Campden BRI group:
Campden BRI (registered no. 510618)
Campden BRI (Chipping Campden) Limited (registered no. 3836922)
Campden BRI (Nutfield) (registered no. 2690377)

Registered office:
Station Road ◆ Chipping Campden ◆ Gloucestershire ◆ GL55 6LD ◆ UK

Campden BRI
food and drink innovation

CONFIDENTIAL

HyServe GmbH & Co. KG
Hechenrainer Strasse 24
82449 Uffing
Germany

MicroVal Study 2007LR05 renewal

Methods comparison study report including evaluation of existing ILS data : summary report

ISO 16140-2:2016 validation study of Compact Dry EC for enumeration of *Escherichia coli* in foods: renewal study for alignment with the requirements of the revised ISO 16140-2:2016 standard

Quantitative method

This report includes 23 pages
Only copies including the totality of this report are authorized.

Version 1
14th June 2017

Contact details:

Linda Everis (linda.everis@campdenbri.co.uk) and Gail Betts (gail.betts@campdenbri.co.uk)
◆- Microbiology Department - Campden BRI (Chipping Campden) Limited
◆ Tel: +44(0)1386 842071 ◆ Fax: +44(0)1386 842100
www.campdenbri.co.uk

Report issued and authorised by:

Campden BRI (Chipping Campden) Limited
Dr Gail Betts ◆ Section Manager

Our ref 2007LR05 renewal 1
Page count: 23

Foreword

Quality assurance documents related to this study can be consulted upon request from Hyserve GmbH & Co KG.

The technical protocol and the result interpretation were carried out according to the EN ISO 16140-2:2016 and the MicroVal technical rules.

✓ **Company**:	HyServe GmbH & Co. KG Hechenrainer Strasse 24, 82449 Uffing, Germany
✓ **Expert Laboratory**:	Campden BRI Station Road, Chipping Campden Gloucestershire, GL55 6LD (UK)
✓ **Studied method:**	Compact Dry EC for enumeration of *Escherichia coli* in foods
✓ **Validation standard**:	EN ISO 16140-2:2016 Microbiology of the food chain —Method validation —Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method
✓**Reference method**:	ISO 16649-2: 2001 Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of β-glucuronidase positive *Escherichia coli* — Part 2: Colony-count technique at 44°C using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide
✓ **Scope:**	A broad range of foods
✓ **Certification organization:**	MicroVal

1. INTRODUCTION

Please note that this study is for a renewal of the Compact Dry EC to align it with the requirements of the ISO 16140-2:2016 standard. The original study was done according to ISO 16140:2003 and did not confirm to the revised study design to test for relative trueness and accuracy profile. All relevant data has therefore been generated in this renewal study. The design of the Inter-laboratory study (ILS) is the same for the 2003 and 2016 versions of ISO16140 and therefore it is acceptable to re-analyse the existing ILS data using the new statistical approach outlined in ISO16140-2:2016. This was done successfully and the results of the ILS analysis are included in this report.

1.1 Alternative method

Compact Dry (Nissui Pharmaceutical Co. Ltd; supplied by Hyserve Gmbh & Co. KG) are ready-to-use dry media sheets comprising culture medium and a cold-soluble gelling agent, rehydrated by inoculating 1 ml diluted sample into the centre of the self-diffusible medium. This is a ready to use, chromogenic plate for the enumeration of *E.coli* and coliforms. These organisms are differentiated by the colony morphology. *E.coli* colonies form blue colonies and other coliforms form red colonies after the required incubation period. The *E.coli* count will therefore be based on a count of blue colonies.

1.2Scope

A broad range of foods.

1.3Restriction of use

None.

1.4 Reference method

The reference method corresponds to the standard ISO 16649-2: 2001 Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of β-glucuronidase positive *Escherichia coli* — Part 2: Colony-count technique at 44°C using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide
The flow diagram is given in Appendix 1.

1.5 Materials and equipment used

Test Kit Information

a) Test name – Compact Dry EC
b) Ordering information HyServe GmbH & Co. KG**,** Hechenrainerstr. 24, 82449 Uffing, Germany. Telefon: +49 (0) 8846 - 13 44, Telefax: +49 (0) 8846 - 13 42,
E-Mail: **info@hyserve.com**, Internet: **http://www.hyserve.com**

Additional supplies, reagents and equipment

a) Stomacher bags
b) Maximum recovery diluents (Peptone saline diluents)
c) Stomacher
d) Balance
e) Pipettes
f) Incubator – 44 ±1ºC
g) Colony counter

Standard Reference Materials

a. Cultures used in this study were obtained from the Campden BRI culture collection
b. Tryptone Bile x-glucuronide Agar (TBX, Oxoid CM0945)

1.6 Safety precautions

Follow the Good Laboratory Practices for running food microbiology analyses.

1.7 Additional information

None.

2 METHOD COMPARISON STUDY

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

2.1 Relative trueness study

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

2.1.1 Number and nature of the samples

Five categories were tested. The number of samples per tested category and type is provided in Table 1:

Table 1 – Categories and types tested

Category	Types	Number of samples
Milk and dairy products	Dry milk product e.g. milk powder, powder for milk based desserts, dried infant formula	5
	Dairy products e.g. ice-cream, yogurts, cream, hard cheese, soft cheese, raw milk cheese	5
	Pasteurised milk products e.g. skimmed, semi-skimmed, full fat and flavoured milks	5
Fresh produce and fruits	Cut ready to eat fruit e.g. fruit mixes, fruit juices	5
	Cut ready to eat vegetables e.g. Bagged pre-cut salads and shredded carrot, cabbage, vegetable juices	5
	Leafy greens/Sprouts e.g. soy, mung, alfalfa,	5
Raw poultry and meats (Combined category raw/ RTC meats and poultry)	Fresh poultry cuts e.g. turkey breast, turkey fillet	5
	Fresh mince e.g. lamb, beef, pork	5
	Processed ready to cook e.g. frozen patties, marinated kebabs, seasoned chicken breasts	5
Ready to eat foods (Combined category RTE/RTRH meats and poultry)	Ready to eat poultry e.g. turkey fillet, chicken sausage, pate	5
	Cooked fish products e.g. prawns, terrine, pate, smoked fish	5
	Cooked meat e.g. ham, salami, pate, corned beef	5
Multi component foods or meal components	Ready to re-heat refrigerated food e.g. cooked chilled foods, rice and pasta, products	5
	Ready to re-heat food frozen e.g. fries, pizza	5
	Composite foods with substantial raw ingredients e.g. .pasta salads, sandwiches, deli-salads	5

75 samples were analysed, leading to 75 interpretable results

2.1.2 Artificial and natural contamination of the samples

Artificial contamination was carried out by spiking or seeding protocols.
Injury efficiency was evaluated by enumerating the pure culture on selective and non-selective agars.

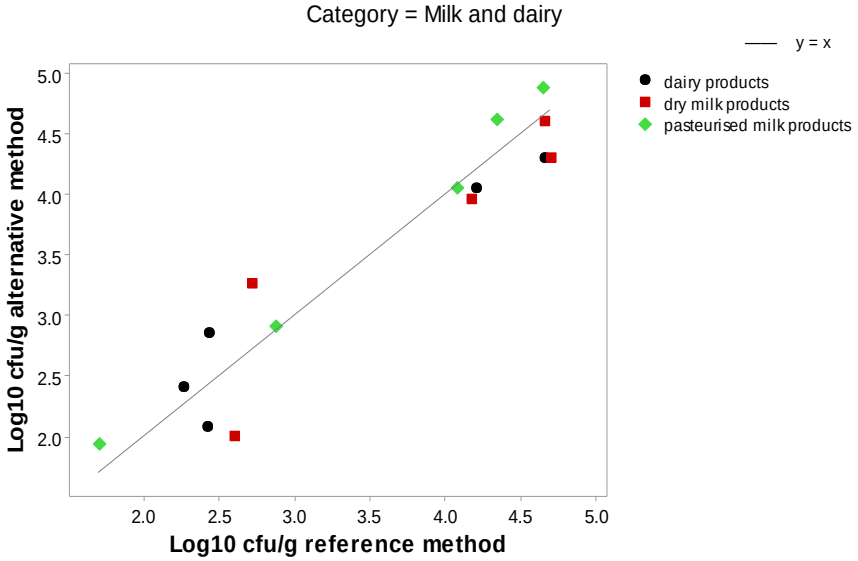
The observed injury measurements varied from 0.30 to more than 0.74 log cfu/g difference between non-selective and selective plates All 75 samples were artificially contaminated in this study because it was not possible to find naturally contaminated samples. In total 44 samples were screened for naturally present *E.coli* but none were found.

2.1.3 Calculation and interpretation

The data for each sample per category and for each sample in all categories were plotted. The line of identity was drawn on which all points would lie if the two methods gave identical results for each sample analysed.

The obtained data were analyzed using the scatter plot. The graphs are provided with the line of identity (y = x). The Figures 1a to 1e shows the data plotted per category and Figure 1f summarises all the data.

Figure 1 a: Dairy products



Campden BRI (Chipping Campden) Limited – part of the Campden BRI group
Station Road◆Chipping Campden◆Gloucestershire◆GL55 6LD◆UK
Providing services under an ISO 9001 registered quality management system

Figure 1b: Fresh produce

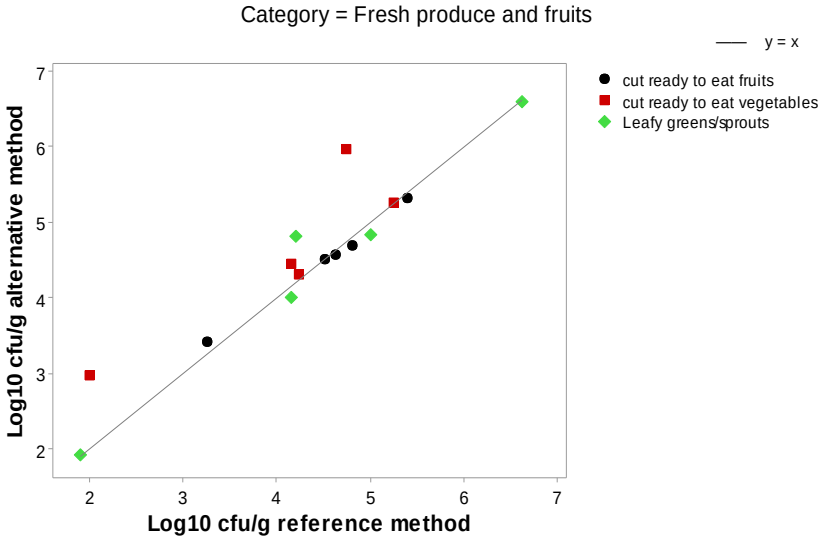


Figure 1c: Raw meat

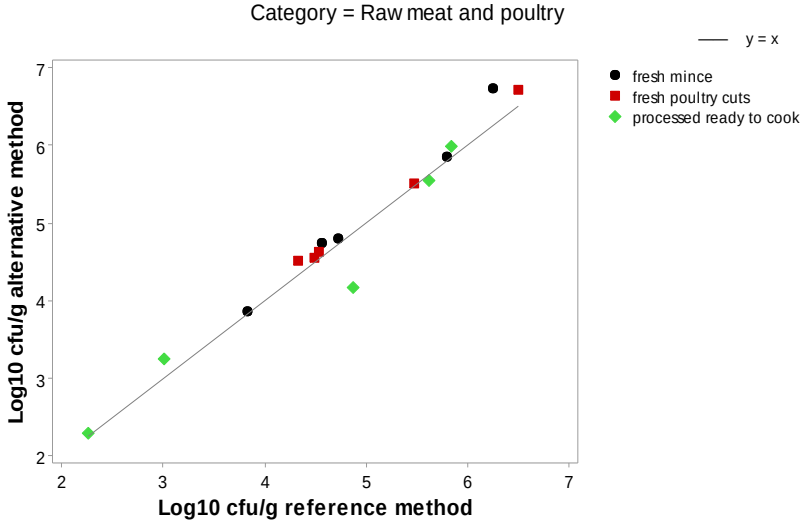


Figure 1d: Ready to eat Foods

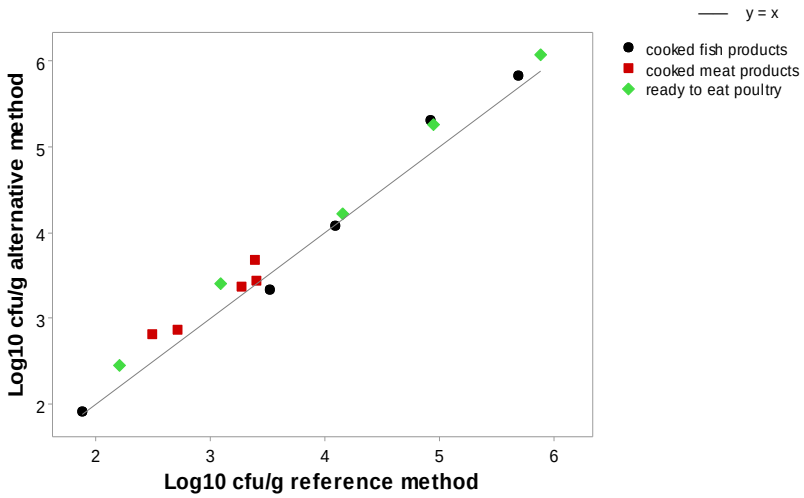


Figure 1e: Multi-component Foods

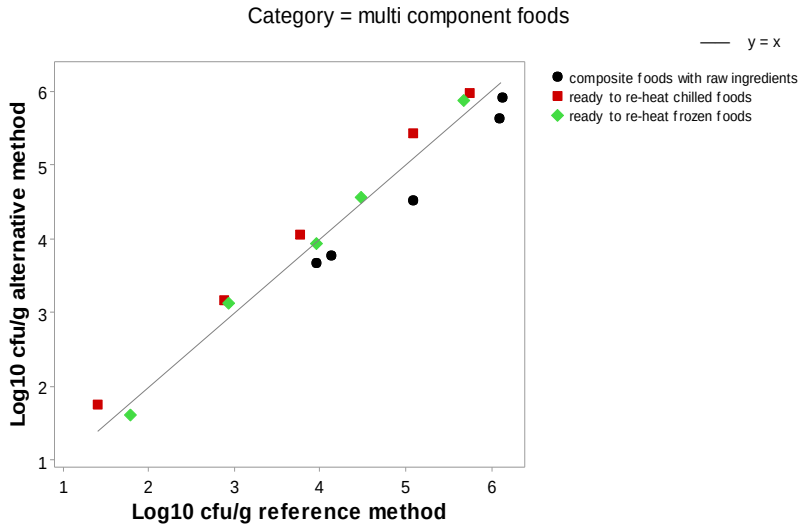
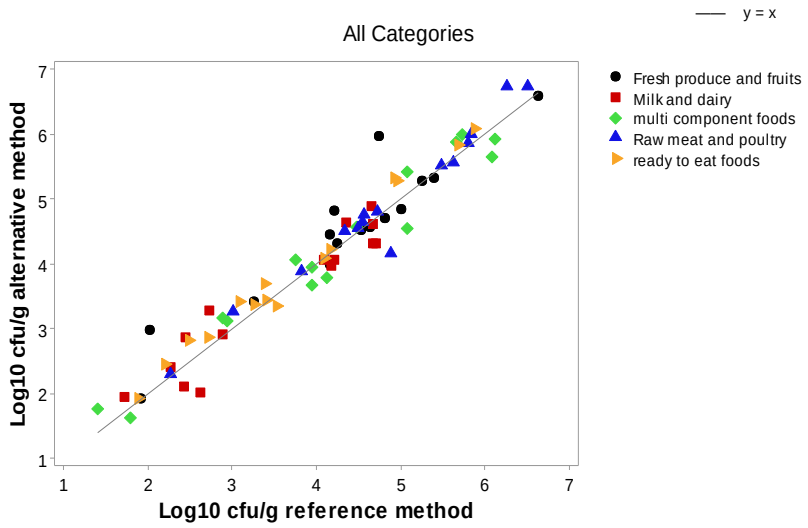


Figure 1f: All categories plot



According to ISO 16140-2:2016 6.1.2.3 the results of the scatter plot are interpreted based on a visual observation on the amount of bias and extreme results. The data appears acceptable on the whole but there is some evidence of a slight positive bias for the alternate method for the dairy products- pasteurized milk (Figure 1a), fresh produce- ready to eat vegetables (Figure1b), ready to eat foods including ready to eat poultry and cooked meat and multi component foods- ready to re-heat products (Figure 1d).

The positive bias was only slight as seen in Table 2, but samples from these categories were responsible for the positive outliers and positive discordant results (Tables 3 and 4).

The data was analysed as described in ISO 16140-2:2016 section 6.1.2.3 in order to produce the Bland –Altman difference plot.

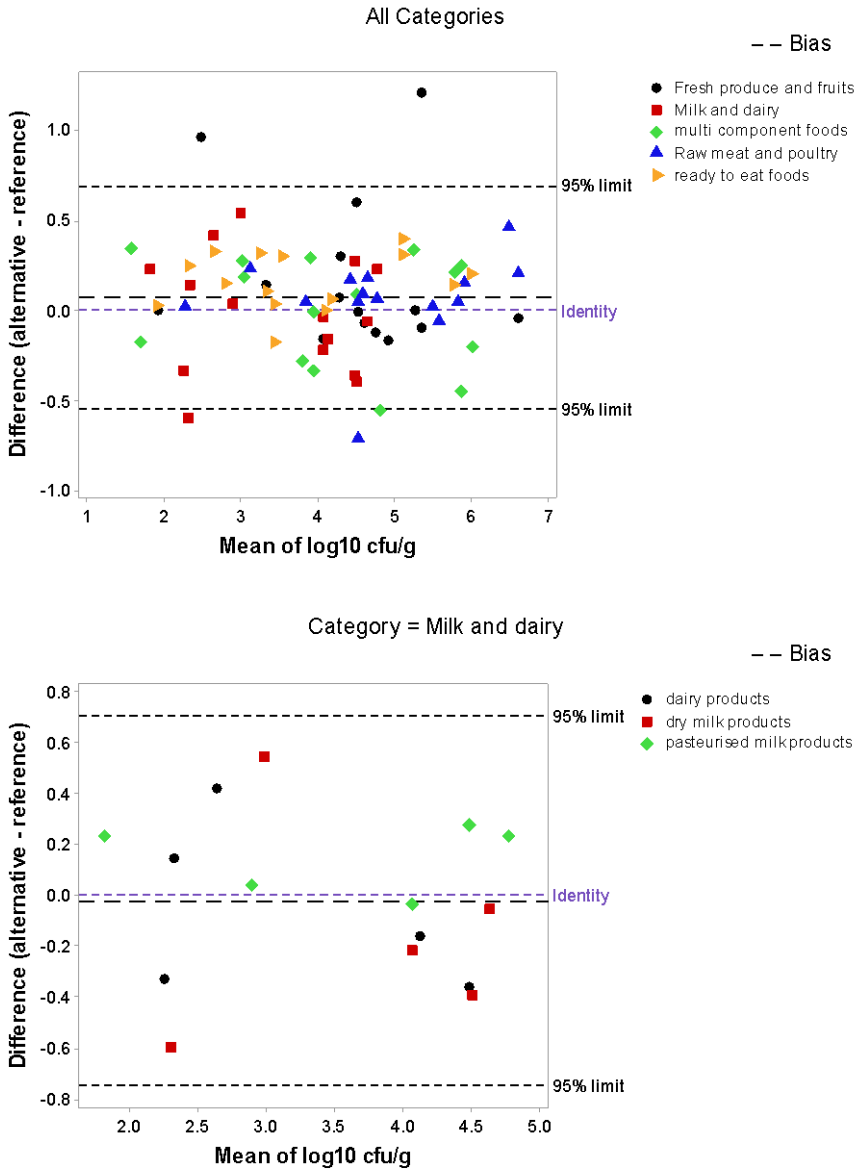
The average difference $\overline{D}$, the standard deviation of difference s_D and the limits of agreement were calculated per category and for all categories (Table 2).The data in Table 2 show the slight positive bias observed in the scatter plot figures with a bias of 0.17 for fresh produce and 0.16 for RTE foods. The overall bias for all categories was 0.073.

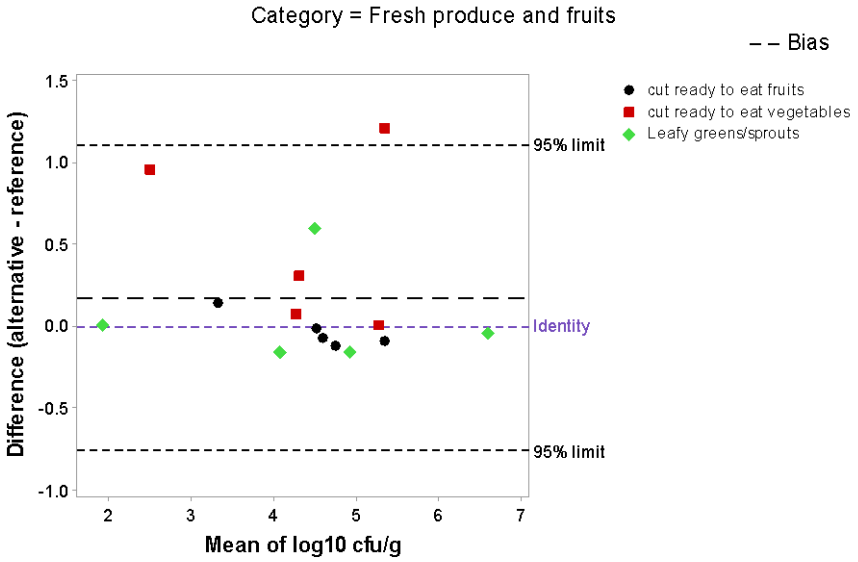
Table 2: Summary of calculated differences

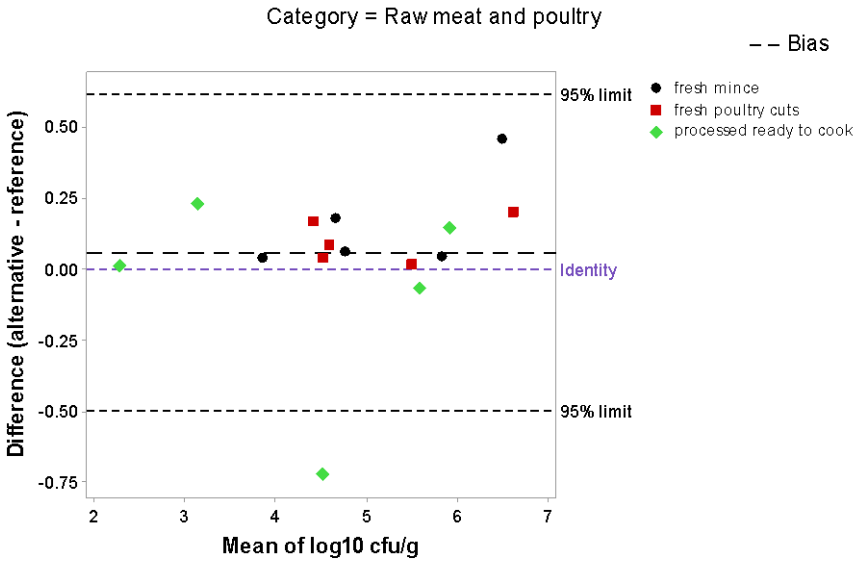
Category	n	$\overline{D}$	s_D	95% Lower limit	95% Upper limit
Fresh produce and fruits	15	0.17284	0.421818	-0.76154	1.107219
Milk and dairy	15	-0.02151	0.327305	-0.74653	0.703512
multi component foods	15	-0.00575	0.308256	-0.68858	0.677073
Raw meat and poultry	15	0.058959	0.250475	-0.49587	0.613793
ready to eat foods	15	0.161741	0.157697	-0.18758	0.511059
All Categories	75	0.073256	0.308554	-0.54564	0.692148

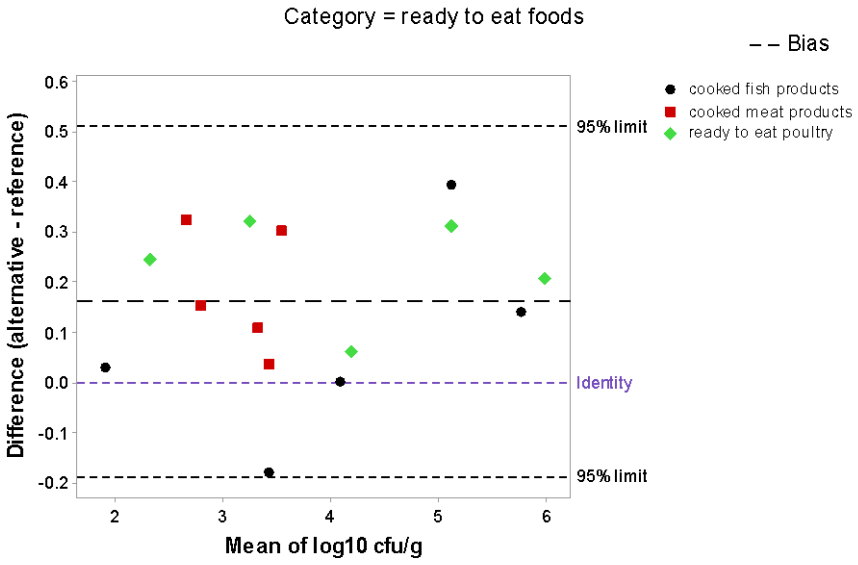
The individual sample differences were plotted against the mean values on a graph that shows the line of identity (zero difference), the line of bias, and the upper and lower 95% confidence limits of agreement of the bias. Although the text specifies four lines, the example in 16140 Figure 2 shows only three. We have plotted the "line of bias" at $\overline{D}$ as well as the line of identity and confidence limits.

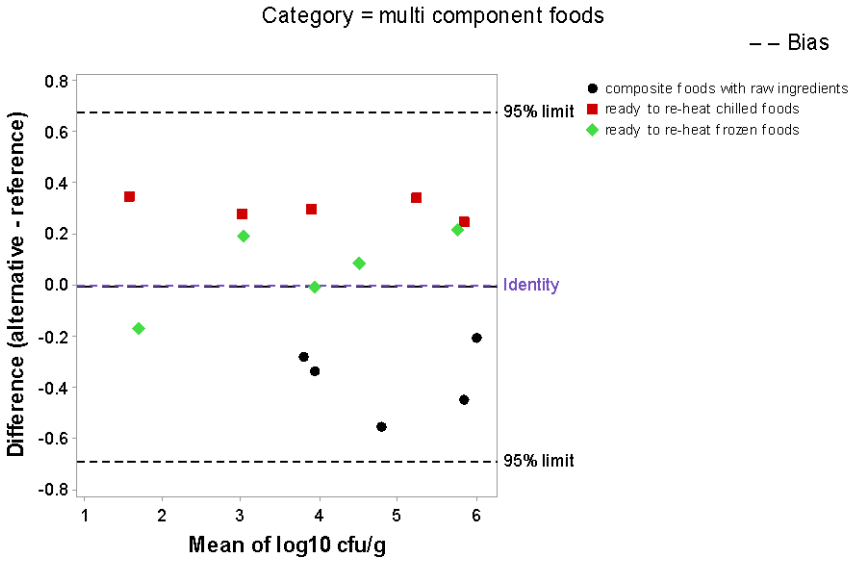
Figure 2: Bland-Altman plot for all categories




Category = Fresh produce and fruits
– – Bias
cut ready to eat fruits
cut ready to eat vegetables
Leafy greens/sprouts
95% limit
Identity
95% limit
Difference (alternative - reference)
Mean of log10 cfu/g


Category = Raw meat and poultry
– – Bias
fresh mince
fresh poultry cuts
processed ready to cook
95% limit
Identity
95% limit
Difference (alternative - reference)
Mean of log10 cfu/g


Category = ready to eat foods
– – Bias
cooked fish products
cooked meat products
ready to eat poultry
95% limit
Identity
95% limit
Difference (alternative - reference)
Mean of log10 cfu/g



The results of the difference and scatter plot were interpreted based on a visual observation on the amount of bias and extreme results. It is expected that not more than one in 20 data values will lie outside the CLs. Any disagreements with the expectation should be recorded.

For 'All Categories' there are five in 75 values which lie outside the CLs. This is a little more than the expectation of less than one in 20.

The five points which were outside of the CLs were shown below in Table 3. There were no identifiable trends in these data and they covered 4 different food categories, 4 different inoculated strains and 3 different seeding/spiking protocols.

Table 3: Results falling outside the confidence limits

Food Category	Food type	Sample code	Food item	Strain	Spiking/seeding protocol	Difference log cfu/g (alternative – reference)
Raw meat and poultry	processed ready to cook	43	southern fried chicken goujons	E.coli 2097	frozen 2 weeks	-0.7231
Milk and dairy	dry milk products	3B	blancmange	E.coli 1253	Ambient 2 weeks	-0.60206
multi component foods	composite foods with raw ingredients	73B	bacon,lettuce, tomato sandwich	E.coli 3385	chill 2-3 days	-0.56067
Fresh produce and fruits	cut ready to eat vegetables	21	mixed leaf salad	E.coli 3379	chill 2-3 days	0.959041
Fresh produce and fruits	cut ready to eat vegetables	24	casserole veg selection	E.coli 3379	chill 2-3 days	1.209027

2.1.4 Discordant results

It is commonly recognized that a bias higher than 0.5 Log CFU/g difference between the compared methods should be explained if possible. It is the case for 7 samples, 4 with positive bias and 3 with negative bias. Of the 4 positive bias samples, 3 were chilled stressed and were from fresh produce samples. For the 3 samples with negative bias, the samples came from 3 different categories, 3 different strains and 3 different stress protocols and so were completely random conditions. The magnitude of the bias was similar with a mean positive bias of 0.82564 and a mean negative bias of -0.62861.

The results showing a HIGHER enumeration with the ALTERNATIVE method than with the REFERENCE method are shown below. (See Table 4).

Table 4 – Discordant results with a positive bias

Sample no.	Product Category	Products	Bias log Alt - log Ref (log CFU/g)	Strain	Stress applied
21	Fresh produce and fruits	mixed leaf salad	0.959041	*E.coli* 3379	chill 2-3 days
24	Fresh produce and fruits	casserole veg selection	1.209027	*E.coli* 3379	chill 2-3 days
2b	Milk and dairy	strawberry whip	0.539269	*E.coli* 1253	Ambient 2 weeks
28	Fresh produce and fruits	pea shoots	0.595221	*E.coli* 6160	chill 2-3 days

The results showing a LOWER enumeration with the ALTERNATE method than with the REFERENCE method are shown below (See Table 5).

Table 5 – Discordant results with a negative bias

Sample no.	Product Category	Products	Bias log Alt - log Ref (log CFU/g)	Strain	Stress applied
43	Raw meat and poultry	southern fried chicken goujons	-0.7231	*E.coli* 2097	frozen 2 weeks
3B	Milk and dairy	blancmange	-0.60206	*E.coli* 1253	Ambient 2 weeks
73B	multi component foods	bacon,lettuce, tomato sandwich	-0.56067	*E.coli* 3385	chill 2-3 days

2.1.5 Conclusion

The relative trueness study of the ALTERNATIVE method is satisfied.

2.2 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. One type per category is tested for this.

2.2.1 Food matrices

For each of 5 food categories, one type of food was tested using 6 samples per type. Of the 6 samples, there were 2 at a low level, 2 at a medium level and 2 at a high level of contamination. For each of the 6 samples per category, 5 replicate test portions were tested. Non –inoculated samples (5) of each product type were also tested. Each sample was bulk inoculated and separate replicate test portions examined

The tested categories, types, items and inoculated strains are provided in the Table 6.

Table 6 - Categories, types and food items

Category	Types	Strain	Item	Target Level*	Test portions
Dairy products	Pasteurised dairy products	*E. coli* CRA 1476 from dried milk	Pasteurised cream	Low 10^2 cfu/g	5
				Medium : 10^4 cfu/g	5
				High : 10^6 cfu/g	5
		E.coli NCTC 8008	Cream cheese	Low 10^2 cfu/g	5
				Medium : 10^4 cfu/g	5
				High : 10^6 cfu/g	5
Fruits and vegetables	Fresh produce	*E.coli* ATCC 25922	Ready to cook Vegetable preparation	Low 10^2 cfu/g	5
				Medium : 10^4 cfu/g	5
				High : 10^6 cfu/g	5
		E. coli NCIMB 700555	Vegetable juice	Low 10^2 cfu/g	5
				Medium : 10^4 cfu/g	5
				High : 10^6 cfu/g	5
Raw poultry and meats (Combined category raw/ RTC meats and poultry)	Fresh meat	*E. coli* CRA 16041 from raw ground mince	Pork mince	Low 10^2 cfu/g	5
				Medium : 10^4 cfu/g	5
				High : 10^6 cfu/g	5
		E. coli CRA 1593 from poultry	Raw bacon	Low 10^2 cfu/g	5
				Medium : 10^4 cfu/g	5
				High : 10^6 cfu/g	5
Ready to eat foods (Combined category RTE/RTRH meats and poultry)	Cooked fish products e.g. prawns	*E.coli* CRA 2003 isolated from fish	Fresh prawns	Low 10^2 cfu/g	5
				Medium : 10^4 cfu/g	5
				High : 10^6 cfu/g	5
		E.coli CRA 1968 isolated from lamb	Fish pate	Low 10^2 cfu/g	5
				Medium : 10^4 cfu/g	5
				High : 10^6 cfu/g	5
Multi component foods	Composite foods with raw ingredients	*E.coli* CRA 16044 isolated from beef	Sandwiches	Low 10^2 cfu/g	5
				Medium : 10^4 cfu/g	5
				High : 10^6 cfu/g	5
		E.coli CRA 1265 dried foods	Cooked chilled rice	Low 10^2 cfu/g	5
				Medium : 10^4 cfu/g	5
				High : 10^6 cfu/g	5

2.2.2 Calculation and interpretation

The statistical results and the accuracy profiles are provided Figure 3a to e.

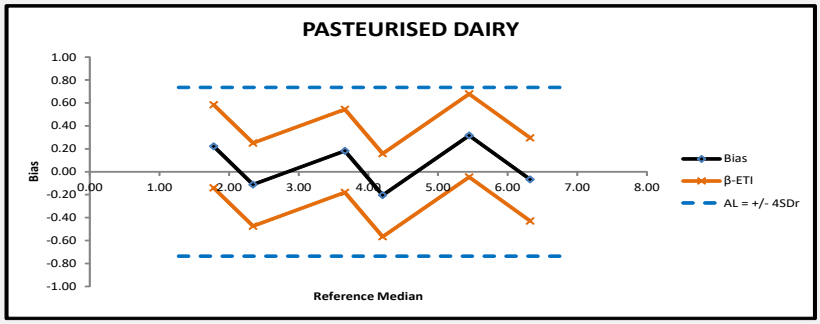
If any of the upper or lower limits exceeded the limits and the standard deviation of the reference method was >0.125, additional evaluation procedure were followed, as described in ISO 16140-2:2016 and the new acceptability limits were calculated as a function of the standard deviation $AL_s = 4 \cdot s_{ref}$. The new AL's are shown in the statistical analysis in Figure 3a to e

If any of the upper or lower limits exceeded the limits and the standard deviation of the reference method was >0.125, additional evaluation procedure were followed, as described in ISO 16140-2:2016 and the new acceptability limits were calculated as a function of the standard deviation $AL_s = 4 \cdot s_{ref}$. The new AL's are shown in the statistical analysis in Figure 3a to e.

For some of the food categories the additional AL calculation was required. For Dairy products , all three levels of pasteurised cream were higher than the upper AL and cream cheese medium level was below the lower AL. When the recalculation was made the final AL was ±0.736. For RTE foods, the medium and high levels for fish pate were above the upper AL. When the recalculation was made the final AL was ±0.772
THE Dairy and Fresh met data showed an even distribution around the line of identity, whereas for Fresh produce, and RTE foods there was a slight positive bias to the data , which is in line the findings from the relative trueness study, although for these categories the 0.5log ALs were met.

Figure 3 a: Dairy products

(Food) Category	DAIRY
(Food) Type	PASTEURISED DAIRY

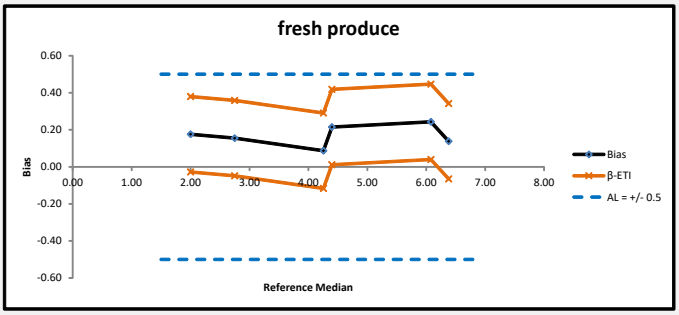


Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
107 45 75 342 322	1.78	0.222	-0.140	0.584	NO	YES
299 41 22 277 230	2.34	-0.112	-0.474	0.250	YES	YES
314 346 25 63 328	3.66	0.182	-0.180	0.545	NO	YES
198 154 231 338 254	4.20	-0.204	-0.566	0.158	NO	YES
58 141 172 83 109	5.45	0.316	-0.046	0.679	NO	YES
130 233 94 162 100	6.32	-0.067	-0.429	0.295	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.184	0.251	NO	+/- 0.736

Figure 3b: Fruit and Vegetable products

(Food) Category	fruit and veg
(Food) Type	fresh produce

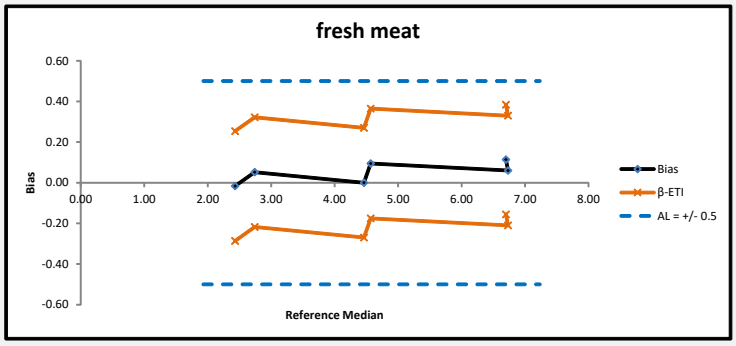


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
164 79 181 213 305	2.00	0.176	-0.027	0.380	YES	YES
119 121 176 150 91	2.75	0.155	-0.049	0.358	YES	YES
276 129 67 3 174	4.26	0.087	-0.116	0.291	YES	YES
116 104 88 201 105	4.40	0.215	0.011	0.418	YES	YES
173 98 44 127 52	6.08	0.243	0.039	0.447	YES	YES
140 326 66 190 343	6.38	0.138	-0.065	0.342	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.132	0.141	NO	+/- 0.500

Figure 3 c: Meat and poultry

(Food) Category	raw poultry and meat
(Food) Type	fresh meat

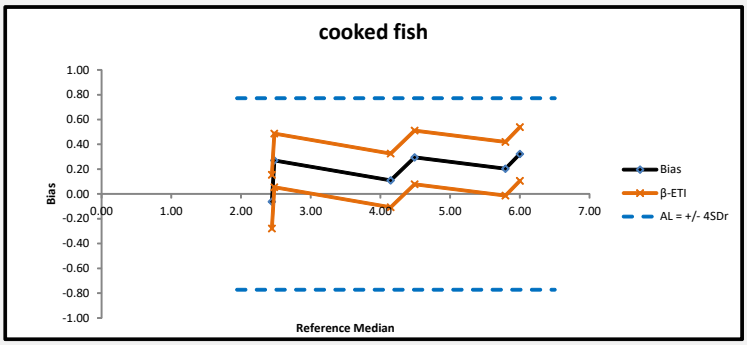


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
206 345 33 210 48	2.43	-0.016	-0.286	0.254	YES	YES
90 81 293 260 286	2.74	0.052	-0.218	0.322	YES	YES
185 199 26 208 178	4.46	0.000	-0.270	0.270	YES	YES
280 220 14 311 50	4.57	0.095	-0.175	0.365	YES	YES
335 191 99 19 193	6.73	0.060	-0.210	0.330	YES	YES
336 237 84 278 221	6.70	0.114	-0.156	0.384	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.159	0.187	NO	+/- 0.500

Figure 3d: Ready to eat foods

(Food) Category	RTE foods
(Food) Type	cooked fish

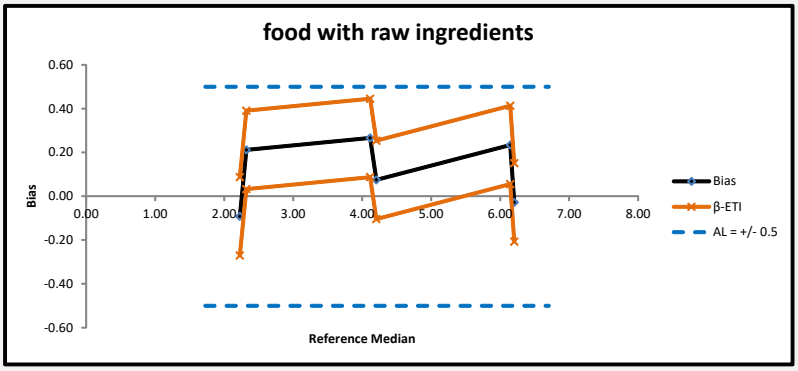


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
51 247 218 87 287	2.44	-0.062	-0.279	0.154	YES	YES
163 241 152 227 125	2.48	0.270	0.054	0.487	YES	YES
289 68 23 309 226	4.15	0.109	-0.107	0.326	YES	YES
24 325 166 70 71	4.49	0.295	0.078	0.511	NO	YES
195 261 42 61 320	5.79	0.203	-0.013	0.420	YES	YES
62 145 266 317 271	6.00	0.322	0.106	0.539	NO	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.193	0.150	NO	+/- 0.772

Figure 3e: Multi component foods

(Food) Category	Mulit component
(Food) Type	food with raw ingredients



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
37 20 28 148 126	2.23	-0.092	-0.271	0.087	YES	YES
122 169 282 60 159	2.32	0.211	0.032	0.390	YES	YES
308 235 239 179 29	4.11	0.266	0.087	0.445	YES	YES
113 318 112 279 27	4.20	0.075	-0.104	0.254	YES	YES
251 128 329 30 146	6.15	0.234	0.055	0.413	YES	YES
265 93 101 302 114	6.20	-0.028	-0.207	0.151	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.170	0.124	NO	+/- 0.500

2.2.3 Conclusion

The observed profiles are within the 0.5log AL or the recalculated AL limit calculated according to ISO16140-2:2015 section 6.1.3.3.

All the accuracy profiles fulfil the performance criteria and the alternative method is accepted as being equivalent to the reference method.

2.3 Quantification limits (LOQ)

As the alternative method is based on counting visible colonies target microorganism, the LOQ was not required to be determined according to ISO 16140-2:2016.

2.4 Inclusivity and exclusivity studies

The inclusivity study is a study involving pure target strains to be detected or enumerated by the alternative method.

The exclusivity study is a study involving pure non-target strains, which can be potentially cross-reactive, but are not expected to be detected or enumerated by the alternative method.

2.4.1 Protocol

After being grown according to appropriate conditions, decimal dilutions were made and the 20 target strains and 10 non-target strains were enumerated by the alternative method, the reference method and a non selective agar (PCA/MRSA depending upon strain used).

2.4.2 Results

2.4.2.1 Old study

The inclusivity results revealed all 31 *E. coli* strains grew and produced typical colonies on the Compact Dry EC medium. By comparison 5 strains failed to grow in the TBX medium (ISO 16649-2:2001) and one strain yielded atypical colonies.

The results from the 21 strains of non-target organisms used to determine the exclusivity of the EC method showed that the majority (19 cultures) failed to grow or produced atypical colonies on the Compact Dry EC medium and in TBX medium. Two strains of *Shigella* did yield typical colonies by both methods which is not surprising because strains of *Shigella* have β-glucuronidase activity which would give rise to typical conies with chromogenic media developed to show this activity.

2.4.2.2 Current Study

Of the 20 inclusivity strains tested all strains were detected using both the alternative and reference method.

Of the 10 exclusivity strains tested, none were detected by either the alternate or reference methods.

3 CONCLUSION

The results from the methods comparison study have shown that

- The Compact Dry EC for enumeration of *E.coli* in foods method shows satisfying trueness
- The Compact Dry EC for enumeration of *E.coli* in foods method shows satisfactory and accuracy profile.
- The Compact Dry EC for enumeration of *E.coli* in foods method was shown to be specific and selective.

These findings are in agreement with those of the original study done according to ISO 16140:2003 and show comparative performance between the reference method and the alternative method

4 INTER-LABORATORY STUDY

The experimental design for the interlaboratory study is the same in ISO16140:2003 and ISO16140-2:2015. However, the statistical analysis of the data is different. It was proposed to use the existing ILS data to recalculate the new statistics using this data as shown below.

4.1 Organisation

There were 8 collaborative laboratories used in this study representing 4 different countries.

Pasteurised milk was used as the food matrix for the interlaboratory study and the samples of milk were artificially contaminated with a single strain of *E. coli* (CCFRA code 11017, NCTC 12241) and a single strain of *Enterobacter aerogenes* (CCFRA 15736, NCTC 10006). Each strain was cultured in 10 ml NB incubated overnight at 37 ± 1°C. Both cultures were serially diluted in MRD to give the desired levels of inoculum for the contamination of the samples and were mixed together in equal concentrations.

A set of 8 x 25 ml samples of pasteurised milk were prepared for each laboratory, including the organising laboratory. Two samples remained uninoculated, whereas the other six samples were inoculated at 3 different contamination levels (low, medium and high). Appropriate dilutions of the mixed culture cocktail were used to individually inoculate 2 x 25ml samples at the low (10^1 – 10^2 CFU/ml), medium (10^2 – 10^3 CFU/ml) and high (10^3 – 10^4 CFU/ml) contamination levels. The samples were blind coded and stored at 2 – 8°C prior to despatch to the collaborative laboratories. Before despatch, each set of eight samples was packed into a suitable container with cool packs. Also, an additional vial containing water was packed with each set of samples. This enabled the laboratories to take a temperature measurement upon receipt.

Upon receipt, each collaborative laboratory tested a 10 ml test portion from each of the eight milk samples by the ISO 4832:2006 method and the Compact Dry EC method. In addition, the organising laboratory tested a set of eight milk samples at the same time as the collaborative laboratories to confirm the presence of the target organism and the contamination levels. This data was not used in the analyses.

The study was done in November 2007

Table 7: Sample receipt data for ILS samples

Laboratory	Date received	Temperature of control sample upon receipt (°C)
1	05/11/07	3.1
2	05/11/07	6
3	05/11/07	7
4	05/11/07	2.65
5	05/11/07	6.5
6	05/11/07	2.9
7	05/11/07	2.1
8	05/11/07	2.7

4.1 Calculations and interpretation of data

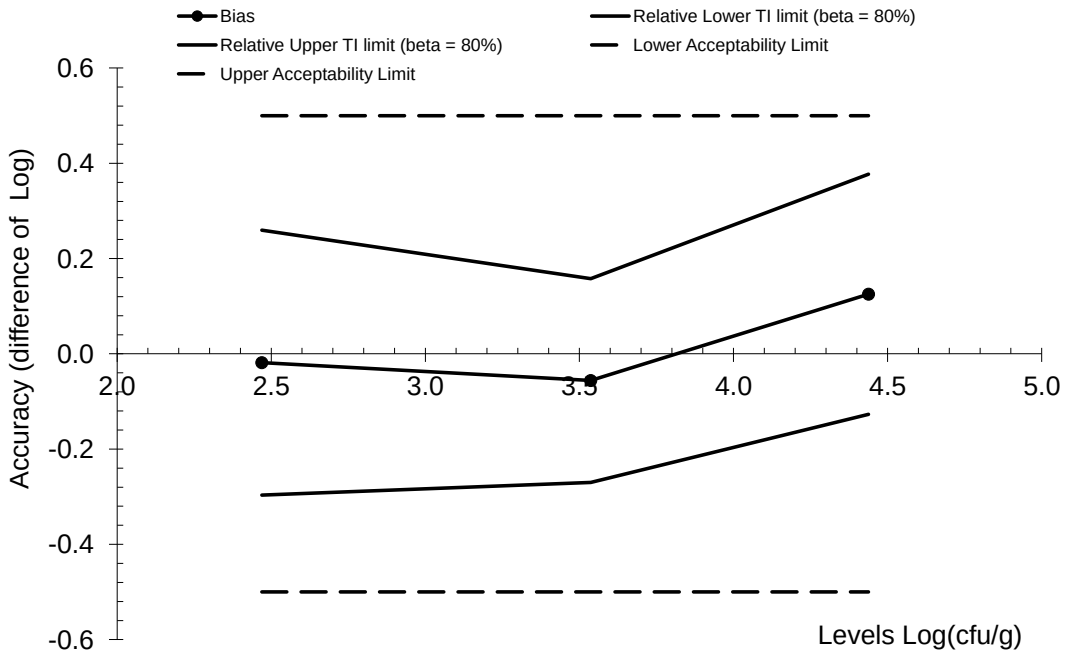
The data from the collaborative trial were calculated and interpreted according to section 6.2.3 of ISO 16140-2:2016 using the freely available Excel® spreadsheet (**http://standards.iso.org/iso/16140**).
The log transformed data from the existing trial is shown in Table 7 below and the Accuracy profile graph is shown in Figure 4.

Table 7: Summary of the results of the interlaboratory study per analyte level (k)

		Reference method x_{ijk}		Alternative method k_{ijk}	
Collaborators (i)	**Level (k)**				
1	Blank	<10	<10	<10	<10
2	Blank	<10	<10	<10	<10
3	Blank	<10	<10	<10	<10
4	Blank	<10	<10	<10	<10
5	Blank	<10	<10	<10	<10
6	Blank	<10	<10	<10	<10
7	Blank	<10	<10	<10	<10
8	Blank	<10	<10	<10	<10
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
1	Low	2.635	2.515	2.571	2.356
2	Low	2.644	2.648	2.611	2.549
3	Low	2.367	2.435	2.490	2.413
4	Low	2.281	2.310	2.238	2.190
5	Low	2.456	2.435	2.502	2.502
6	Low	2.356	2.435	2.225	2.483
7	Low	2.365	2.435	2.301	2.310
8	Low	2.281	2.912	2.477	2.994
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
1	Medium	3.761	3.635	3.428	3.555
2	Medium	3.740	3.888	3.682	3.710
3	Medium	3.549	3.449	3.421	3.508
4	Medium	3.356	3.338	3.248	3.310
5	Medium	3.549	3.477	3.514	3.648
6	Medium	3.560	3.490	3.698	3.428
7	Medium	3.347	3.560	3.435	3.421
8	Medium	3.490	3.397	3.381	3.301
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
1	High	4.686	4.626	4.678	4.587
2	High	4.827	4.940	4.869	4.895
3	High	4.310	4.405	4.562	4.639

		Reference method x_{ijk}		Alternative method k_{ijk}	
Collaborators (i)	**Level (k)**				
4	High	4.201	4.176	4.442	4.347
5	High	4.626	4.597	4.606	4.710
6	High	4.490	4.310	4.477	4.520
7	High	4.630	4.134	4.442	4.463
8	High	3.803	4.248	4.347	4.428

Figure 4: Accuracy profile of the alternative method in the Inter laboratory study



The statistical analysis of the existing ILS data is shown in Table 5 below. It can be seen that the repeatability standard deviation (S_r) was similar for the alternate method and the reference method ranging from 0.052 to 0.156 for Compact Dry and 0.084 to 0.177 for the reference method.

The between-labs standard deviation (S_L) was better for the alternative method (0.119 to 0.162) than the reference method (0.039 to 0.242) and the reproducibility standard deviation (S_R) was better for the alternative method (0.170 to 0.197) than the reference method (0.161 to 0.300).

According to the ISO 16140-2:2016 standard, if any of the values of the β-ETI fall outside of the ±0.5log AL then a further calculation is done to calculate the pooled average S_R of the reference method. This was not required as all values were within the required limits. The data are plotted in Figure 4 and it can be seen that no values lie outside of these AL_s values and therefore the alternative method is accepted as being equivalent to the reference method.

Table 8. Statistical analysis of the ILS data according to the ISO spreadsheet

Accuracy profile			
Study Name	Compact Dry EC E.coli		
Date	Original study data from 2007		
Coordinator	Recalculated June 2017		
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.

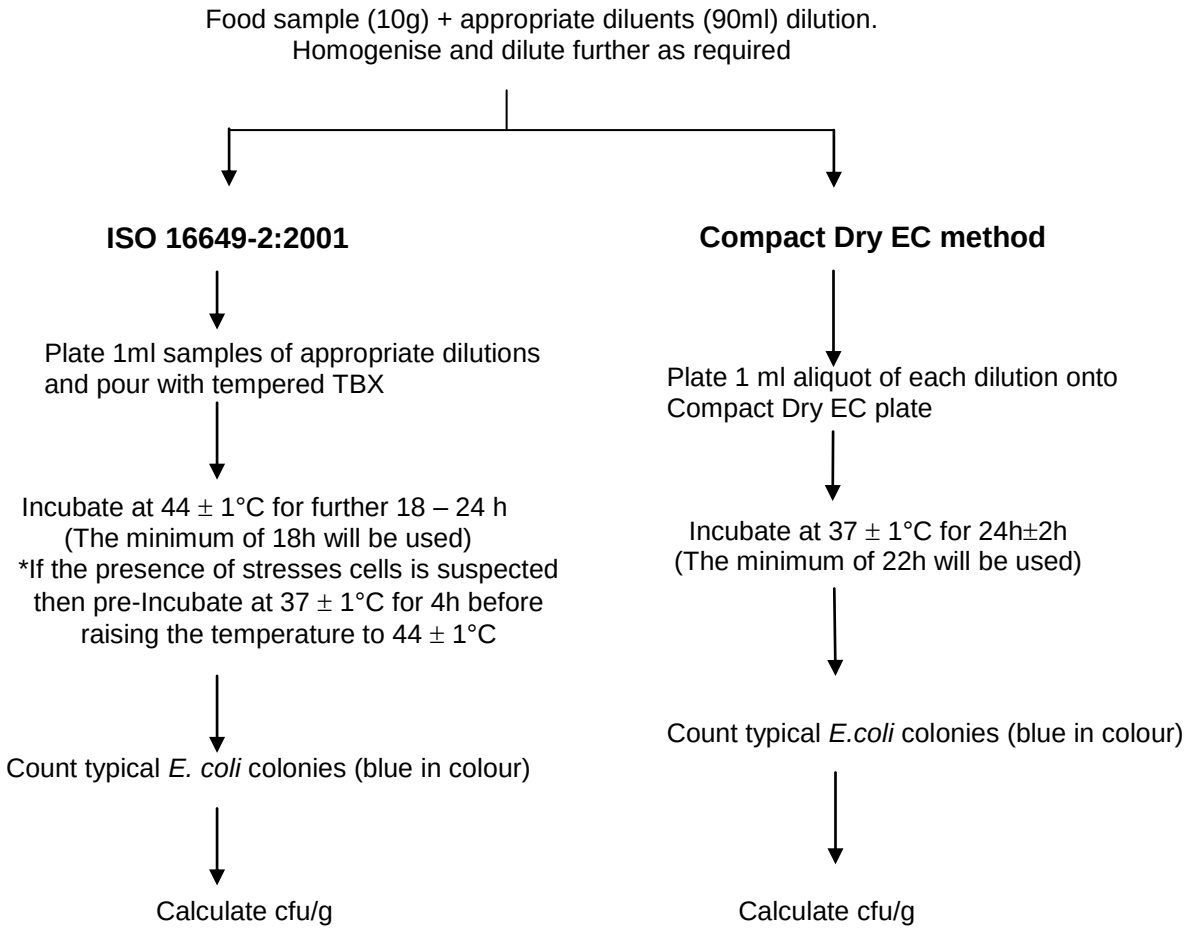
	Alternative method			Reference method		
Levels	Low	Medium	High	Low	Medium	High
Target value	2.469	3.537	4.438			
Number of participants (K)	8	8	8	8	8	8
Average for alternative method	2.451	3.480	4.563	2.469	3.537	4.438
Repeatability standard deviation (sr)	0.156	0.089	0.052	0.164	0.084	0.177
Between-labs standard deviation (sL)	0.120	0.119	0.162	0.039	0.137	0.242
Reproducibility standard deviation (sR)	0.197	0.148	0.170	0.168	0.161	0.300
Corrected number of dof	12.571	9.963	7.694	14.787	9.170	9.892
Coverage factor	1.410	1.441	1.484			
Interpolated Student t	1.353	1.373	1.402			
Tolerance interval standard deviation	0.2056	0.1558	0.1798			
Lower TI limit	2.173	3.267	4.311			
Upper TI limit	2.729	3.694	4.815			
Bias	-0.019	-0.056	0.125			
Relative Lower TI limit (beta = 80%)	-0.297	-0.270	-0.127			
Relative Upper TI limit (beta = 80%)	0.260	0.158	0.377			
Lower Acceptability Limit	-0.50	-0.50	-0.50			
Upper Acceptability Limit	0.50	0.50	0.50			
New acceptability limits may be based on reference method pooled variance						
Pooled repro standard dev of reference	0.219					

FALSE
FALSE

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

Appendix 1 – Diagram of the alternative method and reference method test

Comparison of Reference method (ISO 16649-2:2001) and Alternative Method: HyServe Compact Dry EC for enumeration of *E.coli*



* cells which are heat, acid or osmotically stressed will be pre-incubated at 37°C

Appendix 2 – Compact Dry kit insert

HyServe

HyServe GmbH & Co.KG, Hechenrainerstr. 24, 82449 Uffing, Germany 1 www.hyserve.com v 0813
Compact Dry EC medium for E.coli and coliforms/Medium für E.coli und Coliforme/milieu pour E.coli et coliformes/medio para E.coli y coliformes/ medio per E.coli e coliformi / meio para E.coli e coliformes
40 plates/Platten/plaques/placas/lastre/placas ID-No. 1 000 168 240 plates/Platten/plaques/placas/lastre/placas ID-No. 1 000 169 500 plates/Platten/plaques/placas/lastre/placas ID-No. 1 400 170 1400 plates/Platten/plaques/placas/lastre/placas ID-No. 1 502 880

English Deutsch Français ***Compact Dry EC is a ready to use, chromogenic plate for the enumeration of E.coli and coliforms***

Sample pretreatment
Viable count in water or liquid foodstuff
Drop 1 ml of specimen (dilute if necessary) on the middle of the Compact Dry plate.
Viable count in solid foodstuff
Add buffer solution to the sample and homogenize by stomacher®. Drop 1 ml of specimen (dilute if necessary) on the middle of the dry sheet of the Compact Dry plate.
Viable count in swab test specimen
Use the swab to wipe the surface, put into the device with wiping solution. Drop 1 ml of wiping solution (dilute if necessary) on the middle of the Compact Dry plate. It is recommended to use “Swab for Compact Dry” offered by HyServe Id-No. 1 002 952/3 (40/240 pieces)

Test instructions
1. Open the cap and drop 1 ml of specimen on the middle of the Compact Dry plate.
2. Specimen diffuses automatically and evenly into the sheet and transforms the dried sheet into a gel within seconds.
3. Put the cap again on the plate and write the information needed on the memorandum section.
4. Turn over the capped plate and put in the incubator.
5. After incubation count the number of colored colonies underneath the plate. White paper placed under the plate helps to count the colonies.

Incubation time 24 ± 2 hours
Incubation temperature
MicroVal / NordVal 37 ± 1°C
AOAC 35 ± 2°C
Interpretation of the results
E. coli forms blue colonies, red and blue colonies together are the total coliform group count.
Storage and shelf life
Keep at room temperature (+ 1 – + 30 °C). Total shelf life 24 months after manufacturing.
Notes
□ E. coli 0157 forms pink/red purple colonies.
□ High concentrations on plates (> 250 CFU) will cause the entire growth area to become white/pink. In this case dilute the sample.
□ After use please follow the current disposal regulations.
□ The growth area is 20 cm². The back of the plate has a grid carved of 1 cm x 1 cm to make the colony counting easier. In case of any difficulties to count colonies due to large number of colonies grown, the bacteria count can be obtained by multiplying 20 by an average number of colonies per grid counted from several grids. However, dilutions are recommended.
□ Compact Dry plates are produced at an ISO 9001 / ISO 13485: 2003 certified site.

□ **AOAC approved; certificate No. 110402**
MicroVal approval No.0806-004LR according to ISO 4832 (2006) and No.0806-005LR according to ISO 16649-2:2001
□ **ISO EN 16140:2003**
□ **NordVal International approval No. 036**

Compact Dry EC ist eine gebrauchsfertige, chromogene Platte zum Nachweis von E.coli und Coliformen

Probenvorbereitung Lebendkeimzahl in Wasser oder flüssigen Lebensmitteln
1 ml der Probe (evtl. verdünnen) in der Mitte der Compact Dry Platte aufbringen.
Lebendkeimzahlin festen Lebensmitteln
Zugabe von Pufferlösung und Homogenisierung der Lebensmittelprobe im Stomacher® ist erforderlich. 1 ml der Probe (evtl. verdünnen) in der Mitte der Compact Dry Platte aufbringen.
Lebendkeimzahl aus Tupfer-Proben
Mit dem sterilen, feuchten Wattetupfer kann z.B. die Oberfläche gewischt werden. Der Tupfer wird zurück in die Aufnahmeflüssigkeit überführt. Nach Schütteln wird die gesamte Lösung (1 ml) in der Mitte der Compact Dry Platte aufgebracht. Es wird empfohlen den Swab für Compact Dry von HyServe Id-No. 1 002 952/3 (40/240 Stück) zu verwenden.

Testanweisung
1. Öffnen des Deckels und Auftropfen von 1 ml Probenmaterial in die Mitte der Compact Dry Platte.
2. Das Probenmaterial diffundiert automatisch und gleichmäßig in die Nährsubstanz und rehydriert das Gewebe innerhalb von Sekunden zu einem Gel.
3. Platte mit Deckel verschließen und beschreibbare Fläche zur Kennzeichnung verwenden.
4. Geschlossene Platte umdrehen und in einen Brutschrank legen.
5. Nach Inkubation die Anzahl der farbigen Kolonien von der Rückseite der Platte her zählen. Ein weißes Papier als Unterlage erleichtert den Zählvorgang.
Inkubationszeit 24 ± 2 Stunden
Inkubationstemperatur
MicroVal / NordVal 37 ± 1°C
AOAC 35 ± 2°C
Interpretation des Ergebnisses
E. coli bildet blaue Kolonien aus. Rote und blaue Kolonien zusammengezählt ergibt die Anzahl der Coliformen.
Lagerung und Haltbarkeit
Bei Raumtemperatur aufbewahren (+ 1 bis + 30 °C). Haltbarkeit bis 24 Monate nach Herstellung.
Bemerkungen
□ *E. coli* 0157 bildet rosa/purpurrote Kolonien.
□ Compact Dry Platten können bis zu 250 Kolonien (KBE) pro Platte nachweisen. Höhere Konzentrationen(> 300 KBE) können eine einheitliche Weiß- oder Rosafärbung der Platte verursachen. In diesem Fall ist eine weitere Verdünnung der Probe nötig.
□ Nach Gebrauch entsprechend der gültigen Abfallregelung die Platten entsorgen.
□ Die Plattenfläche beträgt 20 cm². Auf der Plattenrückseite ist ein Raster mit 1cm x 1cm eingraviert, um die Koloniezählung zu erleichtern. Sollte es problematisch sein auf Grund hoher Koloniedichte eine ganze Platte auszuzählen, kann man einzelne Quadrate auszählen und den Mittelwert mit 20 multiplizieren.
□ Compact Dry Platten werden in einem ISO 9001 / ISO 13485: 2003 zertifizierten Betrieb gefertigt.

□ **AOAC zertifiziert; Zertifikat No. 110402**
□ **MicroVal approval No. No.0806-004LR nach ISO 4832(2006); Microval Zertifikat No.0806-005LR nach ISO 16649-2:2001**
□ **ISO EN 16140:2003**
□ **NordVal International Zertifikat No. 036**

Compact Dry EC est une plaque chromogène prête à l'utilisation pour détecter E.coli et des coliformes

Traitement préliminaire de l'échantillon
Nombre de germes revivifiables dans l'eau ou dans des aliments liquides
Appliquer 1 ml de l'échantillon (le diluer si nécessaire) au centre de la plaque Compact Dry.
Nombre de germes revivifiables dans des aliments solides
Il est nécessaire d'ajouter une solution tampon à l'échantillon et de l'homogénéiser par Stomacher®. Appliquer 1 ml de l'échantillon (le diluer si nécessaire) au centre de la plaque Compact Dry.
Nombre de germes revivifiables dans des échantillonsprélevés
Utiliser le tampon pour essuyer la surface, le placer dans l'unité avec la solution d'essuyage. Appliquer 1 ml de la solution d'essuyage (le diluer si nécessaire) au centre de la plaque Compact Dry. Il est recommandé d'utiliser le tampon ‘Swab for Compact Dry’ distribué par la société HyServe Id-No. 1 002 952/3 (40/240 pièces)
Instructions pour le test
1. Ouvrir le couvercle et appliquer 1 ml de l'échantillon sur la plaque Compact Dry.
2. L'échantillon se répand automatiquement et uniformément sur la feuille et en l'espace de quelques secondes, il transforme la feuille sèche en un gel.
3. Refermer le couvercle de la plaque et inscrire les informations nécessaires dans la partie correspondante.
4. Retourner la plaque fermée et la placer dans l'incubateur.
5. Après le temps d'incubation, compter le nombre de colonies de couleur au dos de la plaque. Les colonies peuvent être comptées plus simplement en plaçant du papier blanc sous la plaque.
Temps d'incubation 24 ± 2 heures
Température d'incubation
MicroVal / NordVal 37 ± 1°C
AOAC 35 ± 2°C
Interprétation des résultats
E. coli forme des colonies bleues, le nombre total de colonies rouges et bleues indique le nombre de coliformes.
Stockage et durée de conservation
Stockage à température ambiante (+ 1 à + 30 °C). Durée totale de conservation 24 mois après fabrication.
Remarques
□ E. coli 0157 forme des colonies roses/rouges pourpre.
□ Des concentrations élevées (> 250 CFU) sur les plaques entraînent une coloration blanche/rose de toute la surface. Dans un tel cas, il faut diluer l'échantillon.
□ Après l'utilisation, éliminer les plaques en respectant les règlements correspondants en vigueur.
□ La surface de la plaque est de 20 cm². Une grille de 1 cm x 1 cm est taillée dans le dos de la plaque afin de faciliter le calcul des colonies. S'il est toutefois difficile de compter le nombre de colonies, suite à un grand nombre de colonies, il est possible de déterminer le nombre total de germes revivifiables dans certains carrés de la grille et d'en multiplier par 20 la valeur moyenne obtenue.
□ Les plaques Compact Dry sont fabriquées dans une usine certifiée conforme à ISO 9001 / ISO 13485: 2003.
□ **AOAC approved; certificate No. 110402**
□ **Microval approval No.0806-004LR/ISO 4832; Microval No.0806-005LR ISO 16649-2:2001**
□ **ISO 16140:2003**
□ **NordVal International approval No. 036**