

**Method Comparison Study Report for the ISO 16140-2:2016 validation of
MC Media pad EC, for the detection of coliforms and *E. coli* in a broad
range of foods**

MicroVal study number: 2017LR71

Method/Kit name: MC Media pad EC

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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: MC Media pad EC

Validation standard: ISO 16140-2:2016 Microbiology of the food chain —Method validation —Part 2:
Protocol for the validation of alternative (proprietary) methods against a reference method

Reference methods: ISO 4832:2006 Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coliforms —Colony-count technique

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 of validation: A broad range of foods based on categories

1. Milk and dairy products
2. Fresh produce and fruits
3. Raw poultry and meats (Combined category raw/ RTC meats and poultry)
4. Ready to eat foods (Combined category RTE/RTRH meats, poultry and fish)
5. Multi component foods or meal components

Certification organization: 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
- VRBA	Violet Red Bile Lactose Agar
- PSD	Peptone salt diluent
- TBX	Tryptone bile x-glucuronide agar

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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 *E.coli* and coliforms in five different food categories was carried out by Campden BRI as the MicroVal Expert Laboratory.

This study was also used for an AOAC validation.

The alternative method used was:

- Enumeration of *E.coli* and coliforms on MC Media pad EC, incubated at 35±1°C for 24±h

The reference methods used were:

- ISO 4832:2006 Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coliforms — Colony-count technique
- 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 of the validation study is: A broad range of foods

Categories included :

- Milk and dairy products
- Fresh produce and fruits
- Raw poultry and meats (Combined category raw/ RTC meats and poultry)
- Ready to eat foods (Combined category RTE/RTRH meats and poultry, fish)
- Multi component foods or meal components

Criteria evaluated during the study have been:

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

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

The alternative method MC Media pad EC shows comparable performance to the reference methods (ISO 16649-2:2001, ISO 4832:2006) for the enumeration of coliforms and *E.coli* in a broad range of foods.

2 Method protocols

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

According to ISO 16140-2 the reference method and alternative methods were performed with the same sample. The study was therefore a paired study design.

2.1 Reference method

See the flow diagram in Annex A.

Sample preparations used in the reference method were done according to ISO 6887-series parts 1, 2, 3, 4 and 5. Plating was done according to ISO 7218:2007+A1:2013 section 10.2.2 which says at least one plate per dilution shall be used with at least two successive dilutions. Two plates per dilution may also be used to improve reliability. If only one dilution is used, then two plates of this dilution shall be used to improve reliability of the results. Depending on the sample being tested and the expected contamination level, single or multiple dilutions were used with single or duplicate plates if considered necessary to improve the reliability of the calculated result and ensure at least two relevant plates were available for use in calculations.

2.2 Alternative method

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

See the MC Media Pad EC kit insert in Annex B.

The alternative method principle is based on chromogenic media.

MC Media Pad EC: is a quantitative sheet method intended to simultaneously enumerate coliforms and *E. coli* through a special medium composition and specific chromogenic substrates for both β -galactosidase and β -glucuronidase. Once the liquid sample is inoculated onto the test pad, the sample diffuses to the whole pad through capillary action. The medium re-constitutes automatically. If target organisms are present, coliforms grow as blue-green/blue colonies and *E. coli* grows as purple/navy colonies on the test pad, respectively.

The coliform count is based on a total count of blue-green/blue and red-purple/navy colonies and the *E. coli* count is based on a count of red-purple/navy colonies

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 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 analyzed are presented in Table 1.

Table 1 – Categories, types and number of samples analyzed

Category	Types		Number of samples analyzed	Number of samples with interpretable results
Milk and dairy products	a	Dry milk product e.g. milk powder, powder	5	5
	b	Dairy products e.g. ice-cream, raw milk cheese	5	5
	c	Pasteurised milk products e.g. skimmed, semi-skimmed	5	5
	Total		15	15
Fresh produce and fruits	a	Cut ready to eat fruit e.g. fruit mixes	5	5
	b	Cut ready to eat vegetables e.g. Bagged pre-cut salads	5	5
	c	Leafy greens/Sprouts e.g. soy, mung, alfalfa,	5	5
	Total		15	15
Raw poultry and meats (Combined category raw/ RTC meats and poultry)	a	Fresh poultry cuts e.g. turkey breast	5	5
	b	Fresh mince e.g. lamb, beef, pork	5	5
	c	Processed ready to cook e.g. frozen patties, marinated kebab	5	5
	Total		15	15
Ready to eat foods (Combined category)	a	Ready to eat poultry e.g. turkey fillet, chicken sausage, pate	5	5
	b	Cooked fish products e.g. prawns, terrine, pate, smoked fish	5	5

Category	Types		Number of samples analyzed	Number of samples with interpretable results
RTE/RTRH meats and poultry and fish)	c	Cooked meat e.g. ham, salami, pate, corned beef	5	5
		Total	15	15
Multi component foods or meal components	a	Ready to re-heat refrigerated food	5	5
	b	Ready to re-heat food frozen e.g. fries,	5	5
	c	Composite foods with substantial raw ingredients e.g. pasta salads	5	5
		Total	15	15
		TOTAL	75	75

75 samples were analysed, leading to 75 exploitable results.

3.1.2 Test sample preparation

It is preferable to test naturally contaminated samples. In order to attempt to use naturally contaminated samples, all fifteen samples from each category were first tested for the presence of naturally occurring target organisms making a total of seventy five samples which were tested. From these samples 26 samples (34%) were positive for the coliforms and these samples were used in the data analysis. The remaining 49 samples (66%) were negative for the coliforms and needed to be artificially contaminated.

None of the samples screened had any naturally present *E.coli* present. It was therefore necessary to use artificial contamination procedures for all *E.coli* samples.

Data is not shown for all negative naturally contaminated samples as all results were <10cfu/g on both the reference method and alternative method.

Artificial contaminations were obtained by:

- Seeding with appropriate strains
 - o and storing chilled for minimum 48h at <5°C;
 - o and storing frozen for minimum 2 weeks at <-20°C or
 - o of lyophilised cells, which were freeze dried, mixed into the dry powders and stored ambient for a minimum of 2 weeks before analysis
- Spiking with appropriate strains that have been heated at 55°C for 5 minutes.

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

Injury efficiency was evaluated by enumerating the pure culture on selective and non-selective agars. The observed injury measurements varied from 1.0 to 1.46 log cfu/g difference between non-selective and selective plates.

34 % of the coliform samples were naturally contaminated. None of the *E.coli* samples were naturally contaminated although 75 samples were screened to attempt to find naturally present strains. In order to achieve as wide a range of artificial strains as possible, 15 different strains were used from a range of food types e.g. dried milk powder, flavouring, chocolate, chicken, spinach, bread mix, frozen turkey, fish cakes, cured meat, cooked pork.

3.1.3 Protocols applied during the validation study

A single protocol was applied for the study.

Reference method plates were incubated at $37\pm1^\circ\text{C}$ for $24\pm2\text{h}$ for coliforms and at 44°C for $21\pm3\text{h}$ for *E.coli*

Alternative method plates were incubated at $35\pm1^\circ\text{C}$ for $24\pm2\text{h}$.

In all cases the minimum incubation times were used.

Confirmations if required for the alternative method

No confirmations were needed for the alternative method.

3.1.4 Test results

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

3.1.5 Calculation and interpretation of relative trueness studies

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

Figures 1 to 6 shows the scatter plots for the individual categories and all categories for coliforms and Figures 7 to 12 shows the scatter plots for the individual categories and all categories for *E.coli*.

Figure 1 - Scatter plot of the reference method versus alternative method results for Milk and dairy products - coliforms

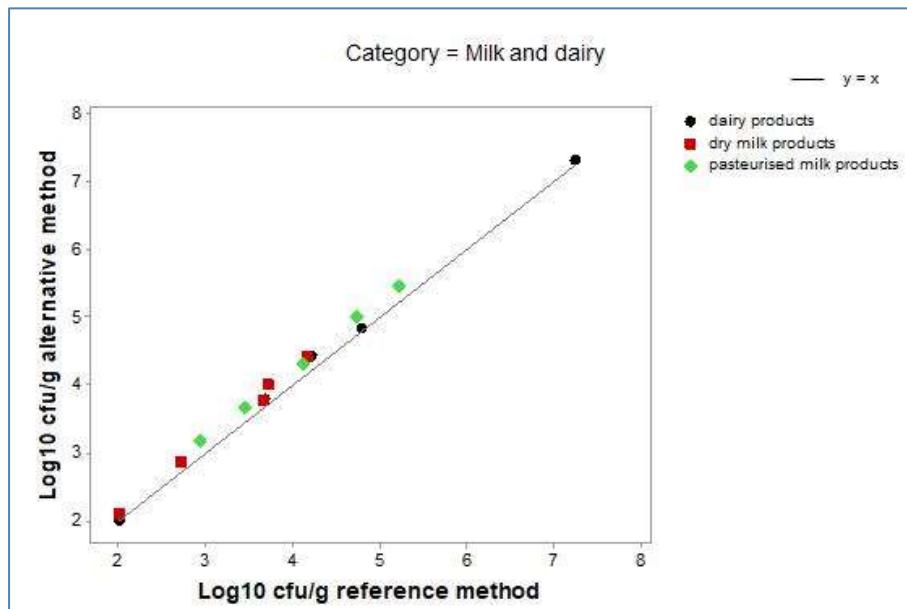


Figure 2- Scatter plot of the reference method versus alternative method results for Fresh produce and fruits - coliforms

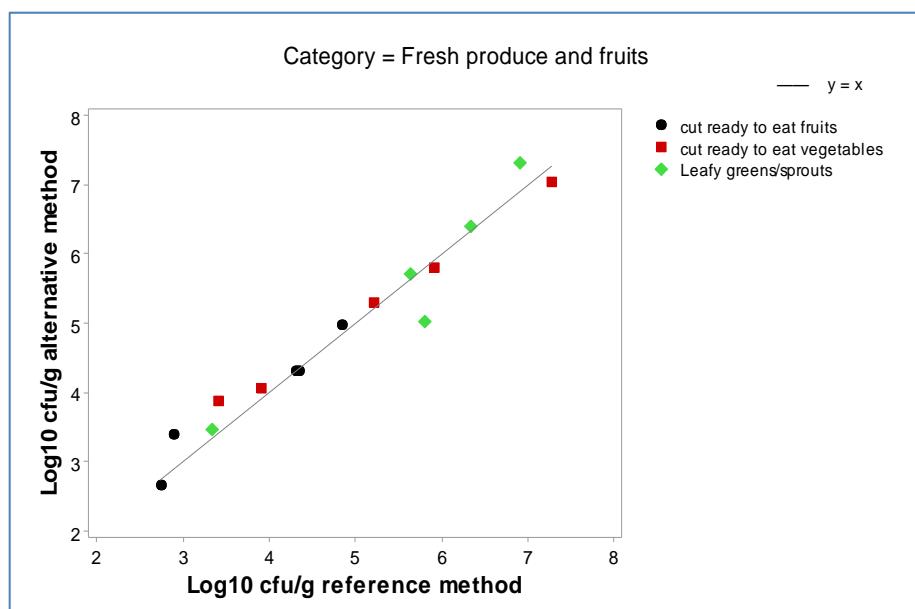


Figure 3- Scatter plot of the reference method versus alternative method results for Raw poultry and meats - coliforms

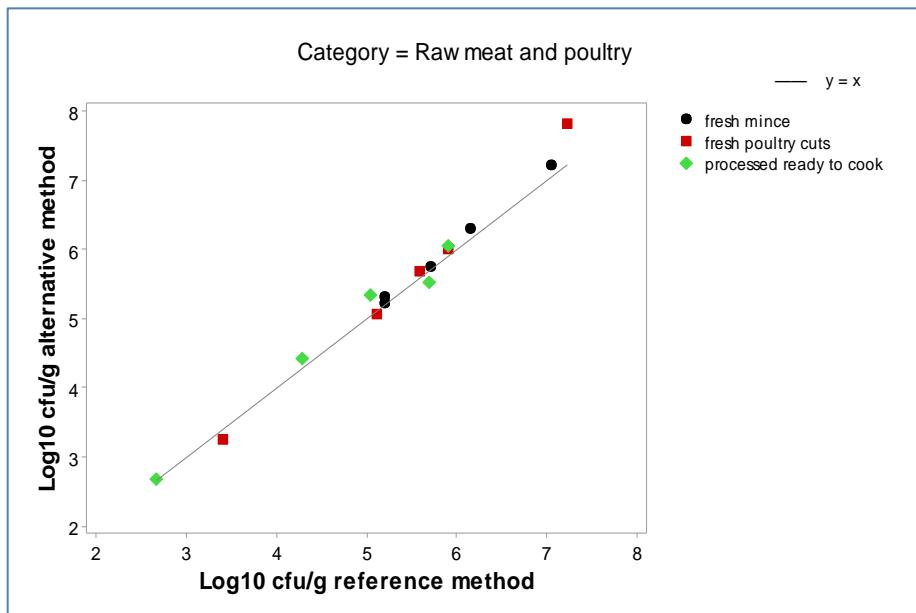


Figure 4- Scatter plot of the reference method versus alternative method results for Ready to eat foods - coliforms

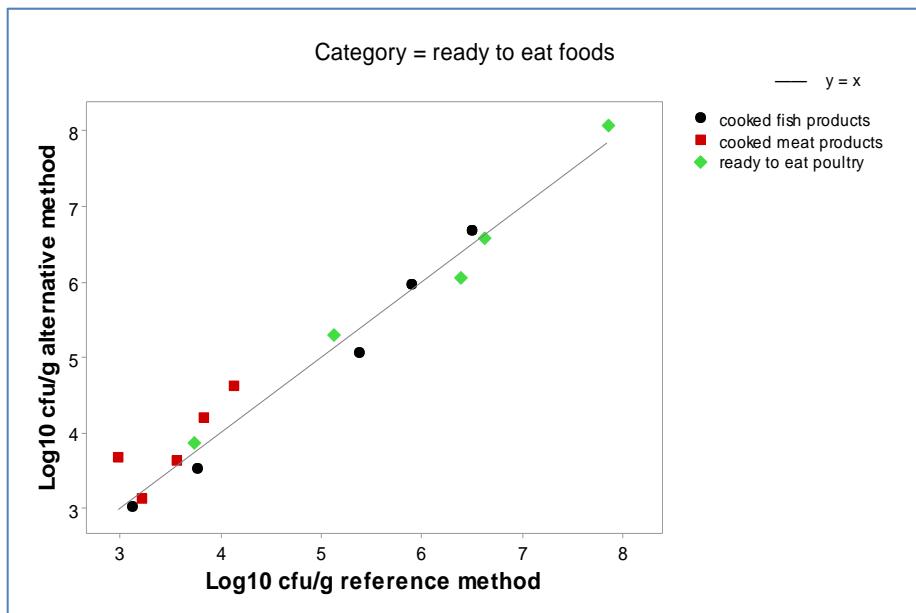


Figure 5- Scatter plot of the reference method versus alternative method results for Multi component foods - coliforms

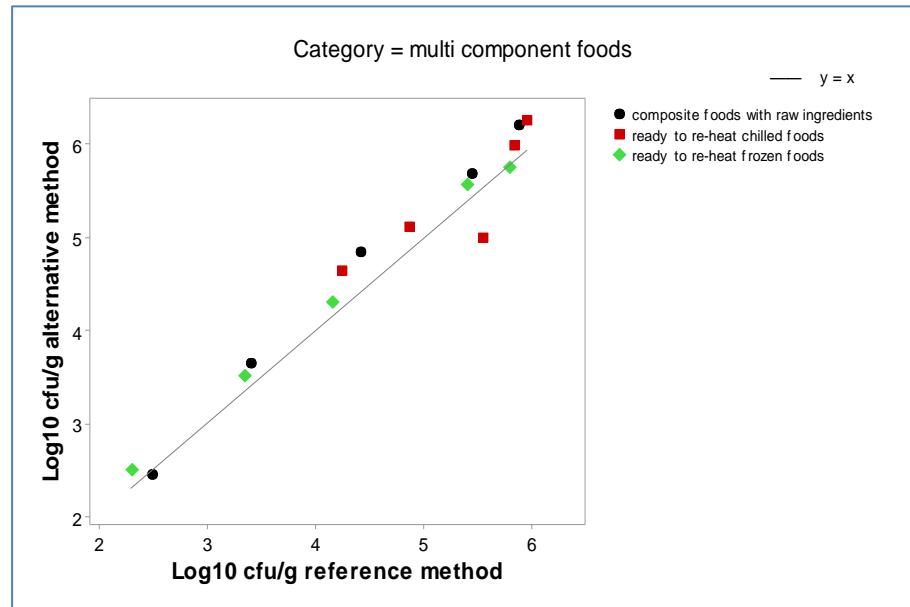


Figure 6 - Scatter plot of the reference method versus alternative method results for all categories -coliforms

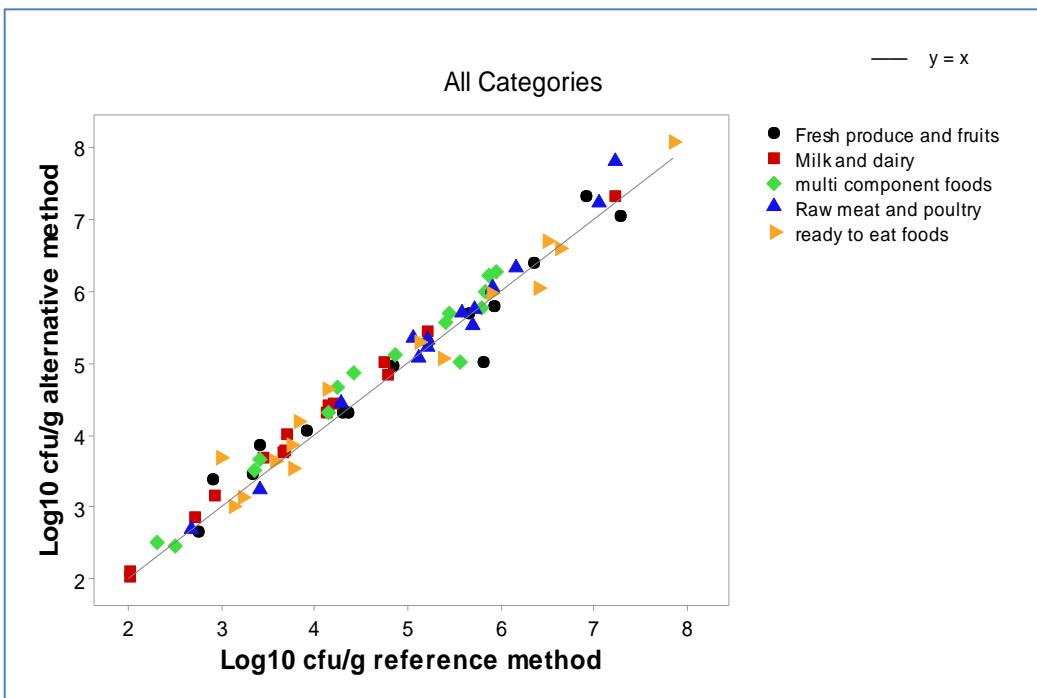


Figure 7 - Scatter plot of the reference method versus alternative method results for Milk and dairy products – *E.coli*

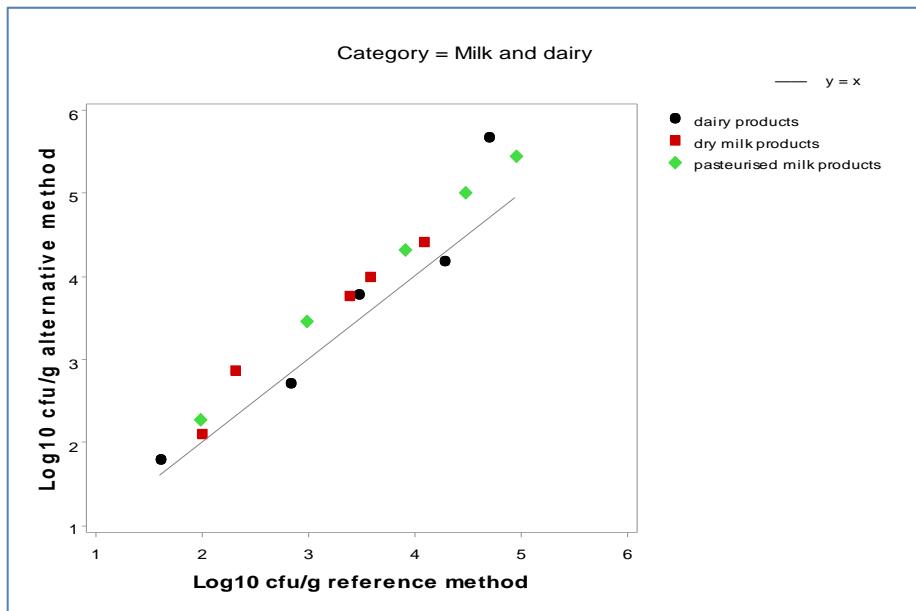


Figure 8- Scatter plot of the reference method versus alternative method results for Fresh produce and fruits - *E.coli*

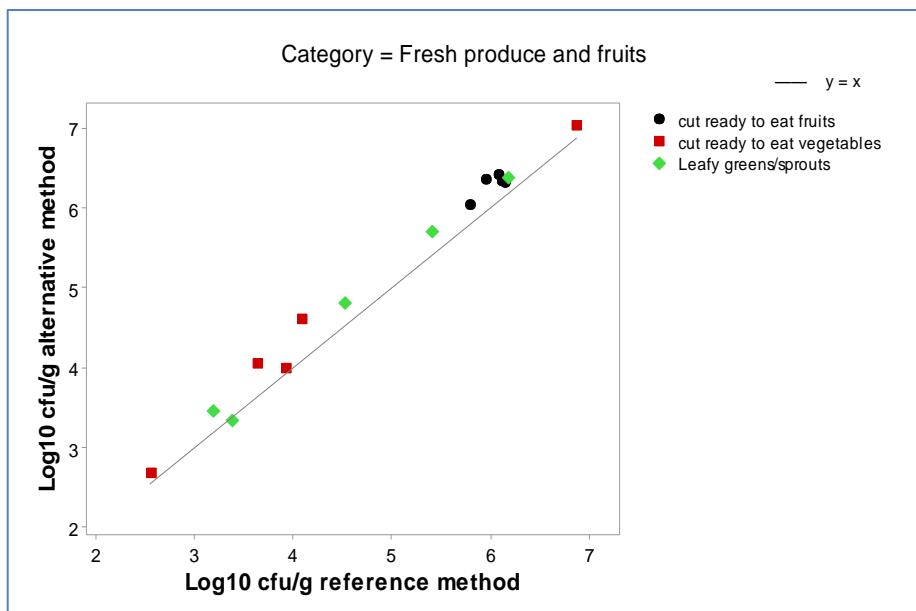


Figure 9- Scatter plot of the reference method versus alternative method results for Raw poultry and meats - *E.coli*

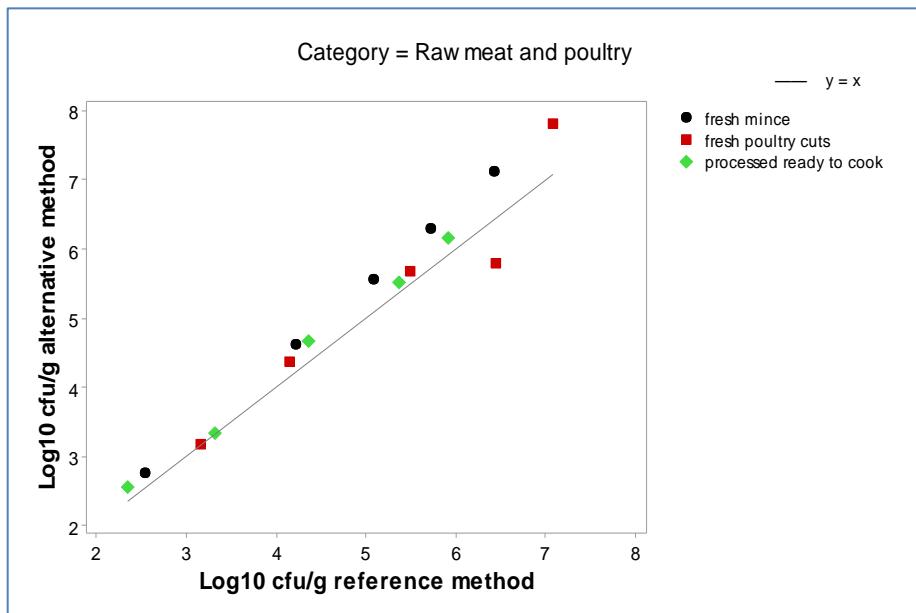


Figure 10- Scatter plot of the reference method versus alternative method results for Ready to eat foods – *E.coli*

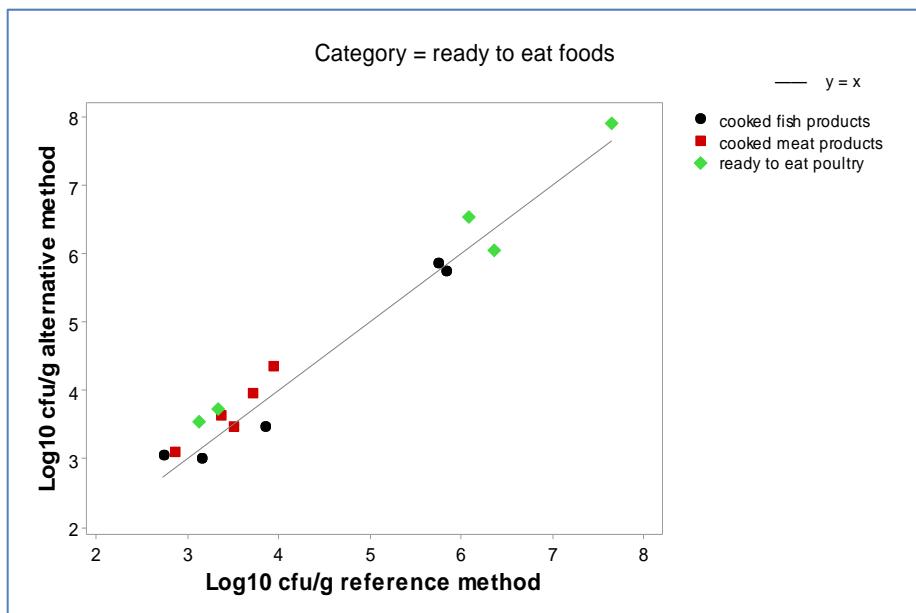


Figure 11- Scatter plot of the reference method versus alternative method results for Multi component foods - E.coli

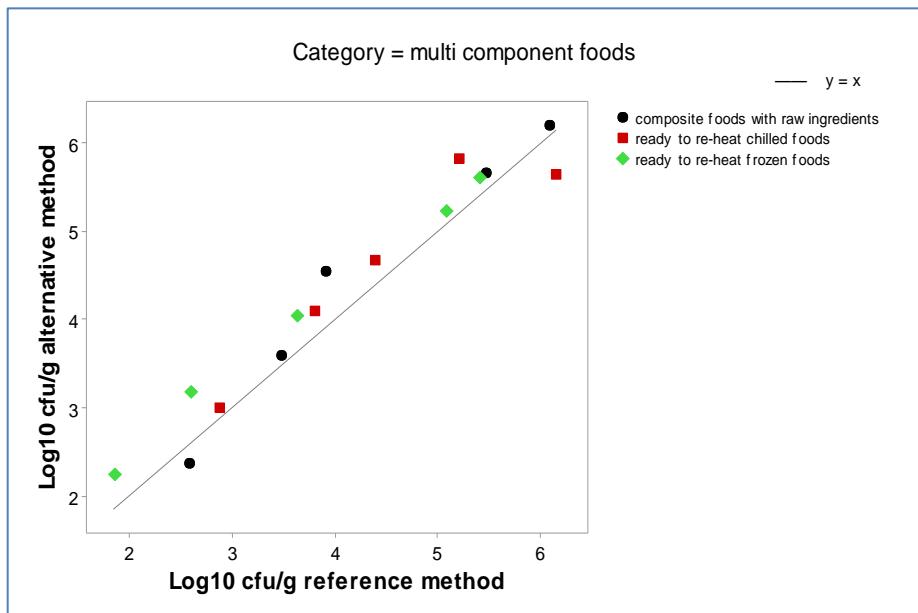
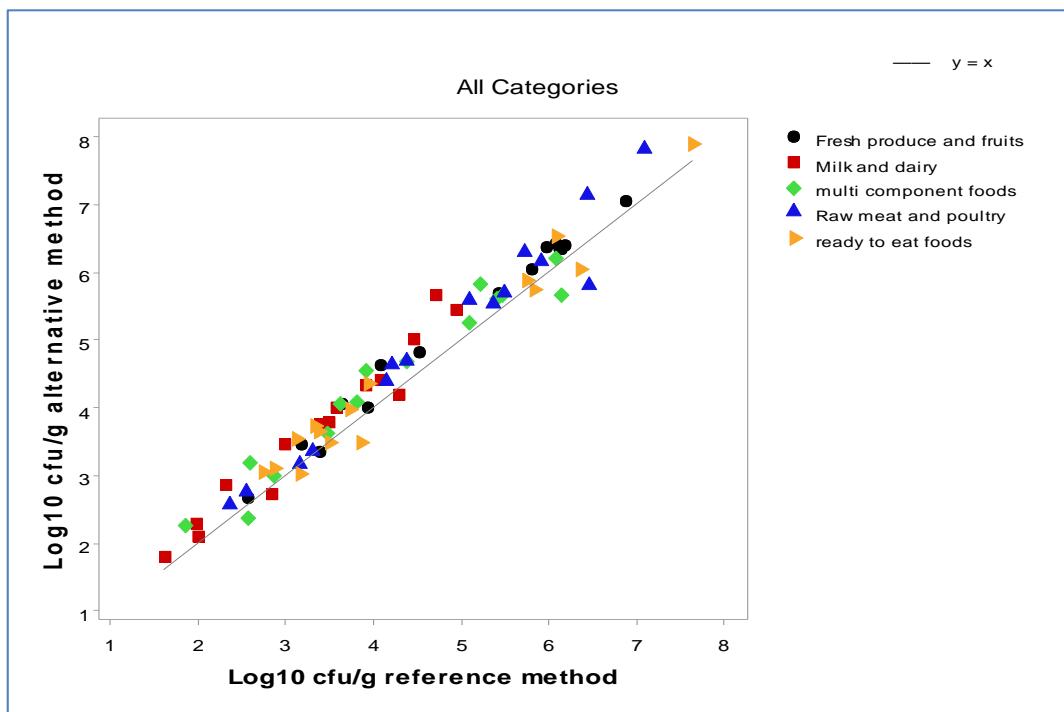


Figure 12 - Scatter plot of the reference method versus alternative method results for all categories- E.coli



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.

For coliforms, there is some evidence of a slight positive bias for the alternative method for dairy foods, and multi component foods. This can be seen from the individual product Figures (1 and 5) and from the all categories Figure (6).

For *E.coli*, the data appears acceptable overall but there is some evidence of a slight positive bias for the alternative method for all categories tested. This can be seen from the individual product Figures (7 to 11) and from the all categories graph. (Figure 12). There was no product type, strain or seeding/spiking protocol associated with this bias, it was a general bias of an average of 0.25 across all categories. This bias may represent better growth of the target organisms at 35°C on the alternate method compared to 44°C for the reference method.

A summary of the calculated values per category is provided in Table 2 for coliforms and Table 3 for *E.coli*.

The Bland-Altman difference plot for all the samples is given Figure 13 for coliforms and Figure 14 for *E.coli*.

Table 2 - Summary of the calculated values per category - coliforms

Category.	n	\bar{D}	s_D	95% Lower limit	95% Upper limit
Fresh produce and	15	0.040	0.313	-0.654	0.733
Milk and dairy	15	0.161	0.090	-0.038	0.359
Multi component	15	0.162	0.239	-0.368	0.691
Raw meat and poultry	15	0.088	0.184	-0.320	0.496
Ready to eat foods	15	0.085	0.291	-0.561	0.730
All Categories	75	0.107	0.236	-0.366	0.580

Table 3 - Summary of the calculated values per category – *E.coli*

Category.	n	\bar{D}	s_D	95% Lower limit	95% Upper limit
Fresh produce and	15	0.241	0.146	-0.081	0.564
Milk and dairy	15	0.339	0.271	-0.261	0.939
Multi component	15	0.230	0.302	-0.439	0.898
Raw meat and	15	0.245	0.332	-0.491	0.981
Ready to eat foods	15	0.139	0.269	-0.458	0.735
All Categories	75	0.239	0.272	-0.306	0.783

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

Figure 13 – Bland-Altman difference plot for all the samples- coliforms

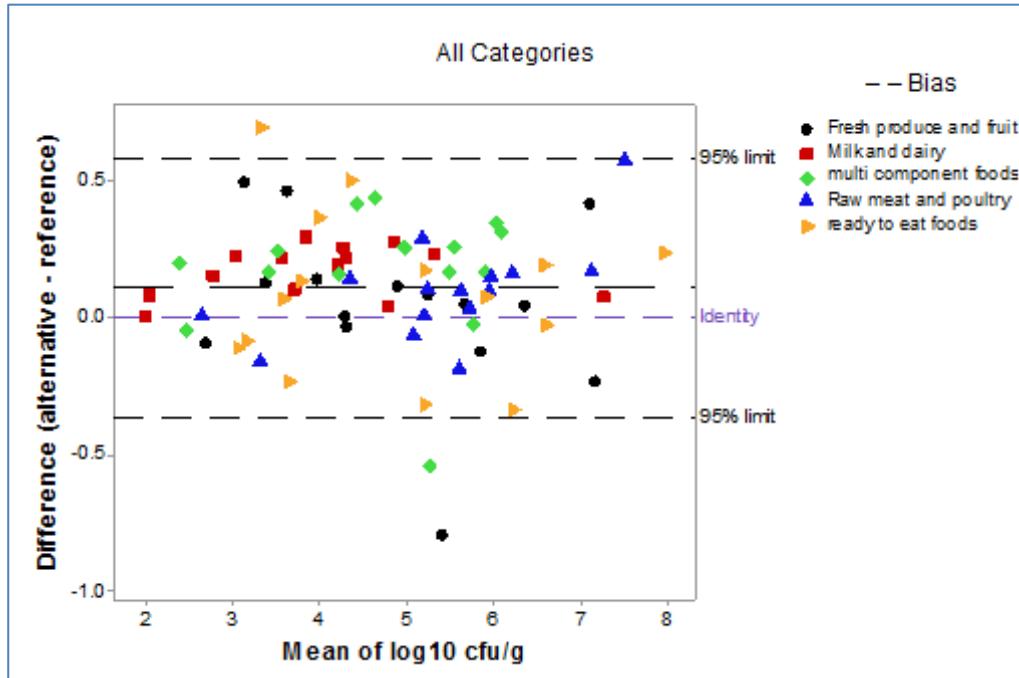
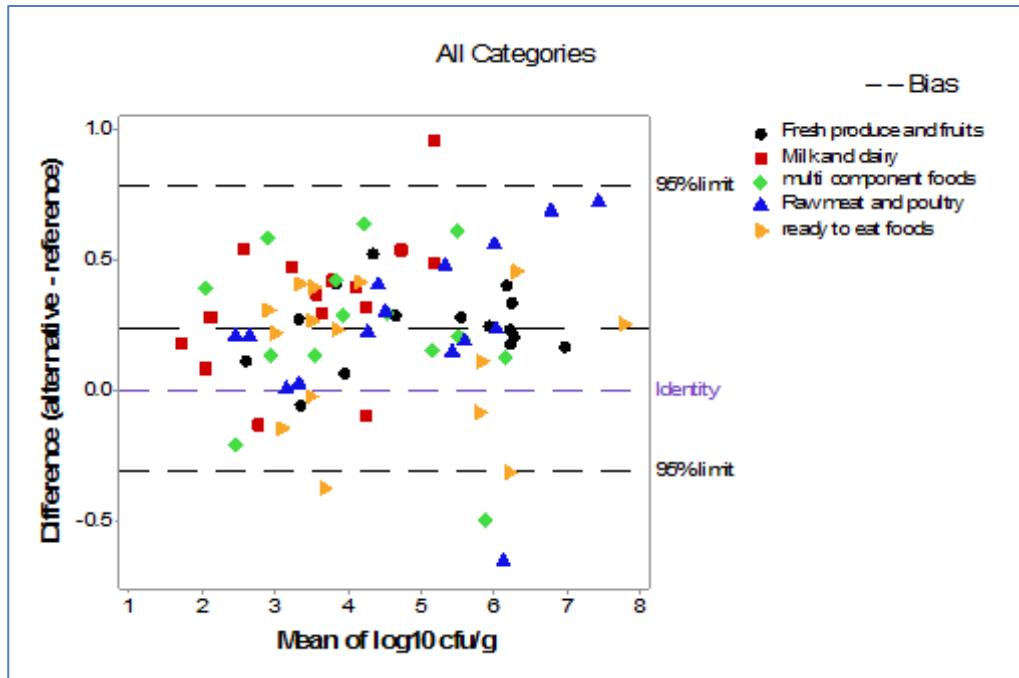


Figure 14 – Bland-Altman difference plot for all the samples- E.coli



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 Tables 4 and 5.

Table 4 - Data which are outside of the accepted limits - coliforms

Category	Types	Code	Food item	strain	Spiking/ seeding	Log (Ref)	Log (Alt)	Mean	Difference
Ready to eat foods	Cooked meat products	57	Ham	<i>E.coli</i> 2077 <i>E.gergoviae</i> NCIMB 13304	Heat	2.97	3.66	3.31	0.68
Multi component foods	Ready to re-heat chilled foods	61	Rice noodles	<i>E.coli</i> 1967 <i>Enterobacter xiangfangensis</i> NCIMB 14836	Chill 2-3 days	5.54	4.99	5.26	-0.54
Fresh produce and fruits	Leafy greens/ sprouts	28	Beansprouts	<i>E.coli</i> 6160 Natural coliforms 10 ⁵	Chill 2-3 days	5.79	5.00	5.39	-0.79
Raw meat and poultry	Fresh poultry cuts	35	Chicken wings	<i>E.coli</i> 1593 Natural coliforms 10 ⁴	Chill 2-3 days	7.23	7.799	7.51	0.56

Table 5 - Data which are outside of the accepted limits – E.coli

Category	Types	Code	Food item	strain	Spiking/ seeding	Log (Ref)	Log (Alt)	Mean	Difference
Milk and dairy	Dairy products	10	Strawberry Trifle	<i>E.coli</i> 1250	ambient 2 weeks	4.69	5.65	5.17	0.95
Multi component foods	Ready to re-heat chilled foods	65	Southern Fried Chicken Goujons	<i>E.coli</i> 3384	chill 2-3 days	6.14	5.64	5.89	-0.50
Raw meat and poultry	Fresh poultry cuts	34	chicken thighs	<i>E.coli</i> 1594	chill 2-3 days	6.44	5.78	6.11	-0.66
Ready to eat foods	Cooked fish products	52	Smoked Salmon Pate	<i>E.coli</i> 108	chill 2-3 days	3.83	3.46	3.65	-0.37
Ready to eat foods	Ready to eat poultry	49	Chicken slices	<i>E.coli</i> 4611	chill 2-3 days	6.36	6.041	6.20	-0.32

Comments

It is expected that not more than one in 20 data values will lie outside the CLs.

In this study for coliforms there were 4 data points from a total of 75 data points which were outside of the accepted limits. This meets the expectation. The data covered 4 different food categories, and 4 different *E.coli* strains, 2 coliform strains and naturally present coliforms.

For *E.coli* there were 5 data points from a total of 75 data points which were outside of the accepted limits. This is slightly outside of the expectation. However, the data covered 4 different food categories, and 5 different *E.coli* strains. In addition, the data was split between negative and positive bias and thus did not indicate a systematic cause for the bias. The all categories scatterplot (Figure 12) showed good agreement between the methods.

3.1.6 Conclusion (RT study)

The relative trueness of the Alternative method for coliforms is satisfied as the expectation of not more than 1 in 20 data points outside of the acceptability limits is met, there was only a small positive bias for the alternate method and the acceptability limits were in the order of 0.5logs

The relative trueness of the Alternative method is satisfied for *E.coli* as it shows comparative performance to the reference method. The expectation of not more than 1 in 20 data points outside of the acceptability limits was not met as there were 5 points outside the acceptability limits (1 more than expected), however, these points covered a wide range of conditions and did not show any systematic root cause for the data points outside the limits. There was only a small positive bias in the data.

3.2 Accuracy profile study

The accuracy profile study is a comparative study between the results obtained by the reference method 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

It is possible to run this study in two different ways. It is possible to use either 2 separate batches of a single item for each food type. Or it is possible to use a single batch of 2 different items for each food type. For joint AOAC studies it is preferable to run the study using a single batch of 2 different items for each food type as this will increase the total number of different food matrices tested. This is important because in AOAC PTM studies the claim is for individual food matrices. This study was a joint AOAC study.

In this study 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.

Each sample was bulk inoculated and five replicate test portions examined from the bulk sample.

As this study was for both coliforms and *E.coli*, each sample tested was co-inoculated with both strains as shown in Table 6.

Table 6 - Categories, types, items, strains and inoculation levels for accuracy profile study

Category	Types	Strain for <i>E.coli</i> study	Strain for coliforms study	Item	Target Level* cfu/g	Test portions
Dairy products	Pasteurised dairy products	<i>E. coli</i> CRA 1476 from dried milk	<i>E. adecarboxylata</i> CRA 5501 from skimmed milk powder	Pasteurised cream	Low 10 ²	5
					Medium : 10 ⁴	5
					High : 10 ⁶	5
			<i>Cream cheese</i>	Low 10 ²	5	
				Medium : 10 ⁴	5	
				High : 10 ⁶	5	
Fruits and vegetables	Fresh produce	<i>E.coli</i> CRA 3779 from frozen spinach	<i>Citrobacter amalonaticus</i> CRA 7458 from beansprouts	Ready to cook Vegetables	Low 10 ²	5
					Medium : 10 ⁴	5
					High : 10 ⁶	5
			Vegetable juice	Low 10 ²	5	
				Medium : 10 ⁴	5	
				High : 10 ⁶	5	
Raw poultry and meats (Combined category raw/ RTC meats and poultry)	Fresh meat	<i>E. coli</i> CRA 3384 from pork	<i>Escherichia fergusonii</i> CRA 7522 from sausages	Pork mince	Low 10 ²	5
					Medium : 10 ⁴	5
					High : 10 ⁶	5
			Raw bacon	Low 10 ²	5	
				Medium : 10 ⁴	5	
				High : 10 ⁶	5	
Ready to eat foods (Combined category RTE/RTRH meats, poultry, fish)	Cooked fish products e.g. prawns	<i>E.coli</i> CRA 2003 isolated from fish	<i>Enterobacter</i> <i>aminigenus</i> NCIMB 2118 from seawater	Fresh cooked prawns	Low 10 ²	5
					Medium : 10 ⁴	5
					High : 10 ⁶	5
			Fish pate	Low 10 ²	5	
				Medium : 10 ⁴	5	
				High : 10 ⁶	5	
Multi component foods	Composite foods with raw ingredients	<i>E.coli</i> CRA 1265 dried foods	<i>E.hermanii</i> CRA 7477 from sesame seeds	Sandwiches	Low 10 ²	5
					Medium : 10 ⁴	5
					High : 10 ⁶	5
			Cooked chilled rice	Low 10 ²	5	
				Medium : 10 ⁴	5	
				High : 10 ⁶	5	

*these are target values only and actual values may be ± 1 log from the target dependent on microbial behaviour

Total number of samples tested= 150

3.2.2 Calculations and interpretation of accuracy profile study

The statistical results and the accuracy profiles are provided in Figures 15 to 24.

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 15 Accuracy profile for Category: Milk and dairy products (type pasteurised)– coliforms

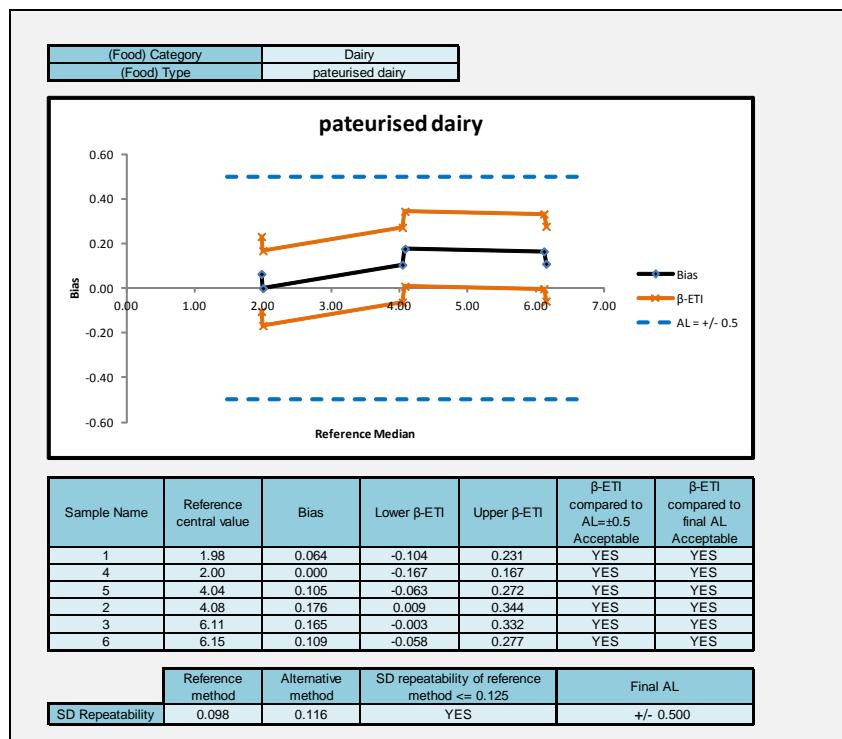


Figure 16 Accuracy profile for Category: Fresh produce and fruits (type fresh produce) – coliforms

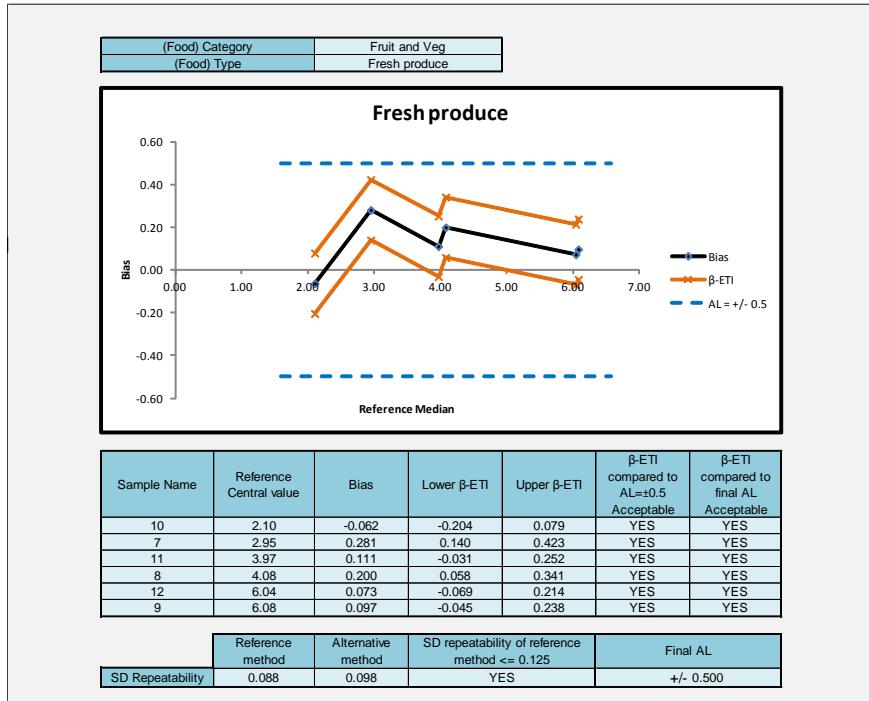


Figure 17 Accuracy profile for Category: Raw poultry and meats (type raw meat) – coliforms

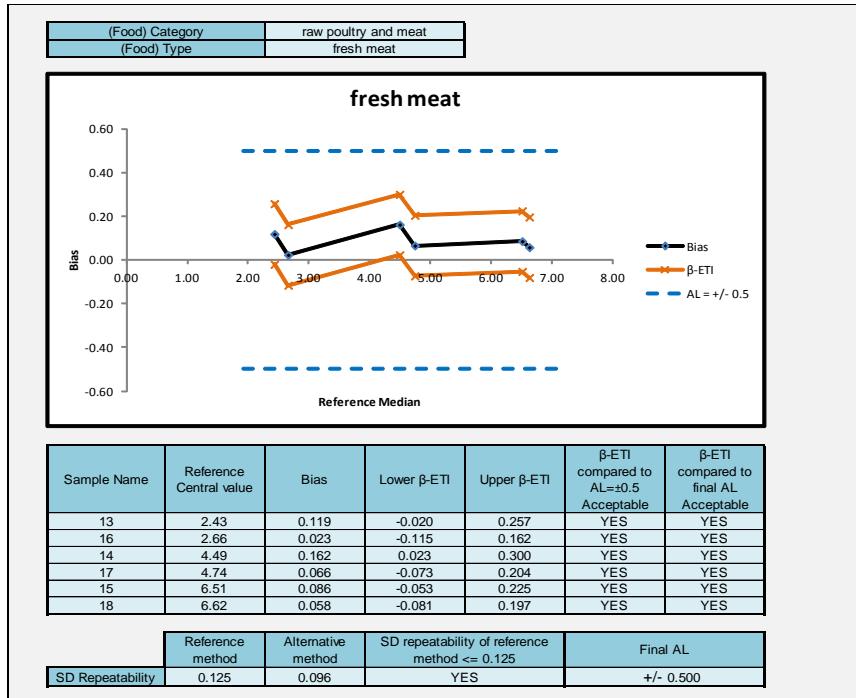


Figure 18 Accuracy profile for Category: Ready to eat foods (type RTE fish) – coliforms

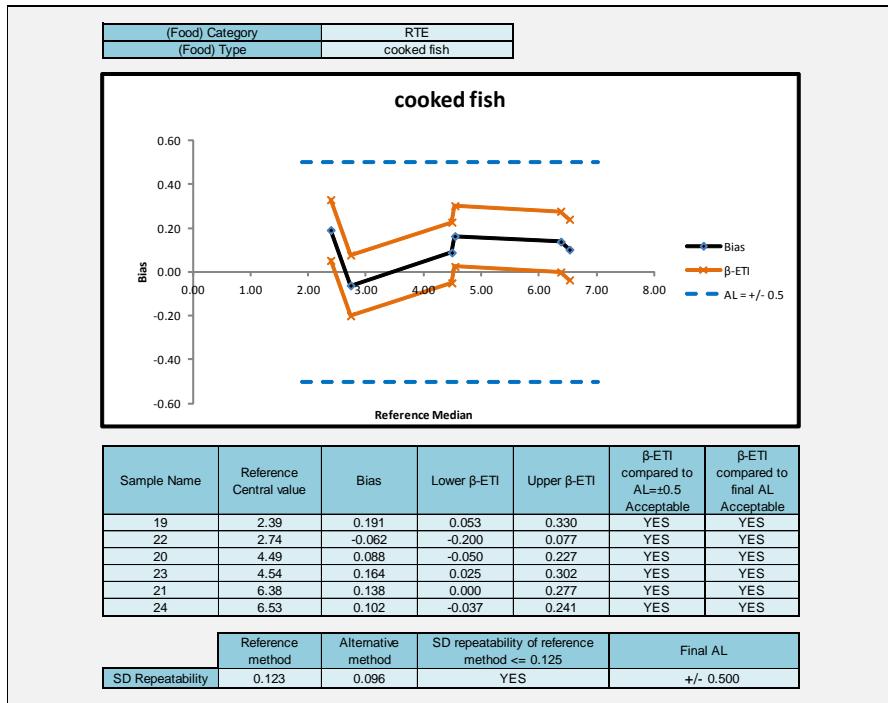


Figure 19 Accuracy profile for Category: Multi component foods (type foods with raw ingredients) – coliforms

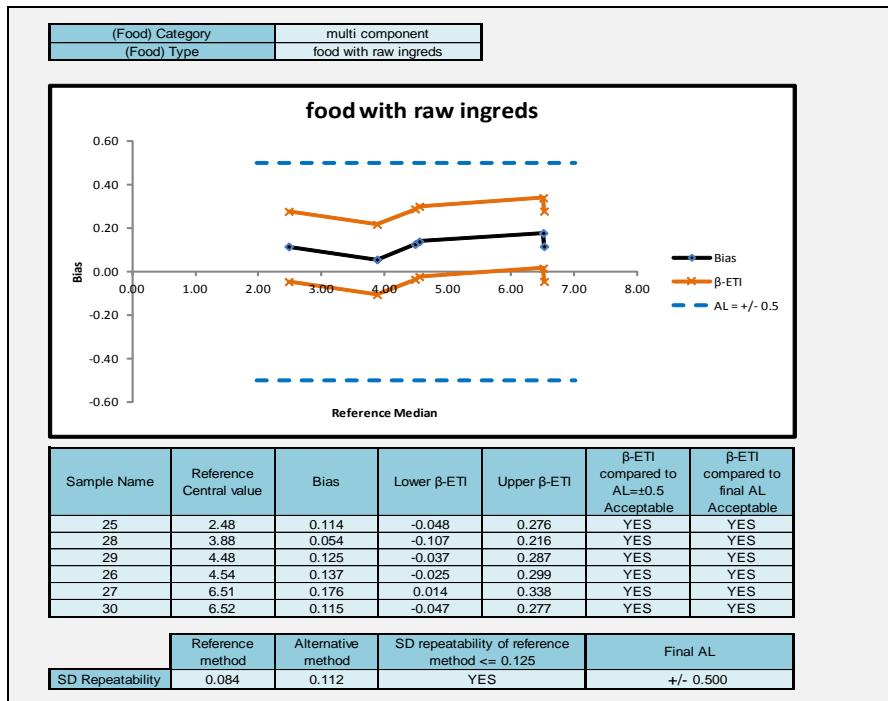


Figure 20 Accuracy profile for Category: Milk and dairy products (type pasteurised) – E.coli

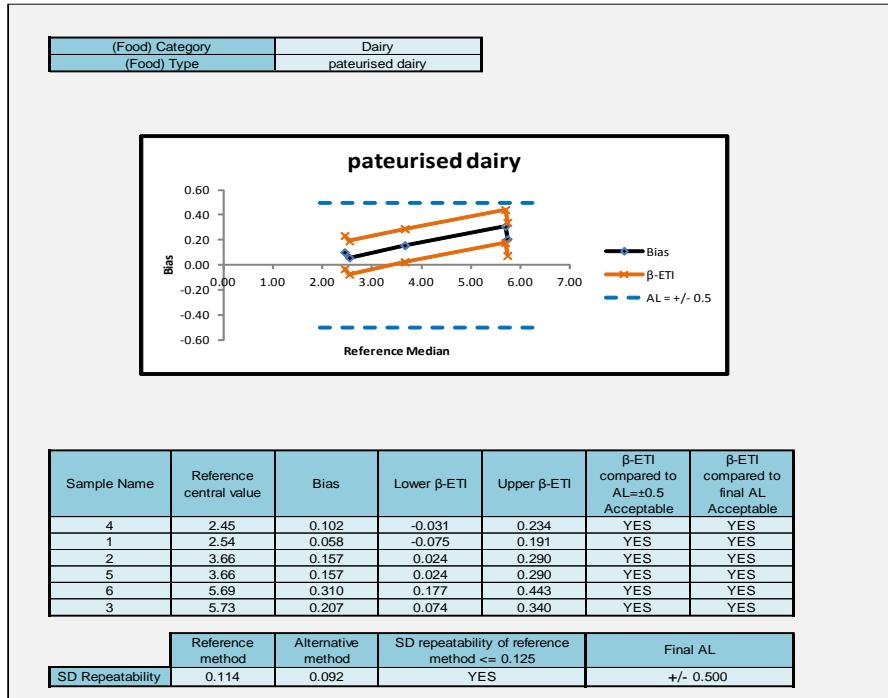


Figure 21 Accuracy profile for Category: Fresh produce and fruits (type fresh produce) - E.coli

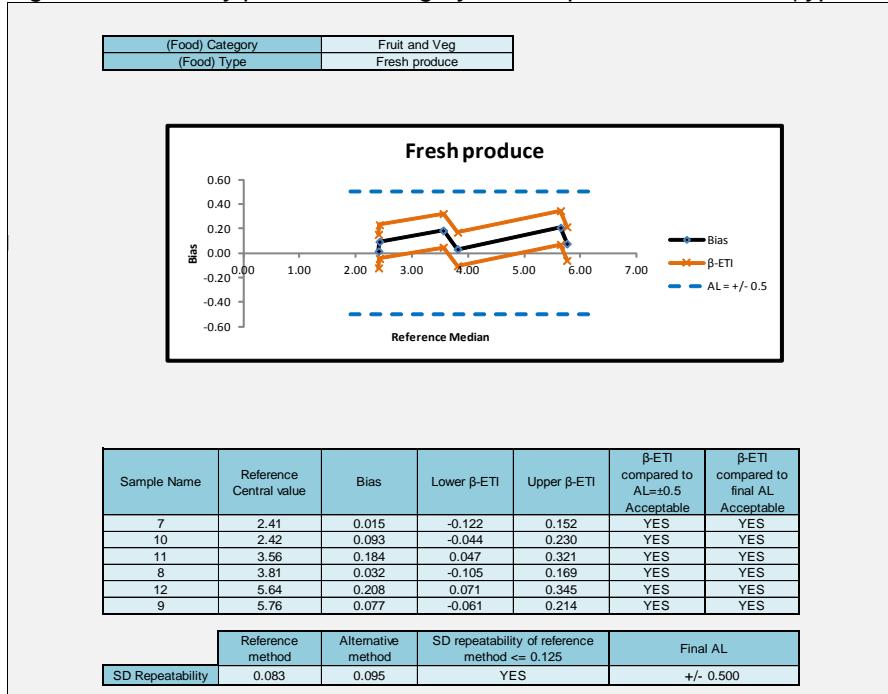


Figure 22 Accuracy profile plot for Category: Raw poultry and meats (type raw meat) - E.coli

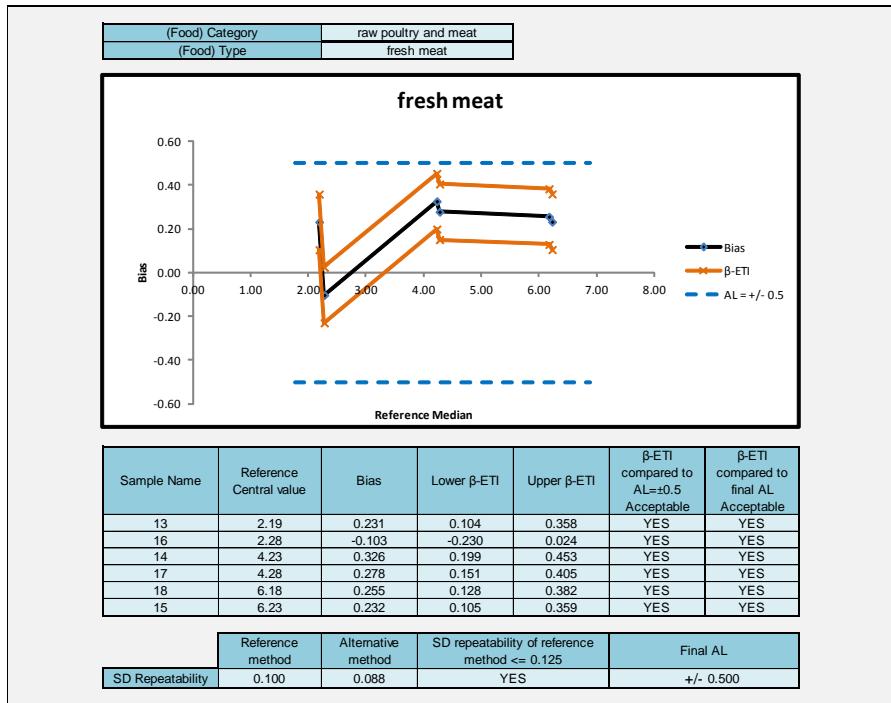


Figure 23 Accuracy profile plot for Category: Ready to eat foods (type RTE fish) – E.coli

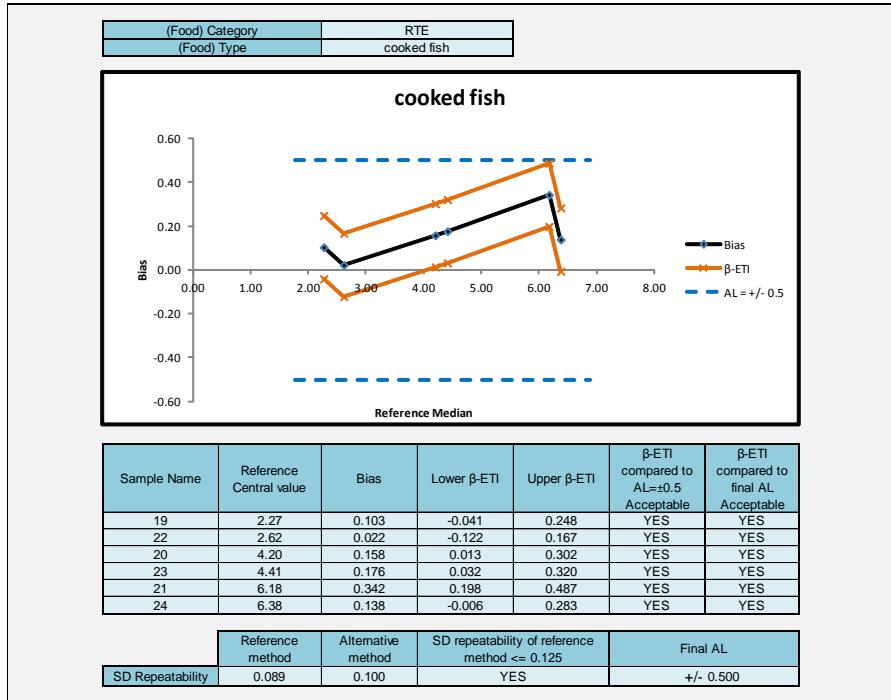
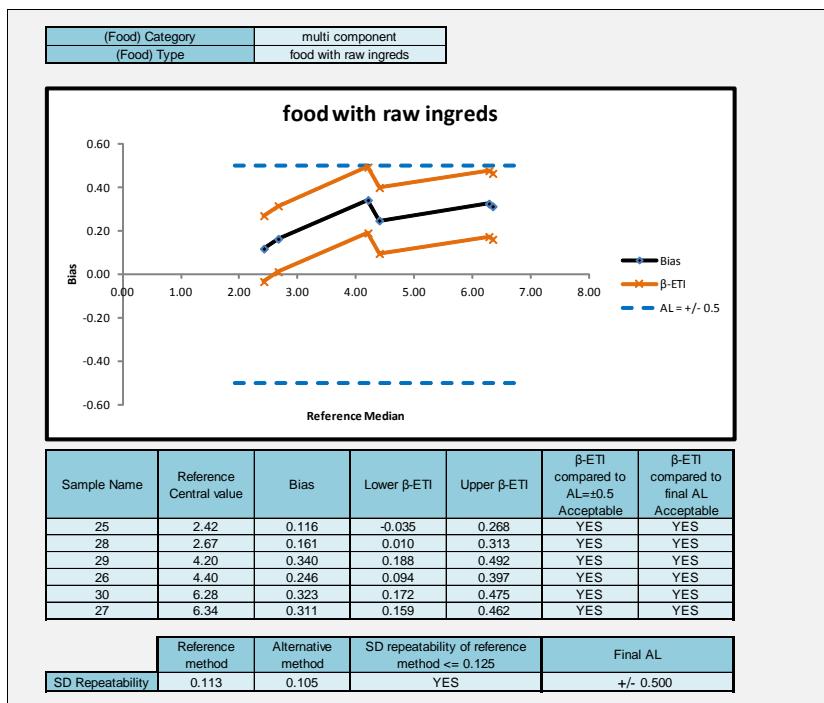


Figure 24 Accuracy profile plot for Category: Multi component foods (type foods with raw ingredients) – *E.coli*



If any of the upper or lower limits exceeded the 0.5log AP limits and the standard deviation of the reference method was >0.125, additional evaluation procedures are required, as described in ISO 16140-2:2016 and the new acceptability limits are calculated

In this study all five categories met the AL of 0.5log for both coliforms and *E.coli*. No additional calculations were necessary. The AP graphs show a slight positive bias for *E.coli* for all categories in line with the level of positive bias seen in the relative trueness study.

The accuracy of the Alternative method is satisfied as all categories met the 0.5log 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 different inclusivity panels were used in this study; one for *E.coli* and one for coliforms.

- 1) Fifty strains of *E.coli* were grown in Nutrient Broth at 37±1°C for 18-24h and appropriate dilutions were made for testing. Each strain was tested once with the alternative method, the reference method and a non-selective agar.
- 2) Fifty strains of coliforms were grown in Nutrient Broth in at 37±1°C for 18-24h and appropriate dilutions were made for testing. Each strain was tested once with the alternative method, the reference method and a non-selective agar.

- Exclusivity

Two different inclusivity panels were used in this study; one for *E.coli* and one for coliforms.

- 1) Thirty strains of coliforms (non-*E.coli*) were grown in appropriate non-selective broths and incubation conditions and appropriate dilutions were made for testing. Each strain was tested once with the alternative method, the reference method and a non-selective agar.
- 2) Thirty strains of non-coliforms were grown in appropriate non-selective broths and incubation conditions and appropriate dilutions were made for testing. Each strain was tested once with the alternative method, the reference method and a non-selective agar.

3.3.2 Results

- Inclusivity

E.coli

Of the 50 inclusivity strains tested one strain *E.coli* 3384 was not detected using either the alternative or reference method. One strain, *E.coli* 1594, was not detected by the alternative method but was detected by the reference method. And one strain, *E.coli* 473, was not detected by the reference method but was detected by the alternative method. The identity of these three strains was checked and confirmed using MALD-ToF or Rapid ID.

Coliforms

Of the 50 inclusivity strains tested 3 strains were not detected using the alternative method; *Enterobacter cloaceae* 1472, *Shimwellia blattae* NCTC 12127, and *Klebsiella rhinoscleromatis* 472. All three strains were detected by the reference method. The identity of these strains was checked and confirmed using MALD-ToF.

- Exclusivity

E.coli

Of the 30 exclusivity strains tested, one strain was detected by both the alternative method and the reference method (*Shigella sonnei* CRA 326) and one (*Shigella sonnei* 326) was detected by the alternative method only. The identity of these strains was checked and confirmed using MALD-ToF.

Coliforms

Of the 30 exclusivity strains tested, six were detected by both the alternative method and the reference method these were *Serratia marcescens* 1521, *Serratia proteamaculans* NCTC 11554, *Shigella sonnei* 10352 and *Shigella sonnei* ATCC 25931, *Serratia liquefaciens* 10670 and *Shigella boydii* NCTC 11321. In addition, *Vibrio mimicus* NCTC 11435 was detected by the alternative method but not the reference method. The identity of these detected strains was checked and confirmed using MALD-ToF.

The coliforms are a poorly defined group and whilst historically this group was based on the four genera used here (*Citrobacter*, *Enterobacter*, *Klebsiella* and *Escherichia*), other related strains which have the ability to ferment lactose due to the β -galactosidase enzyme, will also be detected on the reference medium and alternative medium.

3.3.3 Conclusion

The alternative method Media Pad EC for enumeration of coliforms and *E.coli* in foods was shown to be specific and selective and give comparable performance to the reference method

3.4 Limit of quantification (LOQ)

The limit of Quantification (LOQ) is only required for instrumental measurements. It was not done in this study

3.5 Conclusion (MCS)

Overall, the conclusions for the Method Comparison are:

- The alternative method MC Media Pad EC for enumeration of coliforms and *E.coli* shows satisfactory results for relative trueness;
- The alternative method MC Media Pad EC for enumeration of coliforms and *E.coli* shows satisfactory results for accuracy profile;
- The alternative method MC Media Pad EC for enumeration of coliforms and *E.coli* is selective and specific.

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 6 laboratories in four different countries with 2 collaborators for each laboratory involved in the study

4.1.2 Matrix and strain used

Fish paste was co- inoculated with *E.coli* 2003 isolated from fish and *Enterobacter aeringen* NCIMB 2118 from seawater.

4.1.3 Sample preparation

Samples were prepared and inoculated and despatched 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. For laboratories where there were two different collaborators, a different set of codes were used for each collaborator. A set of samples was also prepared for the EL although the data from these was not used in the data analysis

Samples were inoculated on Tuesday 27th February 2018 and then frozen for 48h prior to despatch.

The target levels and codes are shown below.

Table 7 : Contamination levels

Contamination level	Sample code set 1	Sample code set 2
Uninoculated	4	8
Low (10^2 cfu/g)	1	13
Low (10^2 cfu/g)	5	14
Medium (10^4 cfu/g)	2	10
Medium (10^4 cfu/g)	6	12
High (10^6 cfu/g)	3	9
High (10^6 cfu/g)	7	11

4.1.4 Labelling and shipping

Prior to despatch, each set of samples was removed from the freezer and packed into plastic containers (Air-Sea Containers Limited, code 490). These plastic containers were then placed inside a thermal control unit (Air-Sea Containers Limited, TC-20 code 802) with cool packs (Air-Sea Containers Limited, CP-20 code 405). The samples were packaged frozen so as de-frost occurred during transportation. Each laboratory also received an additional vial containing water "temperature control sample" which was packed with the test samples.

This was used to enable the laboratory to take a temperature measurement, representative of the samples, upon receipt. In addition to this a continuous electronic temperature monitor (Thermochron iButton) was placed in the sample packages. The laboratories were requested to return the ibuttons to the expert

laboratory upon receipt. The target storage conditions were for the temperature to stay lower or equal to 8°C during transport, and between 0°C – 8°C in the labs.

Shipping was arranged so that each laboratory would receive their samples within 72-96h dependent on location and speed of the International courier service. The samples to be sent to Europe were dispatched Thursday, and the samples sent to the UK were dispatched on Monday. The condition of the samples was recorded by each laboratory on a supplied form.

4.1.5 Analysis of Samples

Collaborative study laboratories and the expert laboratory carried out the analyses on Tuesday 6th March 2018 with the alternative and reference methods. 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 *E.coli* and coliforms in the matrix before inoculation

In order to ensure the absence of *E.coli* and coliforms in the food matrix, the reference method was performed on five portions (25 g) before the inoculation. All the results were negative.

4.2.2 Strain stability during transport

Two replicate samples of the low, medium and high inoculation levels of fish paste were enumerated on all media and at time zero (immediately after defrosting) and after 24h, 48h and 6 days storage in the shipping containers stored at 2-8°C.

Table 8: Levels of *E.coli* and coliforms (cfu/g) in stability samples stored at 2-8°C.

Level and time	Reference: coliforms	Alternate: coliforms	Reference: <i>E.coli</i>	Alternate: <i>E.coli</i>
0h				
low a	3.40E+03	4.10E+03	2.80E+03	3.20E+03
low b	7.80E+03	7.50E+03	5.60E+03	4.60E+03
medium a	3.10E+05	4.10E+05	2.70E+05	2.70E+05
medium b	3.20E+05	3.20E+05	2.10E+05	2.50E+05
high a	3.80E+06	3.00E+06	1.60E+06	1.80E+06
high b	2.80E+06	2.60E+06	1.70E+06	1.50E+06
24h				
low a	5.20E+03	9.80E+03	5.20E+03	8.10E+03
low b	8.80E+03	8.50E+03	6.70E+03	7.10E+03
medium a	1.10E+06	2.30E+06	8.50E+05	6.00E+05
medium b	3.90E+05	4.80E+05	3.10E+05	4.00E+05
high a	6.90E+06	7.00E+06	4.10E+06	4.20E+06
high b	2.40E+06	3.20E+06	1.90E+06	2.10E+06

Level and time	Reference: coliforms	Alternate: coliforms	Reference: <i>E.coli</i>	Alternate: <i>E.coli</i>
48h				
low a	1.50E+04	7.40E+03	6.50E+03	5.40E+03
low b	3.10E+03	5.70E+03	2.50E+03	3.90E+03
medium a	1.40E+05	2.90E+05	8.40E+04	1.60E+05
medium b	2.70E+05	3.50E+05	2.40E+05	2.50E+05
high a	2.50E+06	3.10E+06	1.40E+06	1.40E+06
high b	3.80E+06	4.30E+06	1.80E+06	2.50E+06
6 day				
low a	3.80E+03	2.70E+03	2.20E+03	1.70E+03
low b	5.40E+03	5.20E+03	4.30E+03	3.30E+03
medium a	2.40E+05	3.30E+05	1.00E+05	2.60E+05
medium b	1.70E+05	2.60E+05	1.00E+05	1.80E+05
high a	1.50E+06	3.50E+06	9.60E+05	2.20E+06
high b	2.00E+06	3.70E+06	9.50E+05	2.30E+06

The data showed that the levels of *E.coli* and coliforms were not affected by the freezing process and were stable during chill storage with no increase after 6 days at 2-8°C.

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 10.

Table 9 - Sample temperatures at receipt

Organising laboratory	Average Temperature measured by the probe (°C)	Temperature measured at receipt (°C)	Receipt date and time	Analysis date
1	3.7	10	02/03/18	6/03/18
2	Probe not returned	3.9	02/03/18	6/03/18
3	2.4	7.3	06/03/18	6/03/18
4	3	6.1	02/03/18	6/03/18
5	2.3	11.1	06/03/18	6/03/18
6	4	3.6	02/03/18	6/03/18
Expert lab	1.7	2	06/03/18	6/03/18

No problem was encountered during the transport or at receipt for the 12 collaborators.

All the samples were delivered on time and in appropriate conditions.

Temperatures during shipment and at receipt were all correct. The temperature reading at receipt was $<8^{\circ}\text{C}$ for four laboratories. The water temperatures were $>8^{\circ}\text{C}$ for the other two laboratories (1 and 5) but the average temperature measured by the probes as $<3.7^{\circ}\text{C}$

4.3 Calculation and summary of data

4.3.1 MicroVal Expert laboratory results

The results obtained by the expert laboratory are given in Table 10.

Table 10 – Results obtained by the expert lab.

Level	Reference method - <i>E.coli</i>	Alternative method - <i>E.coli</i>	Reference method - coliforms	Alternative method - coliforms
Blank	<10	<10	<10	<10
Low	1.50E+03	1.90E+03	3.30E+03	2.54E+03
Low	5.20E+03	5.10E+03	4.40E+03	5.70E+03
Medium	5.20E+04	4.50E+04	1.10E+05	5.23E+04
Medium	2.40E+04	2.50E+04	6.50E+04	3.22E+04
High	1.80E+06	2.60E+06	2.20E+06	3.50E+06
High	2.00E+06	2.70E+06	5.80E+06	3.57E+06

4.3.2 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 Tables 11 and 12.

The accuracy profile plot is shown in Figures 25 and 26 and the statistical analysis of the data shown in Tables 13 and 14.

Table 11: Summary of the results of the interlaboratory study per analyte level (k – data for coliforms)

Coliforms		Reference method (Log cfu/g)		Alternate method (Log cfu/g)	
Collaborator		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
Lab 01	low	3.11	3.32	3.76	3.46
Lab 02	low	3.48	3.18	3.54	3.52
Lab 03	low	3.52	3.76	3.57	3.74
Lab 04	low	3.64	3.67	3.83	3.72
Lab 05	low	3.66	3.74	3.50	3.88
Lab 06	low	3.65	3.74	3.49	3.52
Lab 07	low	3.63	3.66	3.72	3.66
Lab 08	low	3.59	3.63	3.54	3.54
Lab 09	low	3.56	3.34	3.68	3.37
Lab 10	low	3.28	3.28	3.37	3.44
Lab 11	low	3.65	3.81	3.66	3.68
Lab 12	low	3.83	3.82	3.99	3.85
Lab 01	medium	4.28	4.32	4.56	4.53
Lab 02	medium	4.53	4.15	4.69	4.51
Lab 03	medium	4.78	4.58	4.81	4.69
Lab 04	medium	4.72	4.57	4.81	4.62
Lab 05	medium	4.69	4.51	4.84	4.74
Lab 06	medium	4.51	4.48	4.66	4.54
Lab 07	medium	4.57	4.68	4.76	4.79
Lab 08	medium	4.66	4.62	4.63	4.67
Lab 09	medium	4.49	4.61	4.39	4.74
Lab 10	medium	4.38	4.28	4.63	4.29
Lab 11	medium	4.69	4.74	4.96	4.87
Lab 12	medium	4.76	4.65	4.91	4.98
Lab 01	high	6.20	6.23	6.34	6.30
Lab 02	high	6.36	7.26	6.45	6.25
Lab 03	high	6.64	6.61	6.80	6.79
Lab 04	high	6.54	6.66	6.51	6.60
Lab 05	high	6.51	6.11	6.63	6.24
Lab 06	high	6.11	6.11	6.39	6.20
Lab 07	high	6.68	6.54	6.81	6.59
Lab 08	high	6.46	6.65	6.64	6.60
Lab 09	high	6.59	6.41	6.49	6.56
Lab 10	high	6.34	6.30	6.41	6.21
Lab 11	high	6.57	6.53	6.71	6.65
Lab 12	high	6.53	6.56	6.78	6.63

Table 12: Summary of the results of the interlaboratory study per analyte level (k – data for *E.coli*)

	<i>E.coli</i>	Reference method (Log cfu/g)		Alternate method (Log cfu/g)	
Collaborator		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
Lab 01	low	3.32	3.26	3.32	3.32
Lab 02	low	3.70	3.40	3.46	3.11
Lab 03	low	3.34	3.76	3.40	3.69
Lab 04	low	3.68	3.65	3.72	3.61
Lab 05	low	3.90	3.60	3.46	3.80
Lab 06	low	3.61	3.65	3.38	3.48
Lab 07	low	3.18	3.45	3.60	3.52
Lab 08	low	3.46	3.61	3.40	3.45
Lab 09	low	3.34	3.26	3.63	3.26
Lab 10	low	3.32	3.30	3.32	3.40
Lab 11	low	3.28	3.66	3.56	3.54
Lab 12	low	3.81	3.73	3.85	3.70
Lab 01	medium	4.23	4.26	4.32	4.32
Lab 02	medium	4.34	4.28	4.52	4.30
Lab 03	medium	4.76	4.61	4.74	4.60
Lab 04	medium	4.57	4.53	4.69	4.52
Lab 05	medium	4.62	4.54	4.79	4.68
Lab 06	medium	4.41	4.40	4.59	4.45
Lab 07	medium	4.38	4.58	4.62	4.72
Lab 08	medium	4.38	4.40	4.54	4.56
Lab 09	medium	4.00	4.59	4.30	4.67
Lab 10	medium	4.45	4.20	4.57	4.23
Lab 11	medium	4.52	4.61	4.82	4.79
Lab 12	medium	4.20	4.41	4.81	4.84
Lab 01	high	6.15	6.26	6.20	6.20
Lab 02	high	6.26	6.18	6.36	6.20
Lab 03	high	7.41	6.53	6.40	6.68
Lab 04	high	6.30	6.45	6.23	6.46
Lab 05	high	6.28	5.97	6.52	6.00
Lab 06	high	6.15	5.91	6.28	6.00
Lab 07	high	6.41	6.52	6.64	6.48
Lab 08	high	6.34	6.49	6.52	6.49
Lab 09	high	6.11	6.28	6.36	6.40
Lab 10	high	6.23	6.15	6.23	6.15
Lab 11	high	6.62	6.57	6.62	6.57
Lab 12	high	6.76	6.38	6.76	6.38

Figure 25. Accuracy profile of MC Media Pad EC from the ILS - coliforms

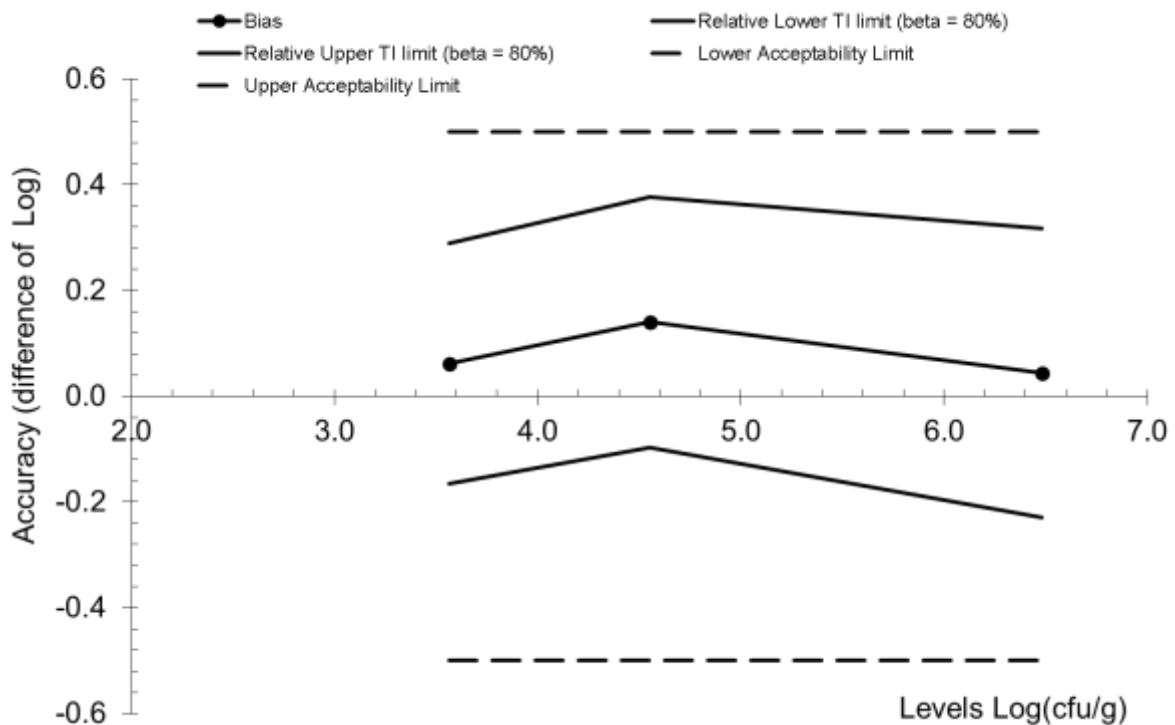


Figure 26. Accuracy profile of MC Media Pad EC from the ILS – *E.coli*

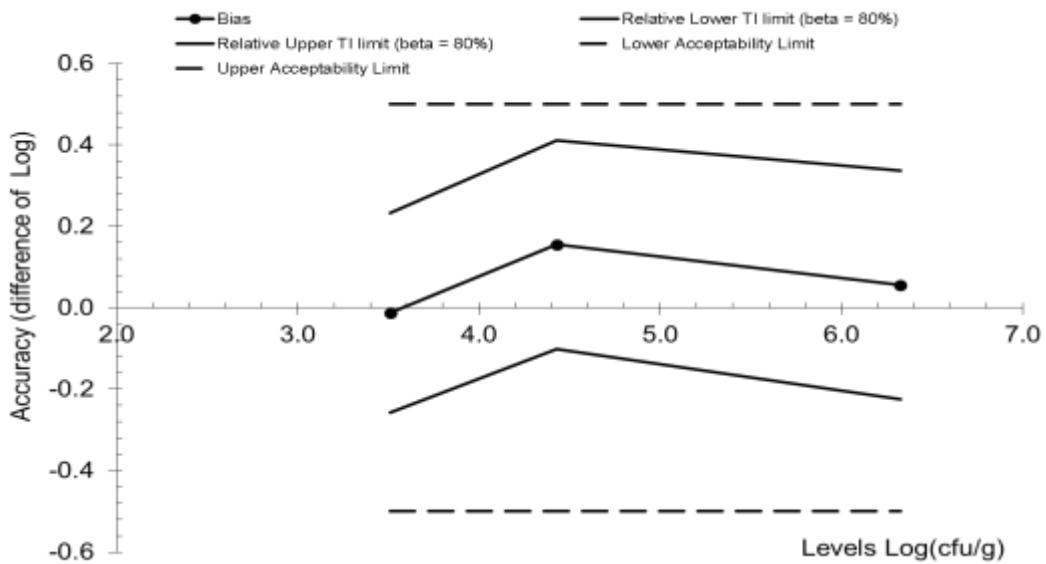


Table 13. Statistical analysis of the ILS data according to the ISO spreadsheet- coliforms

Accuracy profile		0.5	
Study Name	EC coliforms		
Date	11/04/2018		
Coordinator	Campden BRI		
Tolerance probability (beta)	80%	80%	
Acceptability limit in log (lambda)	0.50	0.50	
Alternative method			
Levels	Low	Medium	High
Target value	3.566	4.552	6.483
Number of participants (K)	12	12	12
Average for alternative method	3.627	4.692	6.524
Repeatability standard deviation (sr)	0.131	0.123	0.123
Between-labs standard deviation (sL)	0.103	0.121	0.157
Reproducibility standard deviation (sR)	0.167	0.173	0.198
Corrected number of dof	19.448	17.866	15.856
Coverage factor	1.364	1.372	1.382
Interpolated Student t	1.327	1.331	1.337
Tolerance interval standard deviation	0.1715	0.1780	0.2044
Lower TI limit	3.400	4.455	6.251
Upper TI limit	3.855	4.929	6.798
Bias	0.062	0.140	0.044
Relative Lower TI limit (beta = 80%)	-0.166	-0.097	-0.230
Relative Upper TI limit (beta = 80%)	0.289	0.377	0.317
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	0.213		

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.

Reference method		
Low	Medium	High
12	12	
3.566	4.552	
0.108	0.112	
0.181	0.129	
0.211	0.171	
14.288	16.675	

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

Table 26. Statistical analysis of the ILS data according to the ISO spreadsheet-E.coli

Accuracy profile		
Study Name	Ec E.coli	
Date	11/04/2018	
Coordinator	Campden BRI	
Tolerance probability (beta)	80%	80%
Acceptability limit in log (lambda)	0.50	0.50

Levels	Alternative method		
	Low	Medium	High
Target value	3.512	4.429	6.325
Number of participants (K)	12	12	12
Average for alternative method	3.499	4.583	6.381
Repeatability standard deviation (sr)	0.147	0.128	0.171
Between-labs standard deviation (sL)	0.103	0.136	0.116
Reproducibility standard deviation (sR)	0.180	0.187	0.207
Corrected number of dof	20.183	17.294	20.346
Coverage factor	1.361	1.374	1.360
Interpolated Student t	1.325	1.333	1.325
Tolerance interval standard deviation	0.1847	0.1926	0.2122
Lower TI limit	3.254	4.327	6.100
Upper TI limit	3.744	4.840	6.662
Bias	-0.013	0.155	0.056
Relative Lower TI limit (beta = 80%)	-0.257	-0.102	-0.225
Relative Upper TI limit (beta = 80%)	0.232	0.411	0.337
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	0.240	

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.

FALSE

Reference method

Low	Medium	High
12	12	12
3.512	4.429	6.325
0.160	0.149	0.213
0.136	0.093	0.230
0.210	0.176	0.314
18.936	20.803	17.165

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

FALSE

5 Overall conclusions of the validation study

- The alternative method Media pad EC™ for enumeration of *E.coli* and coliforms shows satisfactory results for relative trueness;
- The alternative Media pad EC™ for enumeration of *E.coli* and coliforms shows satisfactory results for accuracy profile;
- The alternative Media pad EC™ for enumeration of *E.coli* and coliforms is selective and specific.
- The alternative Media pad EC™ for enumeration of *E.coli* and coliforms shows satisfactory performance in the ILS

The alternative Media pad EC™ for enumeration of *E.coli* and coliforms shows comparable performance to the reference methods ISO 16649-2:2001 and ISO 4832:2006 for enumeration of *E.coli* and coliforms in a broad range of foods

Date : 28/03/2019

Signature:



Annexes

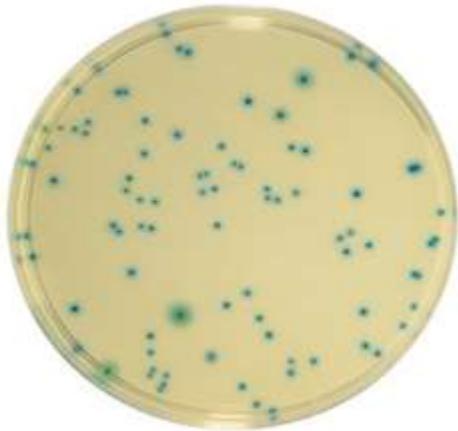
- A. Flow diagram of the reference and alternative method
- B. Test kit insert

ANNEX A: Typical colony morphology and Flow diagram of the alternative method and reference methods

Picture 1: Typical colonies on JNC Media Pad EC: Blue/Green =coliforms Red/Navy = *E.coli*



Picture 2: Typical colonies on TBX

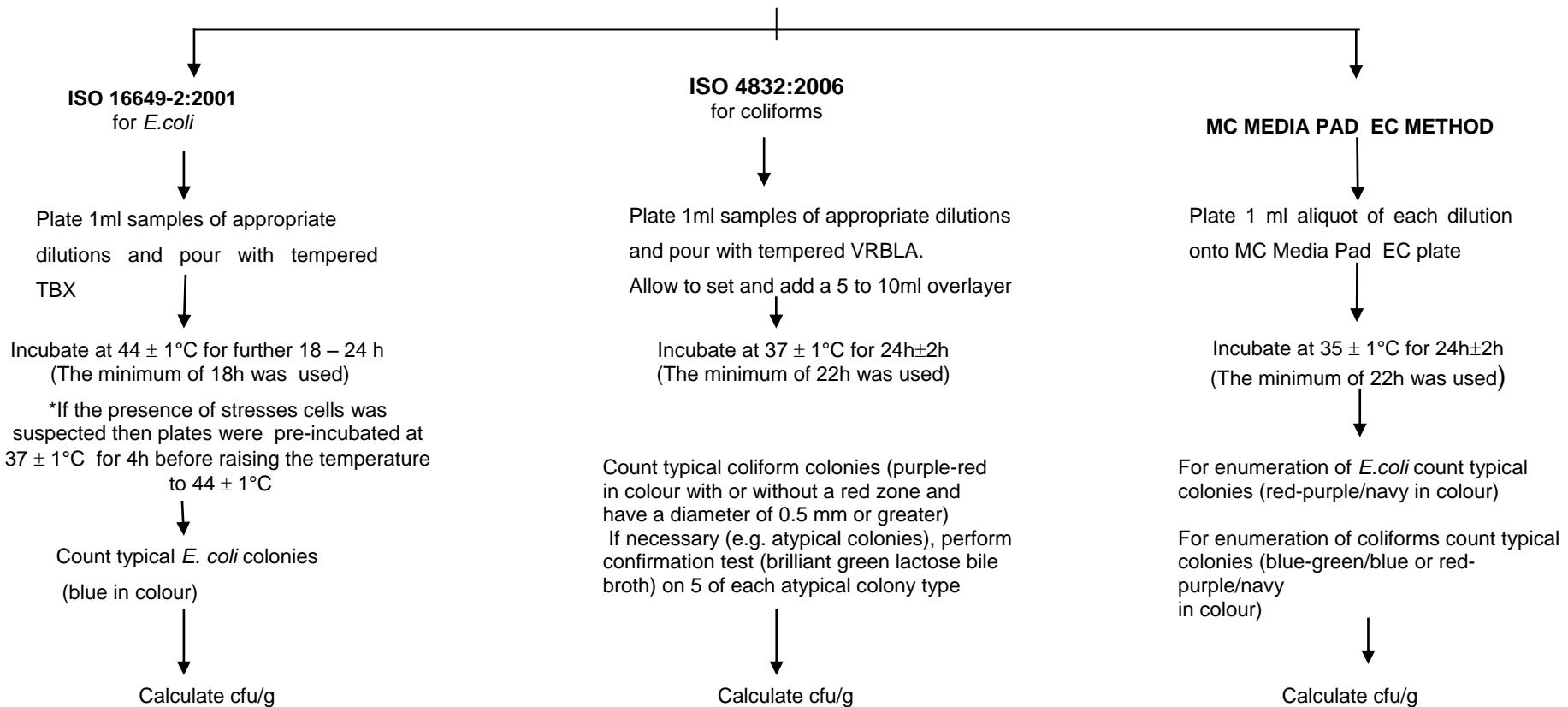


Picture 3: Typical colonies on VRBLA



Food sample (10g) + appropriate diluents (90ml) dilution.

Homogenise and dilute further as required



ANNEX B: Kit insert(s) -latest version provided as a separate document

Instruction Manual

MC-Media E.coli & Coliform

Convenient culture media for simultaneous enumeration of Escherichia coli and coliform bacteria

APPLICATION

For hygiene control, it is important to determine the microbial count in food and beverage products. MC-Media Pad E.coli & Coliform is intended to simultaneously determine coliform and E. coli number through special medium composition and specific chromogenic substrates for both β -galactosidase and β -glucuronidase. MC-Media Pad pre-sterilized, ready-to-use dry culture devices simplify testing and minimize the quantity of waste. MC-Media Pad is composed of a unique adhesive sheet, a test pad coated with medium and water absorption polymer, and a transparent cover film.

TEST PRINCIPLES

MC-Media Pads are coated with selective medium and chromogenic substrate for specific detection. Once the liquid sample is inoculated onto the test pad, the sample diffuses to the whole pad through capillary action. The medium re-constitutes automatically. If target organisms are present, coliform and E. coli grow as blue-green/blue and red-purple/navy colored colonies on the test pad, respectively.

CONTENTS and STORAGE

100 pads (4x25 pads); catalogue number 1323000001

This kit should be stored between 2-15°C. (Refrigerated)

MATERIALS REQUIRED BUT NOT PROVIDED

Incubator (35°C \pm 1)

Stomacher or Blender

Sampling bag (Recommended for Stomacher; bag with filter to eliminate food debris)

Pipette or Pipettor and pipette tips

Phosphate Buffered Saline or appropriate diluents according to EN ISO 6887

SAMPLE PREPARATION

For solid food samples

Homogenize the test sample with 9-fold volume of appropriate diluent (e.g. Phosphate Buffered Saline, Butterfield's Phosphate Buffer, saline or water) with a stomacher. If necessary, make 10-fold serial dilution.

For liquid samples

Sample can be applied directly. If necessary, pH of sample should be adjusted to neutral (pH 7.0 \pm 0.2).

TEST PROCEDURE

General Operation

1. Open the aluminum bag, and remove MC-Media Pad. If necessary, write information on the cover film.
2. Lift the transparent cover film and pipette 1.0 mL of sample solution onto test pad. (It is recommended to lift the cover film diagonally for easy and secure re-sealing.)
3. Close the cover film and lightly press the edges of film to seal.
4. Incubate test plate at 35°C \pm 1 for 24 \pm 2hours.
5. Re-seal the opened bags and store at 2-8°C for up to 4 weeks.

INTERPRETATION

Count all colored colonies (blue-green/blue and red-purple/navy) as coliform regardless of strength of color. For E. coli count, only red-purple/navy colored colonies should be counted. If the large number of colonies is difficult to count, colony counts can be estimated by counting colonies in one grid square and multiplying by 20. If more than 104 of microbes are grown, the entirety of test pad may appear as stained, and it may appear that no individual colonies were formed. If this is the case, dilute the sample further and re-test. If necessary, the target colony can be picked up with a sterile needle from test pad for further analysis.

PRECAUTIONS

- ☒ The test is designed for use by quality control personnel and others familiar with testing samples potentially contaminated with aerobic microbes.
- ☒ Read this instruction manual carefully before use.
- ☒ After opening the aluminum bag, unused pads should be stored in the aluminum bag sealed with tape, and kept in a cool (2-15°C) environment. After opening, use all pads within 1 month.
- ☒ Do not expose unused pads to sunlight or ultraviolet light.
- ☒ Do not use a discolored or damaged pad.
- ☒ A wrinkle on the test pad should not affect detection.
- ☒ Small fragments of fabric on or around the test pad should not affect detection.
- ☒ Do not use the pads after the expiration date. The quality of an expired pad is not warranted.
- ☒ The measurement range is less than 300 cfu/pad. If more than 300 cfu/pad counted, further dilution is recommended.
- ☒ MC-Media Pad Coliform detects coliform bacteria by existence of β -galactosidase. Therefore, certain bacteria (genus Aeromonas etc.) which possess this enzyme may grow as coliform.
- ☒ E. coli serotype O157 is detected as coliform (blue-green/blue) because it lacks β -glucuronidase.
- ☒ In cases where β -galactosidase containing foods are applied (e.g. cheese, lactic drink or liver), the entirety of test pad may appear as stained
- ☒ The used kit must be sterilized by autoclaving or boiling, and disposed according to local regulations for waste.

CONTACT and FURTHER INFORMATION

Merck KGaA, Darmstadt, Germany.

www.millipore.co.de