.796 respectively.

Additional analysis of the two categories (fish and seafood and fresh produce) that had a re-calculated AL showed that only 1 out of the 12 ß-ETI values for the dairy category exceeded the 0.5log AL. This was for frozen fish at medium level. All categories met the amended AL of 0.708. The SD repeatability for both methods was similar being 0.177-0.221.

In the fresh produce and fruit category, 4 out of the 12 ß-ETI values exceeded the 0.5log AL. 2 out of the upper ß-ETI values were outside the AL and these were for mixed leaf at low level and baby leaf at high level. The remaining 2 lower ß-ETI

values outside the AL were for baby leaf at low level, mixed leaf products at the medium level. The fresh produce and fruit met the amended AL of 0.796.

Figure 44 – Accuracy profile Listeria spp. meat and poultry on 0.1ml OPL spread plates

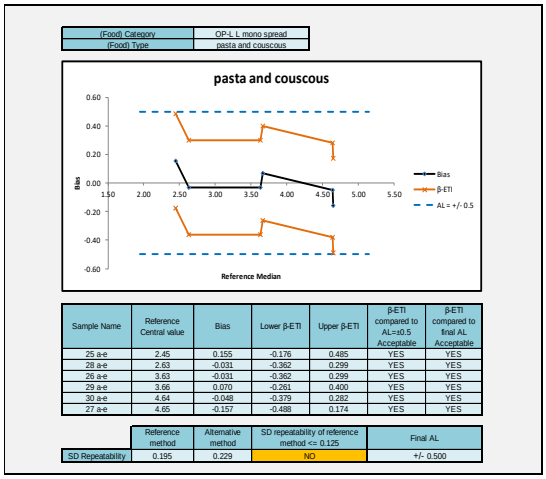


(Food) Category	OPL spread Listeria spp
(Food) Type	meat and poultry

Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
1 a-e	3.08	0.093	-0.155	0.342	YES	YES
4 a-e	3.11	0.007	-0.242	0.255	YES	YES
2 a-e	4.08	0.004	-0.245	0.252	YES	YES
5a-e	4.06	-0.103	-0.351	0.146	YES	YES
3 a-e	4.82	0.180	-0.068	0.429	YES	YES
6 a-e	4.90	0.005	-0.243	0.254	YES	

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.098	0.172	YES	+/- 0.500

Figure 45– Accuracy profile Listeria spp. multicomponent foods 0.1ml OPL spread plates

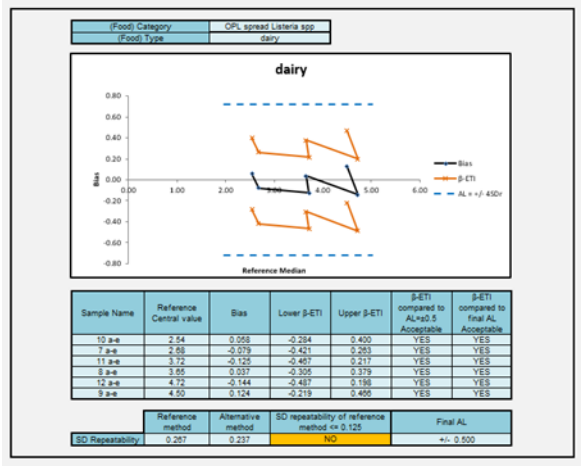


(Food) Category	OP-L L mono spread
(Food) Type	pasta and couscous

Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
25 a-e	2.45	0.155	-0.176	0.485	YES	YES
28 a-e	2.63	-0.031	-0.362	0.299	YES	YES
26 a-e	3.63	-0.031	-0.362	0.299	YES	YES
29 a-e	3.66	0.070	-0.261	0.400	YES	YES
30 a-e	4.64	-0.048	-0.379	0.282	YES	YES
27 a-e	4.65	-0.157	-0.488	0.174	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.195	0.229	NO	+/- 0.500

Figure 46– Accuracy profile Listeria spp.dairy products on OPL 0.1ml OPL spread plates

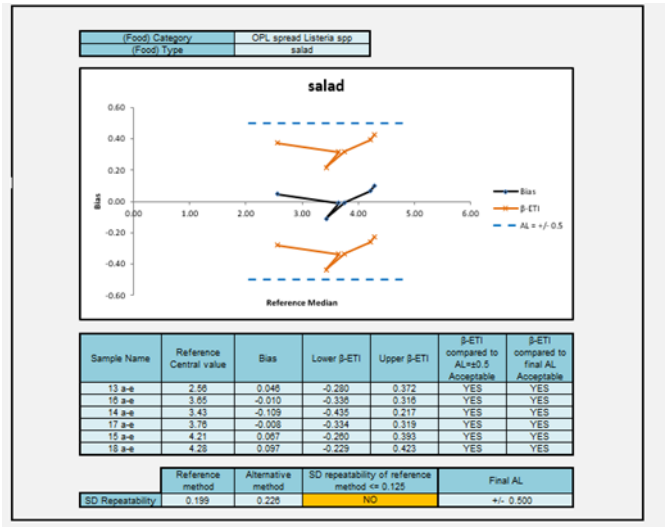


(Food) Category	OPL spread Listeria spp
(Food) Type	dairy

Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
10 a-e	2.54	0.056	-0.284	0.400	YES	YES
7 a-e	2.58	-0.079	-0.421	0.263	YES	YES
11 a-e	3.72	-0.125	-0.467	0.217	YES	YES
8 a-e	3.65	0.037	-0.305	0.379	YES	YES
12 a-e	4.72	-0.144	-0.487	0.196	YES	YES
9 a-e	4.50	0.124	-0.219	0.466	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.267	0.237	NO	+/- 0.500

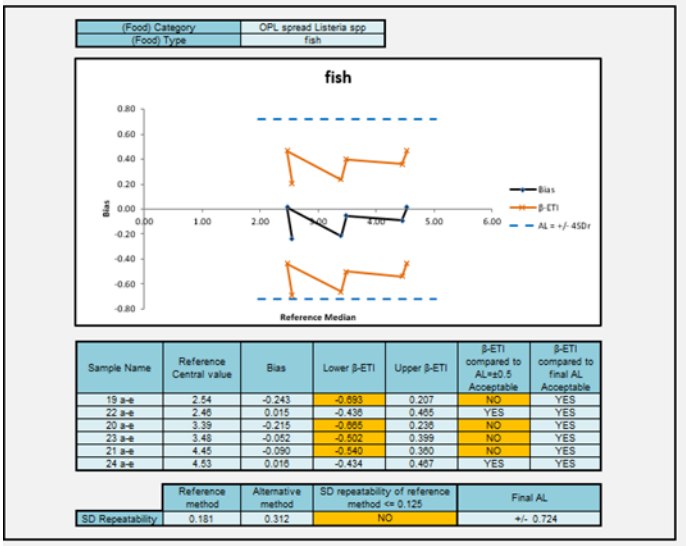
Figure 47 – Accuracy profile Listeria spp. fresh produce and fruits on 0.1ml OPL spread plates



Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
13 a-e	2.56	0.046	-0.280	0.372	YES	YES
16 a-e	3.65	-0.010	-0.336	0.316	YES	YES
14 a-e	3.43	-0.109	-0.435	0.217	YES	YES
17 a-e	3.76	-0.008	-0.334	0.319	YES	YES
15 a-e	4.21	0.067	-0.260	0.393	YES	YES
18 a-e	4.28	0.097	-0.229	0.423	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.199	0.226	NO	+/- 0.500

Figure 48 – Accuracy profile Listeria spp. fish and seafood products on 0.1ml OPL spread plates



Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
19 a-e	2.54	-0.243	-0.693	0.207	NO	YES
22 a-e	2.46	0.015	-0.436	0.465	YES	YES
20 a-e	3.39	-0.215	-0.665	0.236	NO	YES
23 a-e	3.48	-0.052	-0.502	0.399	NO	YES
21 a-e	4.45	-0.090	-0.540	0.360	NO	YES
24 a-e	4.53	0.016	-0.434	0.467	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.181	0.312	NO	+/- 0.724

For the enumeration of Listeria spp with 0.1ml OPL spread plates, the following categories met the AL of 0.5log: meat and poultry, dairy, fresh produce and multicomponent foods. One category required the new AL to be calculated and fish and seafood. This category met the new AL values of 0.724.

Additional analysis of the fish and seafood category that had a re-calculated AL showed that for the meat and poultry category, showed that 4 out of the 12 □-ETI values were above the 0.5log AL. This was for frozen fish at the low medium and high levels and fish cakes at medium level. The relatively high AL was influenced by

the SD repeatability which was between 0.181 and 0.312 for the reference and alternative methods respectively.

The accuracy of the Alternative method (One plate OP-L (pour and spread plate format) enumeration for *L. monocytogenes* and *Listeria* spp.) is satisfied as all categories met the 0.5log AL or the re-calculated AL .

3.3 Inclusivity / exclusivity

Inclusivity is the ability of the alternative method to detect the target analyte from a wide range of strains. Exclusivity is the lack of interference from a relevant range of non-target strains of the alternative method.

3.3.1 Protocols

- Inclusivity

Two sets of *Listeria* cultures were be used

(i) 50 pure cultures of the *L. monocytogenes* were analysed for the *L. monocytogenes panel*
(ii) 30 pure cultures of non-monocytogenes *Listeria* was combined with the 50 pure cultures of the *L. monocytogenes* to give an inclusivity panel of 80 *Listeria* spp. isolates

50 *L. monocytogenes* strains and 30 *Listeria* spp isolates (not belonging to *L. monocytogenes*) were initially incubated in a non-selective broth and diluted so that the inoculum level was at least 100 times greater than the minimum level for quantification of the alternative method being validated. Each strain was tested once with the alternative method, the reference method and a non-selective agar.

- Exclusivity

Two sets of *Listeria* cultures were used

(i) 30 pure cultures of the non-monocytogenes *Listeria* were analysed for the *L. monocytogenes* exclusivity.
(ii) 30 pure cultures of non- *Listeria* were analysed for the *Listeria* spp exclusivity.

30 non-monocytogenes *Listeria* spp strains were initially incubated in a non-selective broth and diluted so that the inoculum level was at least 100 times greater than the minimum level for quantification of the alternative method being validated. Each strain was tested once with the alternative method, the reference method and a non-selective agar.

For *Listeria spp* and additional 30 isolates not belonging to the Genus *Listeria* were initially incubated in an appropriate non-selective broth and diluted so that the inoculum level was at least 100 times greater than the minimum level for

quantification of the alternative method being validated. Each strain was tested once with the alternative method, the reference method and a non-selective agar.

3.3.2 Results

- Inclusivity

A total of 50 strains were tested for inclusivity for *L. monocytogenes*. 49 out of the 50 strains showed a positive result and 1 isolate *L. monocytogenes* CRA 1449 showed a negative result. This isolate also did not grow on the reference media (ALOA).

For *Listeria* spp., 80 strains were tested for inclusivity. 49 out of the 50 *L. monocytogenes* strains and 29 out of the 30 strains showed a positive result. The 2 strains which showed a negative result were *L. monocytogenes* CRA 1449 and *L. weihenstephanensis CRA* 16874, both of which also gave a negaitve result on the reference method.

- Exclusivity

A total of 30 non-monocytogenes *Listeria* spp strains were tested for exclusivity.24 of these strains showed a negative result. 6 strains showed a positive result: *Listeria ivanovii* CRA 1120, 1123, 1126, 3925, 6599 and DSM12491. Analysis of these 6 isolates with MALDI ToF with the Maldi Biotyper complete solution (Bruker Daltonik GmbH) with the microflex LT/SH MALDI-MS system gave an identity of *L. ivanovii* for all 6 isolates. All 6 isolates also gave a positive result on the reference media (ALOA).

Analysis of the 30 non *Listeria* spp excusivity 28 strains showed a negative result and 2 isolates gave a positive result. The isolates showing a positive result were *Bacillus circulans* CRA16385 and *Erysipelothrix rhusiopathiae* CRA7569. These isolates were analysed with MALDI ToF with the Maldi Biotyper complete solution (Bruker Daltonik GmbH) with the microflex LT/SH MALDI-MS system gave an identity of *Bacillus circulans* and *Erysipelothrix rhusiopathiae* respectively. The *Bacillus circulans* also gave a positive reaction on the reference media (ALOA). As the confirmation confirmed that these isolates were not *Listeria* spp then ultimately the correct result of not detected was achieved.

3.3.3 Conclusion

The alternative One plate OP-L enumeration method is selective and specific to *Listeria monocytogenes* and *Listeria* spp. with both the pour and spread plate formats,

3.4 Conclusion (MCS)

Overall, the conclusions for the Method Comparison are:

- The alternative method One plate OP-L (pour and spread plate format) enumeration method for *L. monocytogenes* and *Listeria* spp. shows satisfactory results for relative trueness.

- The alternative One plate OP-L (pour and spread plate format) enumeration for *L. monocytogenes* and *Listeria* spp. shows satisfactory results for accuracy profile.

- The alternative One plate OP-L enumeration method is selective and specific to *Listeria monocytogenes* and *Listeria* spp. with both the pour and spread plate formats,

4 Interlaboratory study

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

4.1 Study organisation

4.1.1 Collaborators
Samples were sent to 13 laboratories.

4.1.2 Matrix and strain used
Cooked sliced chicken was inoculated with *Listeria monocytogenes* CRA 1168 (isolated from cooked turkey)

4.1.3 Sample preparation
Samples were prepared and inoculated on Tuesday 20th April as described below:

For each collaborator, a set of samples was prepared containing 2 samples at a low level, two samples at a medium level, two samples at a high level and a single uninoculated blank sample. The samples were blind-coded so that the collaborators did not know the intended contamination level. A set of samples was also prepared for the EL although the data from these was not used in the data analysis

The target levels and codes are shown below

Table 11 : Contamination levels

Contamination level	Sample code
Uninoculated	1
Low (10^2 cfu/g)	4
Low (10^2 cfu/g)	7
Medium (10^4 cfu/g)	3

Medium (10^4 cfu/g)	6
High (10^6 cfu/g)	2
High (10^6 cfu/g)	5

4.1.4 Labelling and shipping
Blind coded samples were placed in isothermal boxes, which contained cooling blocks, and express-shipped to the different laboratories.

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

Samples were shipped in a frozen condition on Wednesday 21st April 2021 and were received within 24 h to 48 h to the involved laboratories. The temperature conditions had to stay lower or equal to 8°C during transport, and between 0°C – 8°C in the labs. On receipt at the laboratories, 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 26 April 2021. Stability studies had been conducted to show that the required level of target organisms would be present after 6 and 7 days frozen storage. The expert lab analysed a set of samples on Monday 26 April 2021.

4.1.5 Analysis of Samples

Collaborative study laboratories and the expert laboratory carried out the analyses on Monday 26th April. The analyses by the reference method and the alternative method were performed on the same day.

4.2 Experimental parameters controls

4.2.1 Detection of Listeria monocytogenes in the matrix before inoculation
In order to ensure absence of *L. monocytogenes* in the matrix, the reference method was performed on five portions (10 g) before the inoculation. All the results were negative.

4.2.2 Strain stability during transport
Duplicate samples inoculated at low, medium and high levels were tested for enumeration of *L. monocytogenes* after 5, 6 and 7 days storage at -18°C. Samples were thawed under controlled conditions prior to analysis. The data shows good stability under the storage regime tested (Table 12).

Table 12 - L.monocytogenes stability in the matrix

Day	Reference method cfu/g						Alternative method cfu/g					
	Low level		Medium level		High level		Low level		Medium level		High level	
	a	b	a	b	a	b	a	b	a	b	a	b

Day 0	310	320	$3.4x10^3$	$3.1x10^3$	$2.6x10^5$	$2.5x10^5$	245	191	$3.7x10^3$	$3.4x10^3$	$3.1x10^5$	$2.2x10^5$
Day 5	200	220	$1.6x10^3$	$1.2x10^3$	$2.7x10^5$	$1.9x10^5$	270	310	$1.8x10^3$	$1.4x10^3$	$2.4x10^5$	$1.8x10^5$
Day 6	200	330	$3.0x10^3$	$2.9x10^3$	$1.9x10^5$	$2.6x10^5$	240	240	$1.7x10^3$	$1.6x10^3$	$1.5x10^5$	$2.7x10^5$
Day 7	210	216	$1.6x10^3$	$2.7x10^3$	$3.0x10^5$	$1.9x10^5$	140	182	$1.8x10^3$	$1.8x10^3$	$2.0x10^5$	$1.8x10^5$

4.2.3 Logistic conditions

The temperatures measured at receipt by the collaborators, the temperatures registered by the thermo-probe, and the receipt dates are given in Table 13.

Table 13 - Sample temperatures at receipt

Collaborator	Temperature measured by the probe (°C)	Temperature measured at receipt (°C)	Receipt date time	State of the package samples at the	Analysis date
1	n/a	Water frozen	22/4/21, 10.00	Good, samples fully frozen	26/4/21
2	n/a	Water frozen	22/4/21, 09.10	Acceptable	26/4/21
3	n/a	Water frozen	22/4/21, 15.15	Sealed	26/4/21
4	n/a	Water frozen	22/4/21, 09.40	In tact	26/4/21
5	n/a	Water frozen	22/4/21, 09.30	Satisfactory	26/4/21
6	n/a	Water frozen	22/4/21, 09.15	OK	26/4/21
7	n/a	Water frozen	22/4/21, 11.00	Fine	26/4/21
8	n/a	Water frozen	22/4/21, 09.30	frozen	26/4/21
9	n/a	Water frozen	22/4/21, 10.00	frozen	26/4/21
10	n/a	Water frozen	22/4/21, 10.00	Good	26/4/21
11	Not known information not sent		22/4/21	Unknown, details not sent	26/4/21
12	Samples did not arrive, were returned to sender from customs				
13	n/a	Water frozen	23/4/21 21.00	Good	28/4/21

No problem was encountered during the transport or at receipt for the 12 out of 13 collaborators. All the samples were delivered on time and in appropriate conditions to 12 laboratories. Laboratory 12 did not receive the parcels which were returned to the expert laboratory. Temperatures during shipment and at receipt were all correct. Data from 2 labs were excluded from the study, lab 5 did not incubate the samples for the required 48h and lab 8 had error with plating out the samples, with one of the samples being plated Calculation and summary of data

4.2.4 MicroVal Expert laboratory results

The results obtained by the expert laboratory are given in Table 14.

Table 14 – Results obtained by the expert lab.

Level	Reference method	Alternative method
Blank	<10	<10
Low	3.80E+02	4.10E+02
Low	4.00E+02	2.40E+02
Medium	1.04E+05	9.70E+04
Medium	8.00E+04	5.40E+04
High	7.60E+05	5.40E+05
High	5.40E+05	5.70E+05

4.2.5 Results obtained by the collaborative laboratories.

The data from the collaborative trial were calculated and interpreted according to section 6.2.3 of ISO 16140-2:2016 using the freely available Excel® spreadsheet (http://standards.iso.org/iso/16140). Version 14-03-2016 was used for these calculations.

The results obtained by the collaborators are shown in Table 15.

The accuracy profile plot is shown in Figure 49 and the statistical analysis of the data shown in Table 16.

Table 15: Summary of the results of the interlaboratory study per analyte level

Collaborator	**Level**	**Reference method (Log cfu/g)**		**Alternative method (Log cfu/g)**	
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
1	low	2.45	2.54	2.62	2.90
2	low	2.54	2.52	3.10	2.74
3	low	2.41	2.60	2.59	2.49
4	low	2.61	2.40	2.68	2.54
6	low	2.38	2.40	2.78	2.42
7	low	2.52	2.20	2.54	2.41

Collaborator	Level	Reference method (Log cfu/g)		Alternative method (Log cfu/g)	
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
9	low	2.40	2.43	2.79	2.77
10	low	2.45	2.49	2.82	2.76
11	low	2.49	2.54	2.70	2.77
12	low	2.49	2.51	2.65	2.63
1	medium	4.94	4.93	4.97	5.01
2	medium	4.90	4.76	4.76	4.74
3	medium	4.73	4.80	4.69	4.81
4	medium	4.87	4.85	4.80	4.91
6	medium	4.86	5.28	4.96	4.97
7	medium	4.85	4.88	4.85	4.79
9	medium	4.62	4.85	4.70	4.89
10	medium	4.88	4.77	4.72	4.85
11	medium	4.84	4.73	4.70	4.78
12	medium	4.89	4.80	4.98	4.80
1	high	5.89	5.98	5.89	6.04
2	high	5.75	5.79	5.79	5.84
3	high	5.80	5.68	5.78	5.80
4	high	5.85	5.79	5.83	5.94
6	high	6.10	6.11	5.99	6.10
7	high	5.99	5.76	5.89	5.83
9	high	5.78	5.88	5.85	5.79
10	high	5.84	5.70	5.88	5.79
11	high	5.60	5.68	5.76	5.75
12	high	5.85	5.79	5.90	5.88
1	blank	<10		<10	
2	blank	<10		<10	
3	blank	<10		<10	
4	blank	<10		<10	
6	blank	<10		<10	
7	blank	<10		<10	
9	blank	<10		<10	
10	blank	<10		<10	
11	blank	<10		<10	
12	blank	<10		<10	

Figure 49. Accuracy profile of Neogen One plate for Listeria (OP-L) from the ILS

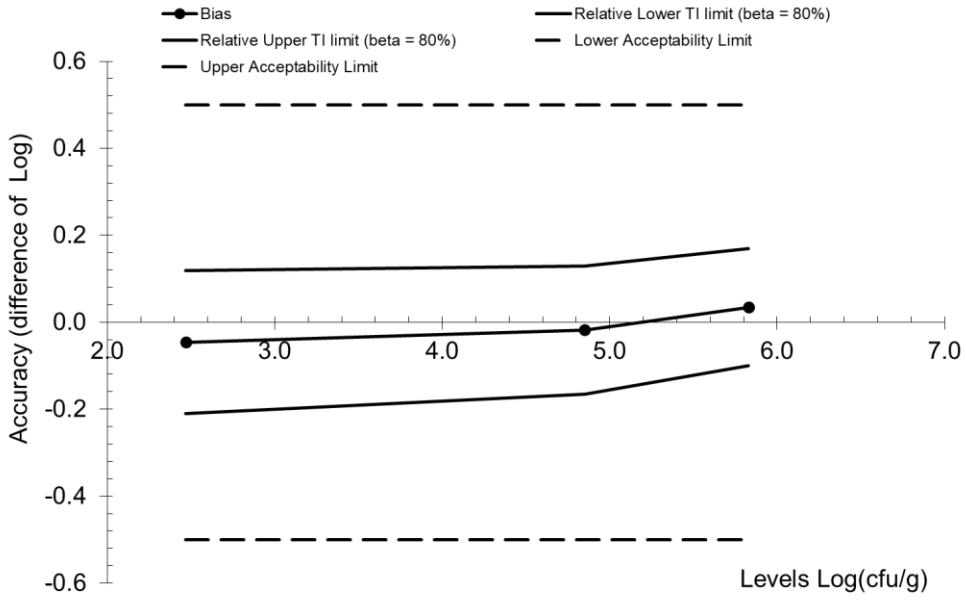


Table 16. Statistical analysis of the ILS data according to the ISO spreadsheet

Accuracy profile	0.5		
Study Name	Neogen OPL		
Date	20/05/2021		
Coordinator	Campden BRI		
Tolerance probability (beta)	80%	80%	80%
Acceptability limit in log (lambda)	0.50	0.50	0.50

FALSE

Application of clause 6.2.3
Step 8: If any of the values for the β-ETI fall outside the acceptability limits, calculate the pooled average reproducibility standard deviation of the reference method.
Step 9: Calculate new acceptability limits as a function of this standard deviation.

	Alternative method			Reference method		
Levels	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
Target value	**2.469**	**4.852**	**5.831**			
Number of participants (K)	10	10	10	10	10	10
Average for alternative method	2.423	4.834	5.865	2.469	4.852	5.831
Repeatability standard deviation (sr)	0.063	0.079	0.057	0.099	0.118	0.077
Between-labs standard deviation (sL)	0.098	0.071	0.077	0.000	0.047	0.112
Reproducibility standard deviation (sR)	0.116	0.106	0.096	0.099	0.127	0.136
Corrected number of dof	11.990	15.216	12.792	18.947	18.353	12.328
Coverage factor	1.413	1.387	1.406			
Interpolated Student t	1.356	1.340	1.351			
Tolerance interval standard deviation	0.1212	0.1098	0.0997			
Lower TI limit	2.259	4.687	5.730			
Upper TI limit	2.587	4.981	6.000			
Bias	**-0.046**	**-0.018**	**0.034**			
Relative Lower TI limit (beta = 80%)	**-0.210**	**-0.165**	**-0.100**			
Relative Upper TI limit (beta = 80%)	**0.118**	**0.129**	**0.169**			
Lower Acceptability Limit	**-0.50**	**-0.50**	**-0.50**			
Upper Acceptability Limit	**0.50**	**0.50**	**0.50**			

FALSE
FALSE

Select ALL blue lines to draw the accuracy profile as illustrated in the worksheet "Graph Profile"

New acceptability limits may be based on reference method pooled variance

Pooled repro standard dev of reference	0.122

5 Overall conclusions of the validation study

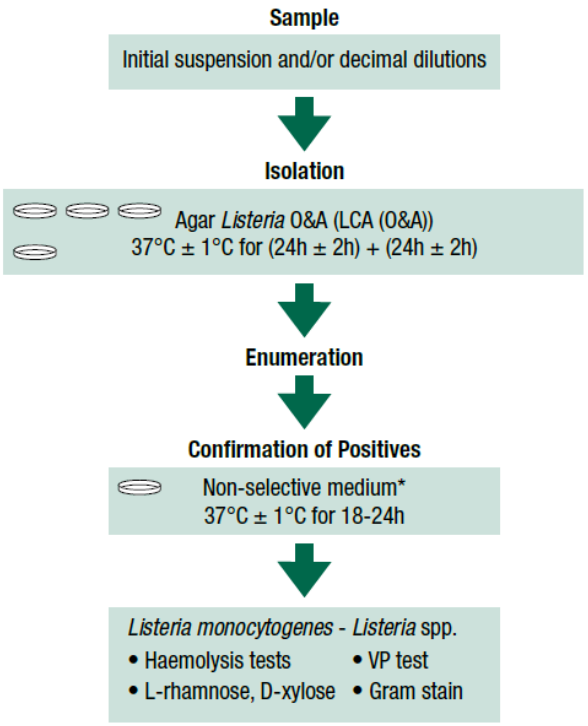
- The alternative method Neogen OPL for enumeration of *Listeria monocytogenes* and *Listeria* spp. shows satisfactory results for relative trueness.
- The alternative method Neogen OPL for enumeration of *Listeria monocytogenes* and *Listeria* spp. shows satisfactory results for accuracy profile.
- The alternative method Neogen OPL for enumeration of *Listeria monocytogenes* and *Listeria* spp. is selective and specific.
- The alternative method Neogen OPL for enumeration of *Listeria monocytogenes* and *Listeria* spp. shows satisfactory performance in the ILS.
- The alternative method Neogen OPL for enumeration of *Listeria monocytogenes* and *Listeria* spp. shows comparable performance to the reference method Microbiology of the food chain — Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria spp.* Part 2: Enumeration method (ISO 11290-2:2017)

The validation of Neogen OPL for enumeration of *Listeria monocytogenes* and *Listeria* spp. Is for a 1ml pour plate and a 0.1ml spread plate format.

Date 02/09/2021

Signature Suzanne Jordan

ANNEX A: Flow diagram of the reference method ISO 11290-2:2017



e.g. 1ml spread over 3 plates and 100µL surface plating from 2 subsequent appropriate dilutions. Plates will be read at 24h plates to compare with the alternative method and will be incubated for a further 24 ±3h to comply with ISO 11290-1 (2017)

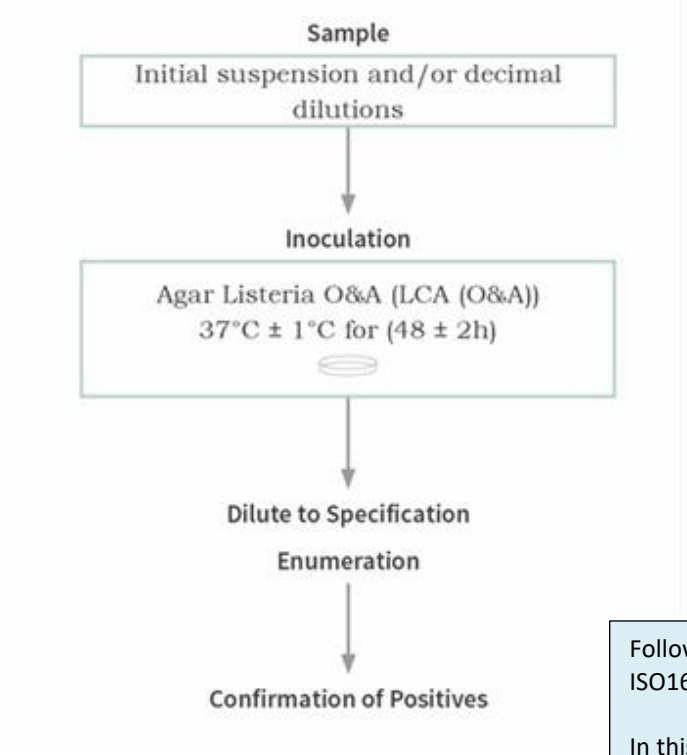
Following ISO 11290 or any ISO16140-6 validated method.

In this study the Bruker MALDI Tof Biotyper will be used

Enumeration range to be used for the quantitative study.

Dilution	Enumeration range 0.1ml spread	Enumeration range 1ml plated
10-1	100-15000	10-3000
10-2	1000-150000	1000-30000
10-3	10000-1500000	1000-300000
10-4	100000-15000000	10000-3000000

ANNEX B: Flow diagram of the alternative method



Following ISO 11290 or any ISO16140-6 validated method.

In this study the Bruker MALDI Tof Biotyper will be used