Method Comparison and Interlaboratory Study Report for the ISO 16140-2:2016 validation of Compact Dry "Nissui"LM (CD LM) for the enumeration of *Listeria monocytogenes* in a broad range of foods and environmental samples

MicroVal study number: 2020LR91(Quantitative)

Method/Kit name: Compact Dry CDLM

Report version:MCS ILS 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

Company:	Nissui Pharmaceutical Co Ltd.
Expert Laboratory:	Campden BRI Station Road Chipping Campden Gloucs, GL55 6LD, UK
Method/Kit name:	Nissui Compact Dry *Listeria* (CDLM)
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:	ISO 11290-2:2017 Microbiology of the food chain — Horizontal method for the detection and enumeration of Listeria monocytogenes and of Listeria spp. Part 2: Enumeration method

Scope of validation: Broad range of foods covering

- Meat and poultry products (RTE/RTRH)
- Dairy products (raw and heat treated)
- Fresh produce and fruit
- Seafood & Fishery products
- Multicomponent foods
- Environmental samples

Certification organisation: 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

Contents

1 Introduction

In this project a MicroVal validation study, based on ISO 16140-2:2016, of alternative method(s) for the enumeration of *Listeria monocytogenes* in a broad range of foods and environmental samples was carried out by Campden BRI as the MicroVal Expert Laboratory.

The study involved the enumeration of *L.monocytogenes* and so the requirements of the Quantitative protocol were carried out.

The alternative method being evaluated was:

Nissui Compact Dry *Listeria* (CDLM). This is a chromogenic medium for the enumeration of *Listeria monocytogenes*. Characteristic colonies of *L. monocytogenes* appear red and may or may not be surrounded by blue coloration. *L.monocytogenes* may also appear orange or reddish brown or reddish purple with or without a blue surround.

Reference method was:

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

Scope of the validation study was: A broad range of foods plus environmental samples.

Categories included:

- Meat and poultry products (RTE/RTRH)
- Dairy products (raw and heat processed)
- Fresh produce and fruit
- Seafood & Fishery products
- Multicomponent foods
- Environmental samples

Criteria evaluated during the study were:

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

The final conclusion on the Method Comparison Study and ILS is summarised below::

The alternative method CDLM shows comparable performance to the reference method ISO 11290-2:2017 for the enumeration of *Listeria monocytogenes* in a broad range of foods and environmental samples.

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 and were therefore treated as paired data.

2.1 Reference method

See the flow diagram in Annex A.

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.

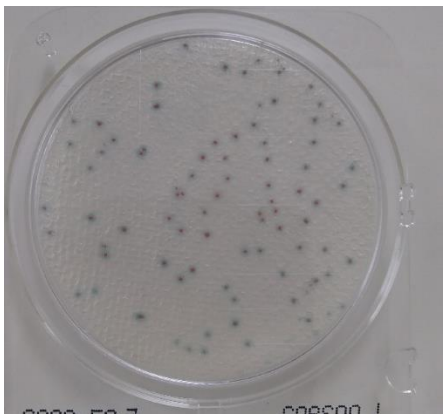
2.2 Alternative method

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

The alternative method principle is based on chromogenic detection of *L.monocytogenes* on ready to use rehydratable films.

Typical colonies grow red with or without a blue surround (Figure 1). These are presumptive *L.monocytogenes* and should be taken forward for further confirmation.

Figure 1. Typical colonies on CDLM



In addition, colonies of *L.monocytogenes* may be orange or reddish brown or reddish purple with or without a blue surround. These colonies are also presumptive *L.monocytogenes* and should also be taken forward for further confirmation.

Other organisms may form white or yellow colonies. These are not presumptive *L.monocytogenes* and do not need further confirmation.

Confirmations were carrried out by streaking presumptive positve colonies 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.

2.3 Study design

Samples of product containing the target organism were diluted 1 in 10 with an appropriate diluent according to ISO 6887 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 6 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

Categories	Types	Items (examples)	Minimum Samples	Preparation
1) Meat and poultry products (RTE/RTRH)	Cooked meat and poultry	Cooked hams, pate, cooked poultry,	5	ISO 6887-1 &2
	Fermented or dried products	Salami, chicken sausage	5	
	Raw cured products	Dry cured hams, smoked turkey products	5	
2) Dairy products (pasteurised and raw)	Pasteurised dairy products	Milk based desserts Ice cream, Drinks, Dry milk	5	ISO 6887-1 & 5
	Pasteruised milk based products	Yogurts, Milk, Cream, hard cheese, soft cheese	5	
	Raw milk products	Raw milk and cream, Raw milk yogurt, raw milk cheese	5	
3) Fresh produce and fruits	Ready to eat fruit	Fruit mix Fruit drinks	5	ISO 6887-1 & 4
	Cut ready to eat vegetables/sprouts	Bagged pre-cut salads Vegetable juices Bean sprouts	5	
	Leafy greens	Basil, Lettuce, Parsley	5	

Categories	Types	Items (examples)	Minimum Samples	Preparation
4) Seafood & Fishery products	Unprocessed	Raw chilled or frozen fish	5	
	RTE	Smoked fish, pates, terrines, crustaceans	5	
	(Processed RTC	fish/seafood meals/mixes)	5	ISO 6887-1 & 3
5) Multicomponent foods	Composite foods with subtsantial raw ingredients	Refrigerated pasta salads, sandwiches	5	
	Composite processed foods	Ready meals	5	ISO 6887-1
	Mayonnaise based deli salads	Sandwich spread, raw vegetables with dressing	5	
6) Environmental samples	Surface samples	Equipment, floors, walls	5	ISO 6887-1 ISO 18593:2008
	Process water	Wash water, cooling water	5	
	Dust wipes and residues	Food manufacturing environments	5	
Total			**90**	

105 samples were analysed, leading to 105 exploitable results.

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.

Samples were inoculated with *L. monocytogenes.*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.

In addition, 5 samples of Pasteurised milk products, 5 samples of Pasteurised dairy products and 5 samples of cooked meat and poultry were spiked with heat treatd strains of *L.monocytogenes* (10min @55°C) The injury level achieved was between 1.2 and 1.5logs.

Eighteen *L. monocytogenes* isolates 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 at the range usually associated with the test organisms and within the capabilities of the test methods, covering the range 10^2cfu/g to 10^7cfu/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 24±2h (22h was used) plus an additional 24±2 h at 37°C if colonies were not distinct. A total incubation period of 44h (22h + 22h) was used for this validation as not all colonies were disitnct after the first 22h.

Confirmations 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 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).

Figures 2-7 shows the scatter plots for *L. monocytgenes* in the 6 individual categories

Figure 8 shows the scatter plot for all the categories for *L. monocytgenes* plated onto CDLM

Figure 2 - Scatter plot of the reference method versus alternative method results for L. monocytogenes in Meat and poultry products

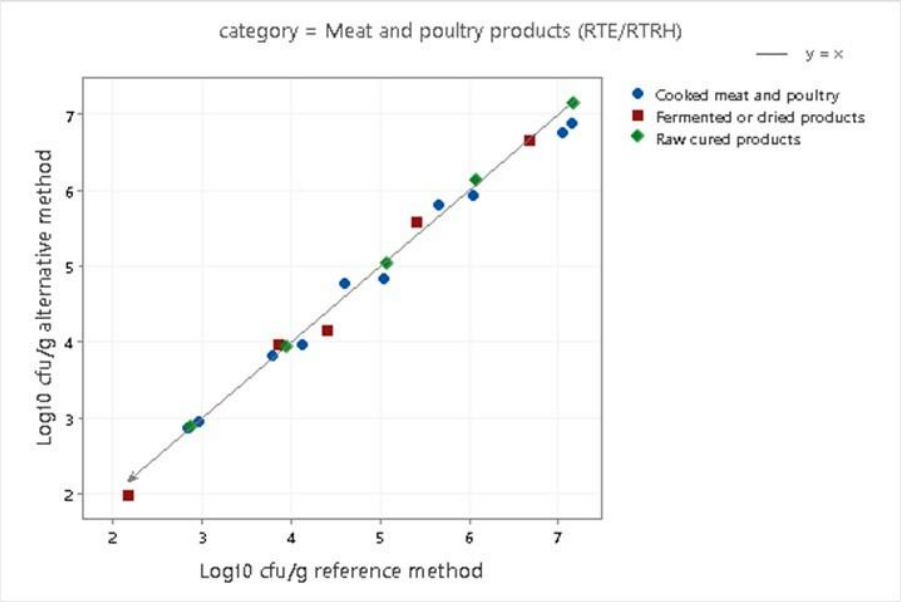


Figure 3- Scatter plot of the reference method versus alternative method results for L. monocytogenes in Dairy products

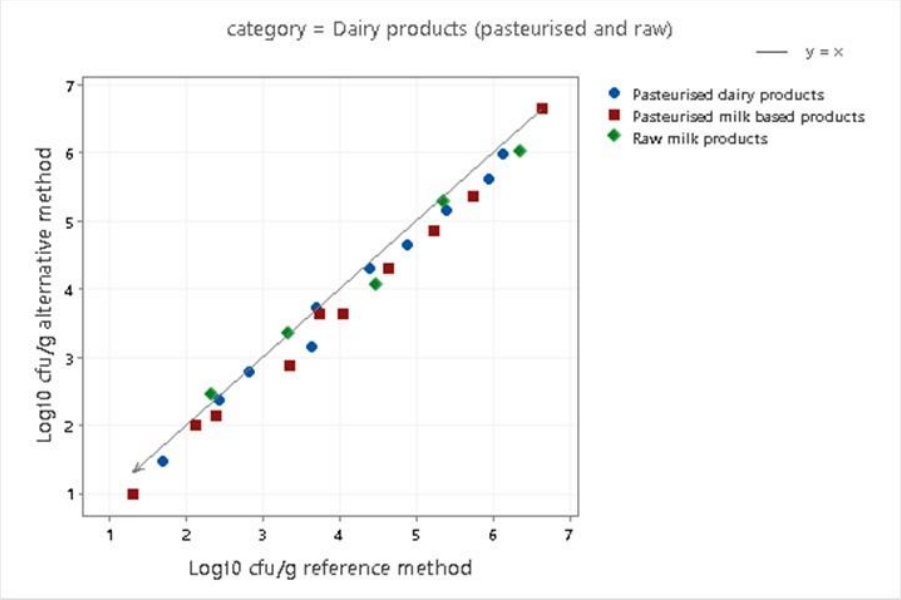


Figure 4- Scatter plot of the reference method versus alternative method results for L. monocytogenes in Fresh produce and fruit

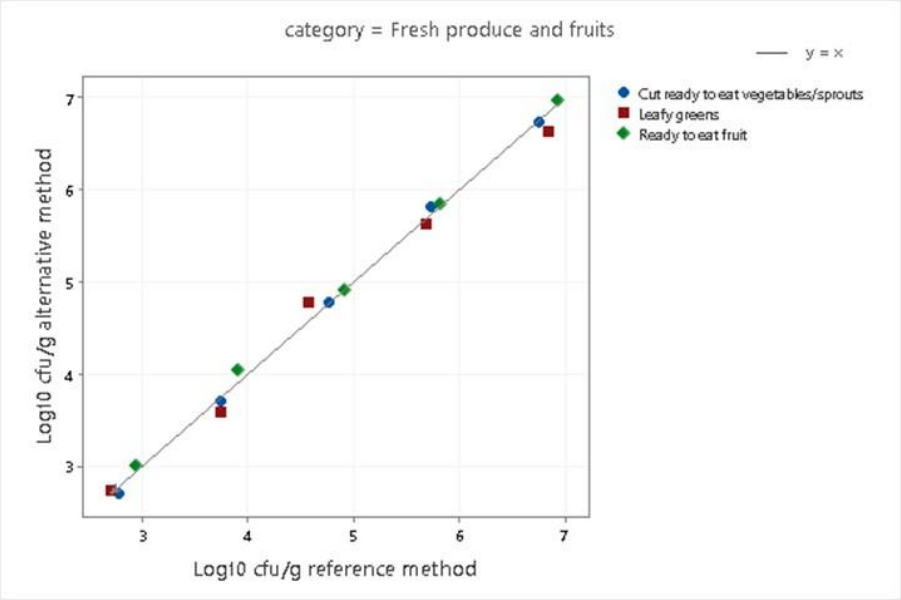


Figure 5- Scatter plot of the reference method versus alternative method results for L. monocytogenes in Seafood and fishery products

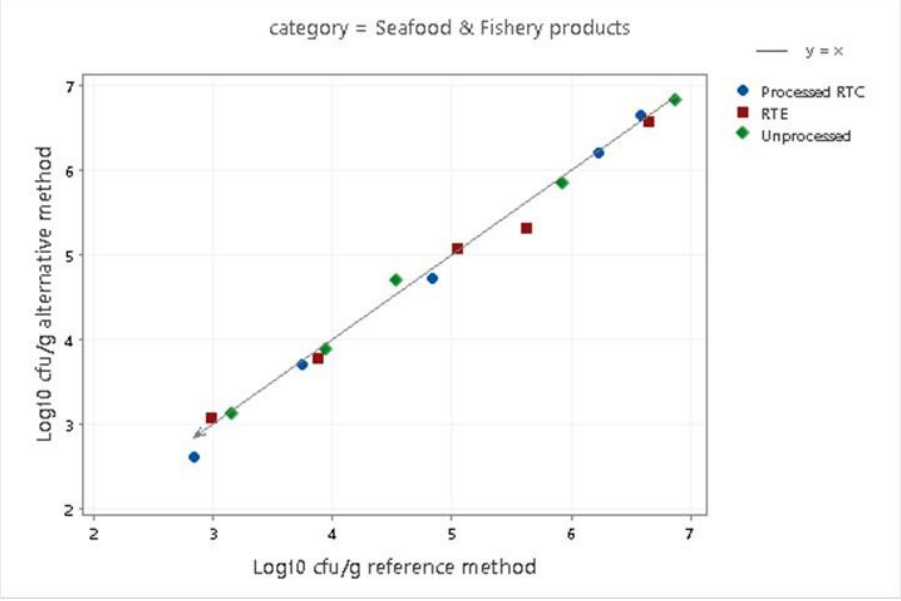


Figure 6- Scatter plot of the reference method versus alternative method results for L. monocytogenes in Multicomponents foods

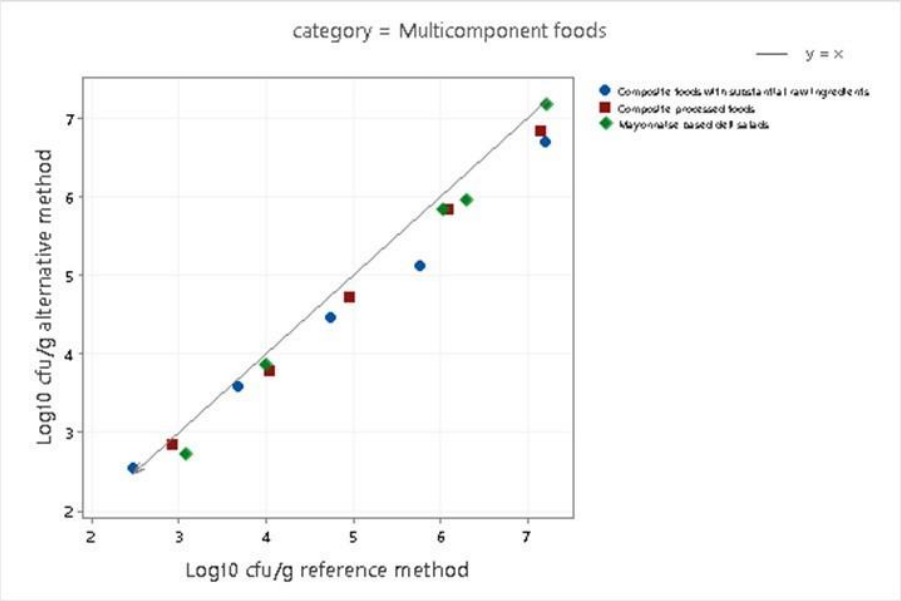


Figure 7- Scatter plot of the reference method versus alternative method results for L. monocytogenes in Environmental samples

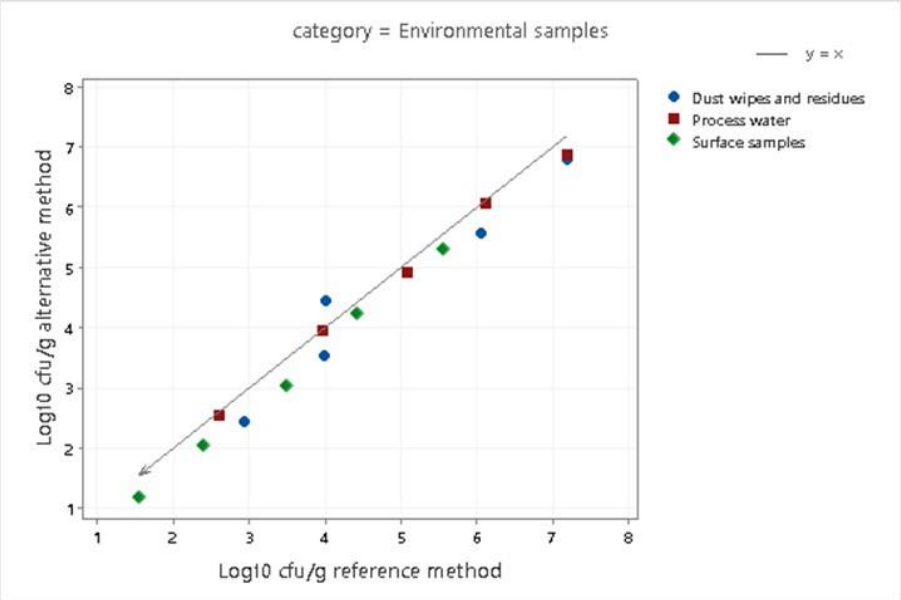
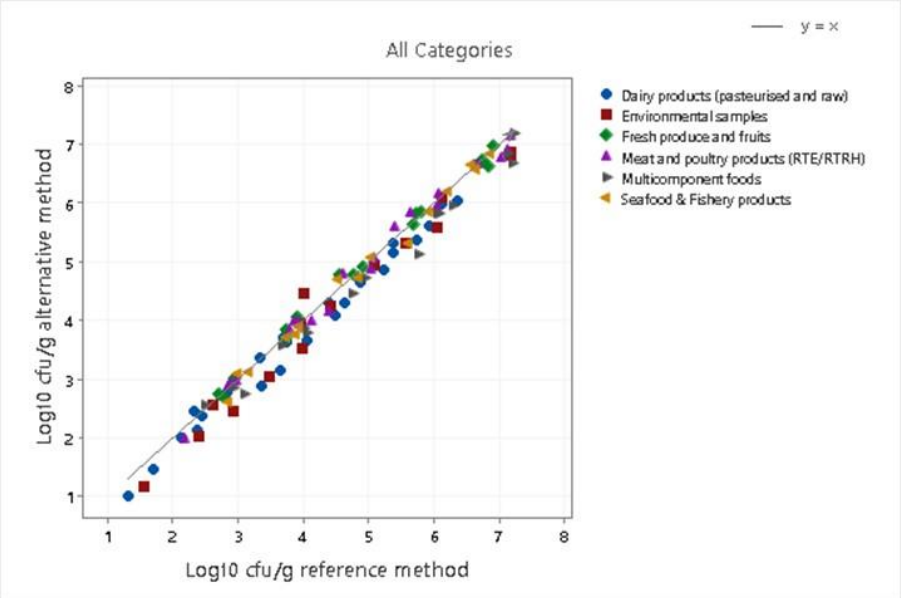


Figure 8 - Scatter plot of the reference method versus alternative method results for all the categories for L. monocytogenes



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 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 9.

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

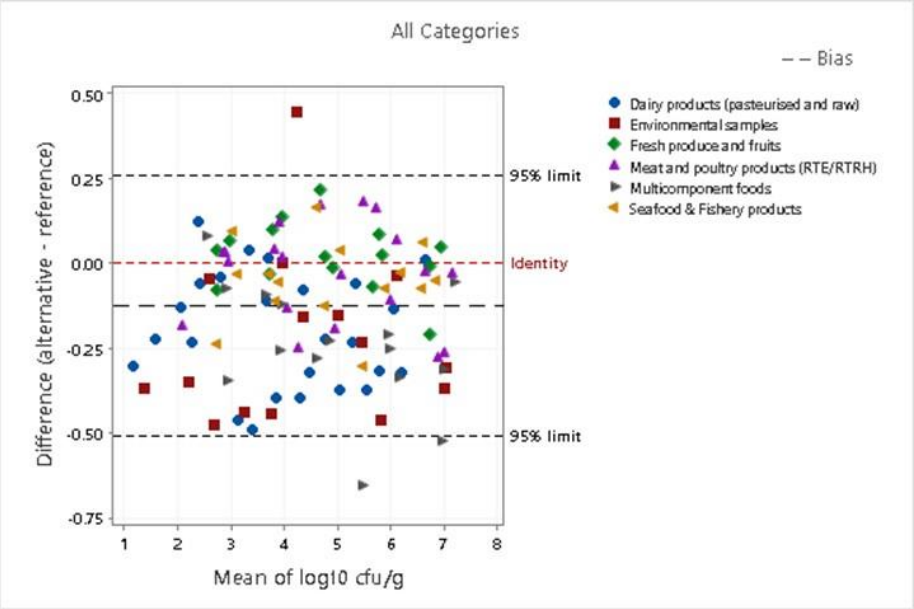
Table 2 - Summary of the calculated values per category plates.

Category.	**n**	$\bar{D}$	**sD**	**95% Lower limit**	**95% Upper limit**
Dairy products (pasteurised and raw)	25	-0.203	0.170	-0.561	0.154
Environmental samples	15	-0.226	0.247	-0.773	0.321
Fresh produce and fruits	15	0.022	0.101	-0.201	0.246
Meat and poultry products (RTE/RTRH)	20	-0.036	0.145	-0.347	0.276
Multicomponent foods	15	-0.244	0.186	-0.656	0.167
Seafood & Fishery products	15	-0.050	0.119	-0.314	0.214
All Categories	105	-0.126	0.193	-0.510	0.258

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

The Bland-Altman difference plot for all the samples is given Figure 9.

Figure 9 – Bland-Altman difference plot for all the samples



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

Category	**Type**	**Item**	**N° Sample**	**Reference method Log cfu/g**	**Alternative method Log cfu/g**	**Mean Log cfu/g**	**Diff Alt – ref**
Mulitcomponent food	Substantial raw ingredients	Cheese coleslaw	M9	5.77	5.11	5.44	-0.6
Mulitcomponent food	Substantial raw ingredients	Egg and bacon salad	M10	7.20	6.68	6.94	-0.5
Environmental samples	Dusts and residues	Conveyor belt	E14	4.00	4.45	4.22	0.45

Comments

The Bland Altman showed good agreement between the Reference method and the Alternative method. There were 3 data points from a total of 90 data points which were outside of the accepted limits representing two different categories.

There was a slight negative bias for the alternate method with an overall bias from all the categories of -0.115 which means that overall the alternate method gave good agreement with the reference method. For 3 of the categories there was a negative bias of -0.203 to -0.244. The categories affected were Dairy, Environmental and Multicomponent. A root cause analysis was carried out to determine possible reasons for the negative bias seen in the alternative method, although no explanation for this negative devition was identifed.

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 in the scatterplots and Bland Altman analyses.

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 4)

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 4- Categories, types, items, strains and inoculation levels for accuracy profile study

Category	Types	Strain of *L. monocytogenes*	Target Level
Meat and poultry products (RTE/RTRH)	Cooked sliced chicken (batch 1 and 2)	*L. monocytogenes* 3b (CRA 1168 from cooked turkey)	100-250 cfu /g
			1000- 5,000 cfu/g
			10,0000- 60,0000 cfu/g
Dairy products (pasteurised and raw)	Raw milk (batch 1 and 2)	*L. monocytogenes* 4b (CRA 1177 from ice-cream)	100-250 cfu /g
			1000- 5000 cfu/g
			10,0000- 60,0000 cfu/g
Fresh produce and fruits	Bagged salads (batch 1 and 2)	*L. monocytogenes* 1/2a (CRA 1102 from lettuce)	500-1,000 cfu /g
			5,000- 10,000 cfu/g
			10,0000- 50,0000 cfu/g
Seafood & Fishery products	RTC frozen fishcakes (batch 1 and 2)	*L. monocytogenes* (CRA 5219) from salmon fish cakes	100-250 cfu /g
			1000- 10,000 cfu/g
			100,000- 1,000,000 cfu/g
Multicomponent foods	Pasta salad (batch 1 and 2)	*L. monocytogenes* 3c (CRA 1173 from chicken and lettuce sandwich)	100-250 cfu /g
			1000- 5000 cfu/g
			10,0000- 50,0000 cfu/g
Environmental samples	Process water (batch 1 and 2)	*L. monocytogenes* 4a (CRA 1191 industrial isolate)	100-250 cfu /g
			500- 1,000 cfu/g
			50,000- 50,0000 cfu/g

Preparation of samples were done as a bulk inoculation. A 100g sample was inoculated with 1ml of appropriate dilution of inoculating strain and homogenised by hand massaging or stomaching to evenly

distribute the inoculum. For all matrices, the 100g samples was inoculated and stored at 2-8°C for 48-72h prior to analysis, or at -20°C for 2 weeks for frozen foods.

Five separate 10g test portions were removed from the bulk sample and mixed with appropriate diluent (BPW ISO formulation) and enumerated on both methods.

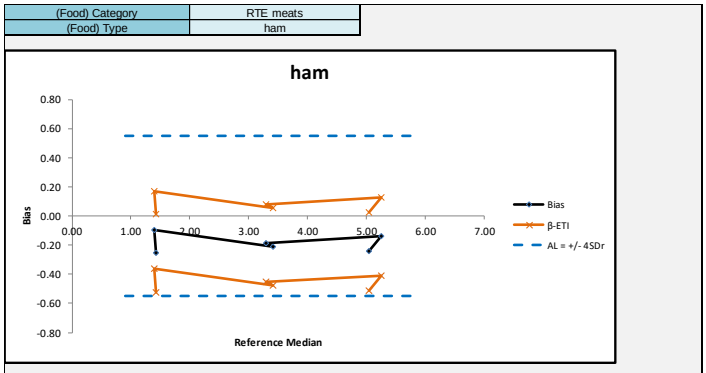
All results were tabulated, calculated and interpreted according to ISO 16140-2.

3.2.2 Calculations and interpretation of accuracy profile study

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

The statistical results and the accuracy profiles are provided Figures 10-15.

Figure 10 – Accuracy profile for reference method versus alternative method results for L. monocytogenes in Meat and poultry products

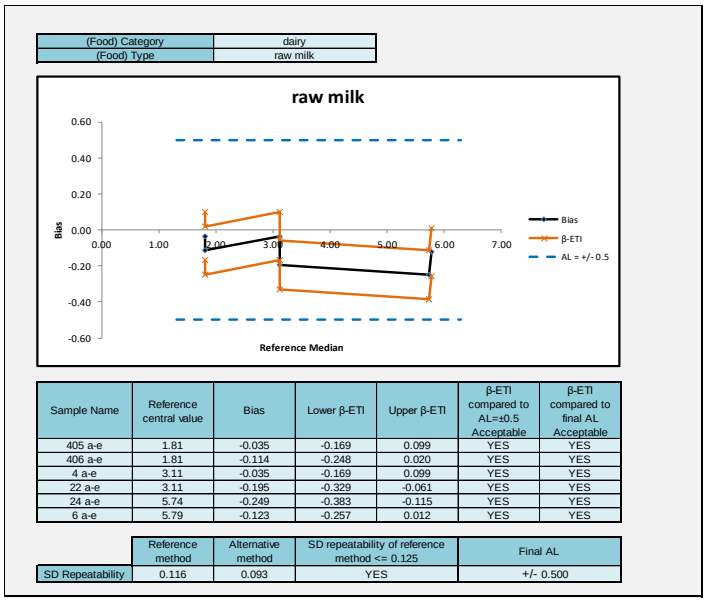


(Food) Category	RTE meats
(Food) Type	ham

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	1.43	-0.255	-0.522	0.012	NO	YES
19 a-e	1.40	-0.097	-0.364	0.170	YES	YES
2 a-e	3.41	-0.211	-0.478	0.056	YES	YES
20 a-e	3.30	-0.187	-0.454	0.080	YES	YES
3 a-e	5.26	-0.141	-0.408	0.126	YES	YES
21 a-e	5.05	-0.243	-0.510	0.024	NO	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.137	0.185	NO	+/- 0.548

Figure 11– Accuracy profile for reference method versus alternative method results for L. monocytogenes in Dairy products

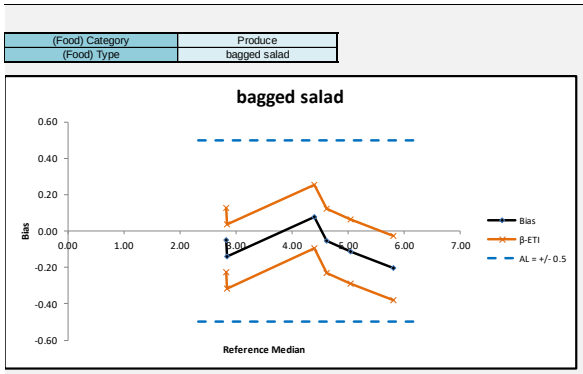


(Food) Category	dairy
(Food) Type	raw milk

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
405 a-e	1.81	-0.035	-0.169	0.099	YES	YES
406 a-e	1.81	-0.114	-0.248	0.020	YES	YES
4 a-e	3.11	-0.035	-0.169	0.099	YES	YES
22 a-e	3.11	-0.195	-0.329	-0.061	YES	YES
24 a-e	5.74	-0.249	-0.383	-0.115	YES	YES
6 a-e	5.79	-0.123	-0.257	0.012	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.116	0.093	YES	+/- 0.500

Figure 12– Accuracy profile for reference method versus alternative method results for L. monocytogenes in Fresh produce and fruits

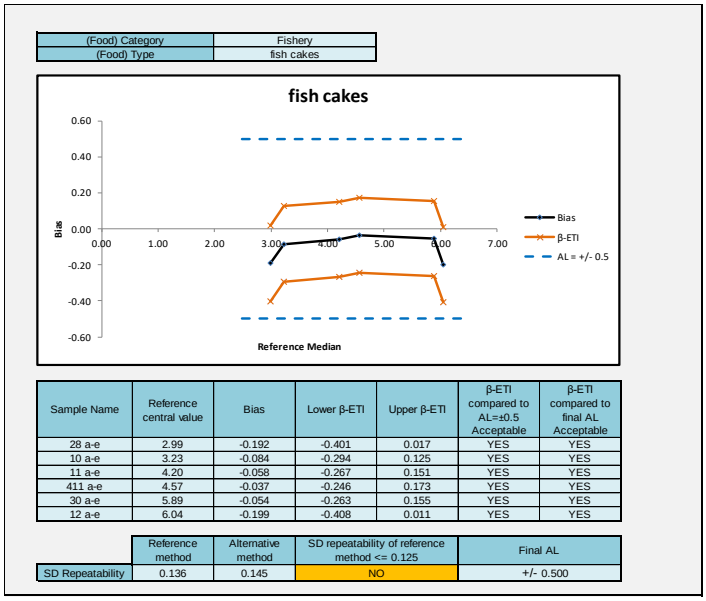


(Food) Category	Produce
(Food) Type	bagged salad

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	2.83	-0.049	-0.225	0.127	YES	YES
7 a-e	2.84	-0.140	-0.316	0.036	YES	YES
8 a-e	4.40	0.079	-0.097	0.255	YES	YES
26 a-e	4.62	-0.055	-0.231	0.121	YES	YES
27 a-e	5.04	-0.112	-0.288	0.064	YES	YES
9 a-e	5.81	-0.204	-0.380	-0.028	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.133	0.122	NO	+/- 0.500

Figure 13 – Accuracy profile for reference method versus alternative method results for L. monocytogenes in Seafood and Fishery products

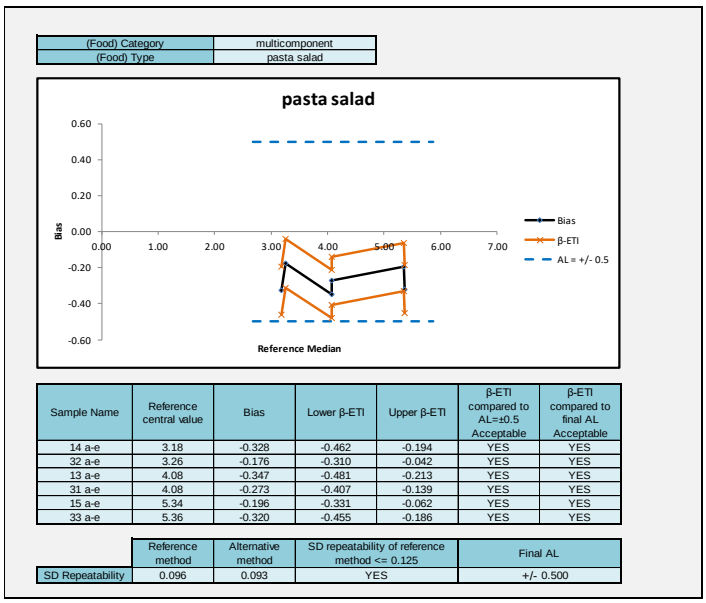


(Food) Category	Fishery
(Food) Type	fish cakes

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
28 a-e	2.99	-0.192	-0.401	0.017	YES	YES
10 a-e	3.23	-0.084	-0.294	0.125	YES	YES
11 a-e	4.20	-0.058	-0.267	0.151	YES	YES
411 a-e	4.57	-0.037	-0.246	0.173	YES	YES
30 a-e	5.89	-0.054	-0.263	0.155	YES	YES
12 a-e	6.04	-0.199	-0.408	0.011	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.136	0.145	NO	+/- 0.500

Figure 14 – Accuracy profile for reference method versus alternative method results for L. monocytogenes in Multicomponent Foods

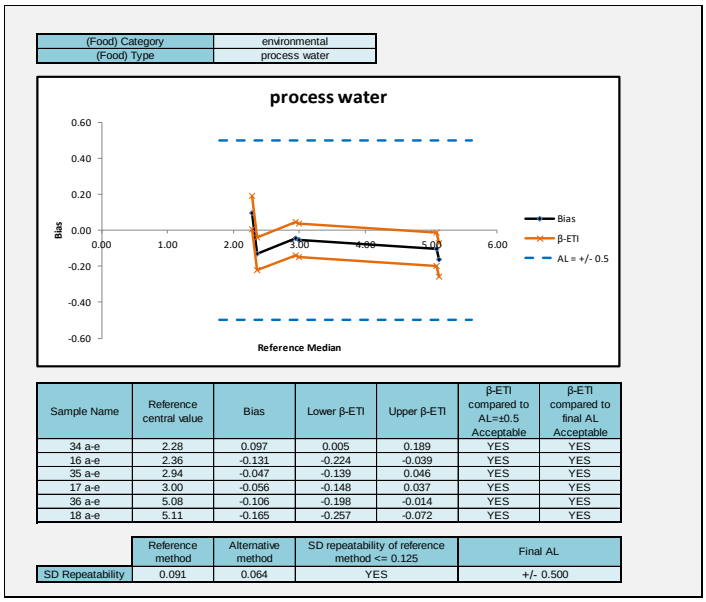


(Food) Category	multicomponent
(Food) Type	pasta salad

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
14 a-e	3.18	-0.328	-0.462	-0.194	YES	YES
32 a-e	3.26	-0.176	-0.310	-0.042	YES	YES
13 a-e	4.08	-0.347	-0.481	-0.213	YES	YES
31 a-e	4.08	-0.273	-0.407	-0.139	YES	YES
15 a-e	5.34	-0.196	-0.331	-0.062	YES	YES
33 a-e	5.36	-0.320	-0.455	-0.186	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.096	0.093	YES	+/- 0.500

Figure 15 – Accuracy profile for reference method versus alternative method results for L. monocytogenes in Environmental samples



(Food) Category	environmental
(Food) Type	process water

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
34 a-e	2.28	0.097	0.005	0.189	YES	YES
16 a-e	2.36	-0.131	-0.224	-0.039	YES	YES
35 a-e	2.94	-0.047	-0.139	0.046	YES	YES
17 a-e	3.00	-0.056	-0.148	0.037	YES	YES
36 a-e	5.08	-0.106	-0.198	-0.014	YES	YES
18 a-e	5.11	-0.165	-0.257	-0.072	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.091	0.064	YES	+/- 0.500

Five of the six categories met the AL of 0.5log (dairy, fresh produce, fish and seafood, multicomponent foods, environmental samples. 1 category (meat and poultry required the new AL to be calculated. All data met the new AL value of 0.548.

The multicomponent foods (pasta salad) showed a slight negative bias for the alternate method although all samples met the 0.50 log AL. Other categories did not show any systematic bias between methods

Conclusion accuracy profile study

The accuracy of the Alternative method (CDLM)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:

Fifty seven strains of *L. monocytogenes* were analysed. The analysis was carried out once with the Alternative method, the Reference method and a non selective method. All inclusivity strains were grown

overnight in BHI and enumerated on the reference method and alternative method at a level 10- 100 times greater than the minimum level of detection following protocols described in Annex A.

Exclusivity:

Fifty seven strains of non-target organisms was analysed. This consisted of 24 strains of non-*Listeria* species and 33 strains of non-*monocytogenes Listeria* species.

Each test was performed once with the Alternative method, the Reference method and a non slective agar.

3.3.2 Results inclusivity and exclusivity study

Inclusivity

For the inclusivity study , all 57 strains of *L.monocytogenes* gave typical colonies on the alternate and reference method and all colonies were confirmed by MALDI-Tof.

The level enumerated on the reference method and alternative method were similar with no negative or positive bias shown.

Exclusivity

For the exclusivity strains, there were 2 of the 24 non non-*Listeria* species which gave typical colonies on CDLM but not the ISO reference method. These were *Bacillus cereus* CRA 16662 isolated from dried potato and *Bacillus thuringiensis* CRA 16616 isolated from broccoli. The final confirmation result confirmed the identity of the strain as the target *Bacillus* species and therefore showed the correct results as negative for *L.monocytogenes*.

For the 33 non-*monocytogenes Listeria* strains, several gave typical colonies on both CDLM and the reference method agars. These were as follows:

- 2 *L.innocua* strains; CRA 1110 from pate and CRA1111 from camembert
- 9 *L.ivanovii* strains; CRA 1120 from radish, CRA 1122 from salami, CRA1123 from soft cheese, DSM 12491 (*L.ivanovii* subsp. londoniensis) from food and 4 strains from industrial food environment (CRA 1835, CRA 3925, CRA 5931, CRA 6085)
- 1 *L.murrayi* strain; CRA 8603 from industrial food environment
- 1 *L.seeligeri* strain; CRA 1145 from corned beef
- 1 *L.welshimeri* strain; CRA 1130 from raw chicken

In addition, 1 strain of *L.weihenstephanensis* CRA 16874 from a water plant gave typical colonies on CDLM but not the reference agar.

All colonies were correctly identified by the Bruker Biotype MALDI-Tof and therefore gave the correct result. This shows the importance of using confirmation procedures for identification of *L.monocytogenes* from other *Listeria* species which have similar morphological characteristics on selective agar plates.

3.3.3 Conclusion

All 57 *Listeria* strains were correctly identified following the alternative method.

All 57 non target strains were correctly identified as non-*L.monocytogenes* following the alternative method detection and confirmation procedures.

The alternative method gave comparable performance to the reference method and is therefore selective and specific to *Listeria monocytogenes*.

3.4 Limit of Quantification

The LOQ applies only to instrumental methods. It does not apply to methods based on counting visible colonies. It may also not apply to instrumental methods where it is not possible to get blank samples e.g. instrumental methods for total plate counts.

The alternate method is based on visible colonies.

The LOQ does not have to be calculated for the alternative method in this study.

3.5 Conclusion (MCS)

Overall, the conclusions for the Method Comparison are:

- The alternative method CDLM enumeration method for *L. monocytogenes* shows satisfactory results for relative trueness.
- The alternative method CDLM enumeration for *L. monocytogenes* shows satisfactory results for accuracy profile.
- The alternative method CDLM enumeration is selective and specific to *Listeria monocytogenes*.

4 Interlaboratory study

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

4.1 Study organization

Collaborators
Samples were sent to 11 laboratories.

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

Sample preparation
Samples were prepared and inoculated on 8 November 2021 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 13 : Contamination levels

Contamination level	**Sample code**
Uninoculated	4
Low (10^2 cfu/g)	1
Low (10^2 cfu/g)	5
Medium (10^4 cfu/g)	2
Medium (10^4 cfu/g)	6
High (10^6 cfu/g)	3
High (10^6 cfu/g)	7

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 9 November 2021 and were received within 24 h to 72 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 15 November 2021. Stability studies had been conducted to show that the required level of target organisms would be present after 7 and 8 days frozen storage. The expert lab analysed a set of samples on Monday 15 November 2021.

Analysis of Samples

Collaborative study laboratories and the expert laboratory carried out the analyses on Monday 15 November 2021. The analyses by the reference method and the alternative method were performed on the same day.

Experimental parameters controls

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 the inoculation. All the results were negative.

Strain stability during transport
Duplicate samples inoculated at low, medium and high levels were tested for enumeration of *L. monocytogen* 7 and 8 days storage at -18°C. Samples were thawed under controlled conditions prior to analysis. The data shows good s under the storage regime tested (Table 14).

Table 14 - L.monocytogenes stability in the matrix

	Reference method cfu/g						Alternative method cfu/g					
	Low level		Medium level		*High level*		Low level		Medium level		*High level*	
Day	a	b	a	b	a	b	a	b	a	b	a	b
Day 0	$1.1x10^3$	$1.5x10^3$	$1.2x10^5$	$1.2x10^5$	$8x10^5$	$8x10^5$	964	855	$5.8x10^4$	$6.7x10^4$	$3.1x10^5$	$6x10^4$
Day 7	$1.5x10^3$	$1.1x10^3$	$1.0x10^5$	$9.5x10^4$	$7x10^5$	$7.3x10^5$	$1.2x10^3$	$1.2x10^3$	$1x10^5$	$7.5x10^4$	$6x10^5$	$7.1x10^4$
Day 8	$1.3x10^3$	$1.7x10^3$	$1.3x10^5$	$1.1x10^5$	$9.8x10^5$	$8.6x10^5$	$1.3x10^3$	$1.2x10^3$	$1x10^5$	$1.1x10^5$	$8.4x10^5$	$6x10^5$

Logistic conditions
The temperatures measured at receipt by the collaborators, the temperatures registered by the thermo-probe, and the rece are given in Table 15.

Table 15 - Sample temperatures at receipt

Collaborator	Temperature measured by probe (°C)	Temperature measured at receipt (°C)	Receipt date and time	State of the package and samples at receipt	Analysis date
1	4.7	N/A	12/11/2021 13:45	1x box damaged	15/10/2021
2	5.5	5.3	10/11/2021 12:00	Good	15/10/2021
3	2.7	N/A	11/11/2021 09:30	Satisfactory	15/10/2021
4	Data not received		11/11/2021 15:00	Data not received	15/10/2021
5	Data not received				
6	4.5	7.5	10/11/2021	OK	15/10/2021
7	5.9	6.6	10/11/2021 14:00	Good	15/10/2021
8	Data not received	7.0	11/11/2021	Data not received	15/10/2021
9	Data not received				
10	N/A	Water blank frozen	10/11/2021 10:00	Good	15/10/2021
11	N/A	Water blank frozen	10/11/2021 10:00	Good	15/10/2021

No problem was encountered during the transport or at receipt for 9 out of 11 collaborators. All the samples were delivere and in appropriate conditions to 11 laboratories. Temperatures during shipment and at receipt were all correc the study was not recieved from 2 participants (labs 5 and 9) and therefore these were excluded from the study.

Calculation and summary of data

MicroVal Expert laboratory results
The results obtained by the expert laboratory are given in Table 16.

Table 16 – Results obtained by the expert lab.

Level	Reference method cfu per g	Alternative method cfu per g
Blank	<10	<10
Low	1.20E+03	1.00E+03
Low	1.50E+03	945
Medium	9.30E+03	9.00E+03
Medium	8.50E+03	8.10E+03
High	8.40E+04	6.30E+04
High	7.10E+04	4.50E+04

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 17.

The accuracy profile plot is shown in Figure 16 and the statistical analysis of the data shown in Table 18.

Table 17: 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	3.49	3.93	3.49	2.93
2	low	3.04	3.04	2.67	3.08
3	low	3.95	4.66	3.46	4.72
4	low	2.66	2.76	2.57	2.81
5	low	Data not received			
6	low	3.48	2.62	2.88	2.81
7	low	2.95	3.15	2.76	3.08
8	low	2.90	2.92	2.86	2.77
9	low	Data not received			
10	low	2.87	3.04	2.90	3.00
11	low	3.04	3.18	3.04	3.15
1	medium	4.57	4.49	4.38	4.34
2	medium	3.94	3.99	3.40	3.70

3	medium	5.11	4.99	4.77	4.98
4	medium	3.72	3.71	3.77	3.96
5	medium	Data not received			
6	medium	4.08	4.04	4.00	3.92
7	medium	3.86	3.70	3.76	3.94
8	medium	3.98	3.89	3.82	3.86
9	medium	Data not received			
10	medium	3.78	4.04	3.83	3.82
11	medium	3.98	4.08	3.83	3.91
1	high	5.53	5.59	5.41	5.40
2	high	4.86	4.66	4.88	4.72
3	high	5.89	5.89	6.08	5.97
4	high	4.46	4.85	4.71	4.72
5	high	Data not received			
6	high	5.94	4.79	5.65	4.65
7	high	5.11	4.94	4.95	4.94
8	high	3.88	3.81	4.82	4.64
9	high	Data not received			
10	high	5.11	4.61	4.95	4.72
11	high	5.18	4.77	4.97	4.73
1	blank	<1		<1	
2	blank	<1		<1	
3	blank	<1		<1	
4	blank	<1		<1	
5	blank	<1		<1	
6	blank	<1		<1	
7	blank	<1		<1	
8	blank	<1		<1	
9	blank	<1		<1	
10	blank	<1		<1	
11	blank	<1		<1	

Figure 16. Accuracy profile of Nissui Compact Dry LM from the ILS

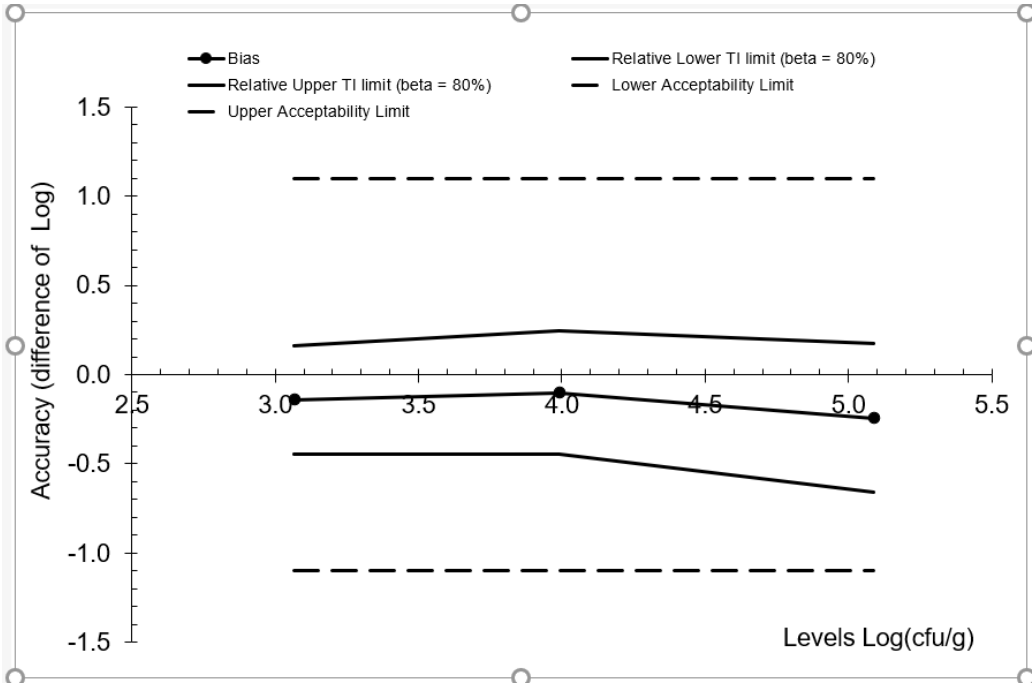


Table 18. Statistical analysis of the ILS data according to the ISO spreadsheet

Levels	Alternative method Low	Medium	High	Reference method Low	Medium	High
Target value	3.066	3.990	5.090			
Number of participants (K)	8	8	8	8	8	8
Average for alternative method	2.925	3.890	4.847	3.066	3.990	5.090
Repeatability standard deviation (sr)	0.206	0.105	0.087	0.252	0.087	0.126
Between-labs standard deviation (sL)	0.073	0.212	0.267	0.227	0.239	0.371
Reproducibility standard deviation (sR)	0.218	0.237	0.281	0.339	0.255	0.392
Corrected number of dof	14.541	8.529	7.700	11.842	7.876	7.771
Coverage factor	1.388	1.466	1.483			
Interpolated Student t	1.343	1.390	1.402			
Tolerance interval standard deviation	0.2257	0.2495	0.2975			
Lower TI limit	2.622	3.543	4.430			
Upper TI limit	3.228	4.237	5.264			
Bias	**-0.141**	**-0.100**	**-0.242**			
Relative Lower TI limit (beta = 80%)	**-0.444**	**-0.447**	**-0.659**			
Relative Upper TI limit (beta = 80%)	**0.162**	**0.246**	**0.175**			
Lower Acceptability Limit	**-1.10**	**-1.10**	**-1.10**			
Upper Acceptability Limit	**1.10**	**1.10**	**1.10**			
New acceptability limits may be based on reference method pooled variance						
Pooled repro standard dev of reference	0.333					

TRUE
FALSE

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

5 Overall conclusions of the MCS/ILS study

- The alternative method CDLM for enumeration of *Listeria monocytogenes* shows satisfactory results for relative trueness.
- The alternative method CDLM for enumeration of *Listeria monocytogenes*. shows satisfactory results for accuracy profile.
- The alternative method CDLM for enumeration of *Listeria monocytogenes* is selective and specific.
- The alternative method CDLM for enumeration of *Listeria monocytogenes* shows satisfactory performance in the ILS.
- The alternative method CDLM for enumeration of *Listeria monocytogenes* 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)

Date 25/01/2022

Signature Suzanne Jordan

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