Method Comparison Study Report for the ISO 16140-2:2016 validation of MC Media pad CC, for the enumeration of coliforms in a broad range of foods

MicroVal study number: 2017LR70

Method/Kit name: MC Media pad CC

Report version:MCS ILS Summary report 28/03/2019

MicroVal Expert Laboratory: Campden BRI (Linda Everis and Gail Betts gail.betts@campdenbri.co.uk)

Foreword

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

Company: JNC Corporation,
Yokohama Research Center
5-1, Ookawa,
Kanazawa-ku,
Yokohama, Kanagawa,
Japan, 236-8605

Expert Laboratory: Campden BRI

Method/Kit name: MC Media pad CC

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

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 meat, poultry and fish)
5. Multi component foods or meal components

Certification orgnization: Lloyd's Register

List of abbreviations

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

Contents

1 Introduction

In this project a MicroVal validation study, based on ISO 16140-2:2016, of alternative method(s) for the enumeration of 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 coliforms on MC Media pad CC, incubated at 35±1°C for 24±h

The reference method used was:

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

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 summarized below:

The alternative method *MC Media pad CC* shows comparable performance to the reference methods (ISO 16649-2:2001, ISO 4832:2006) for the enumeration of coliforms 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 method 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 CC kit insert in Annex B.

The alternative method principle is based on chromogenic media

MC Media pad CC: is a quantitative sheet method intended for selective enumeration of coliforms. It has a special medium composition and specific chromogenic substrate for β-galactosidase. 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 coliform organisms are present, they grow as blue-green/blue colonies on the test pad.

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 analyzed, 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 occuring target organisms making a total of seventy five samples which were tested. From these samples 26 samples (34%) were positive for coliforms and these data were used in the analysis. The remaining 49 samples (66%) were negative for the coliforms and needed to have artificial contamination.

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
  - and storing chilled for minimum 48h at <5°C; stored
  - and storing frozen for minimum 2 weeks at <-20°C or
  - 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 55ºC for 5minutes.

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

The observed injury measurements varied from 1.0 to 1.46 log cfu/g difference between non-selective and selective plates.

3.1.3 Protocols applied during the validation study

A single protocol was applied for the study.

Reference method plates were incubated at 37±1ºC for 24±2h.

Alternative method plates were incubated at 35±1ºC for 24±2h.

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 method in order to have 15 interpretable results per incubation protocol, and 5 interpretable results per tested type by the two methods.

3.1.5 Calculation and interpretation of relative trueness study

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.

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

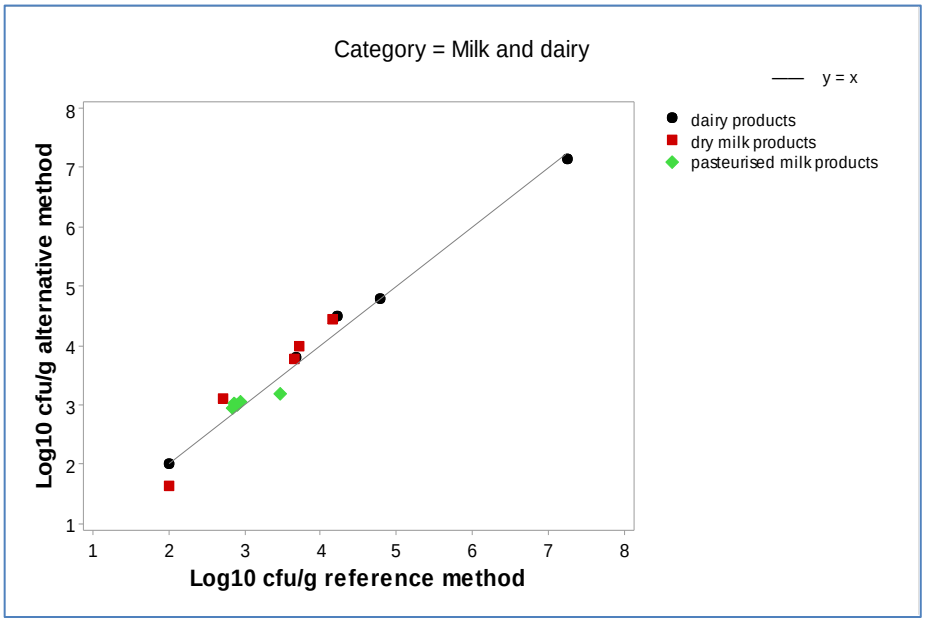


Figure 2- Scatter plot of the reference method versus alternative method results for Fresh produce and fruits

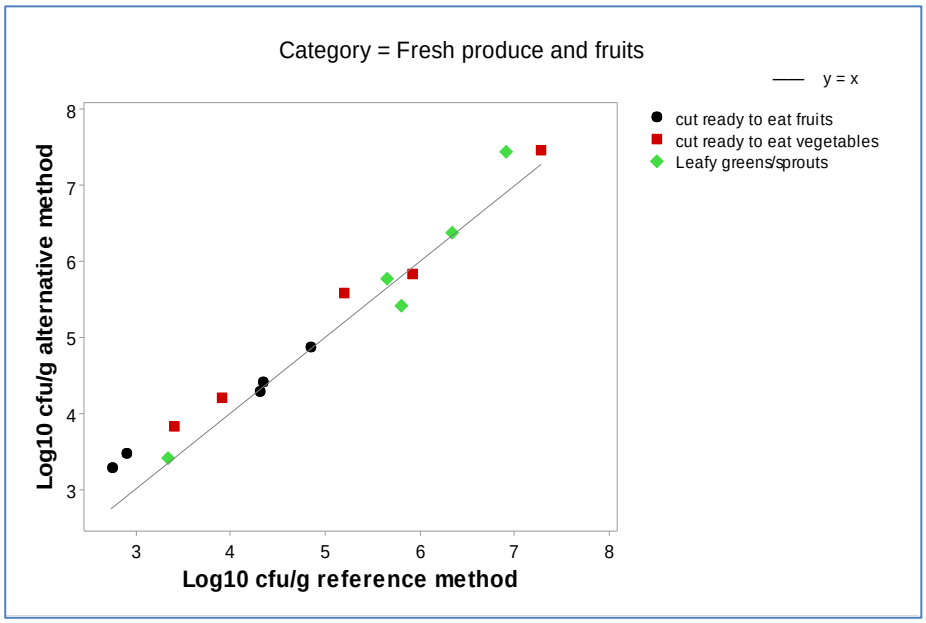


Figure 3- Scatter plot of the reference method versus alternative method results for Raw poultry and meats

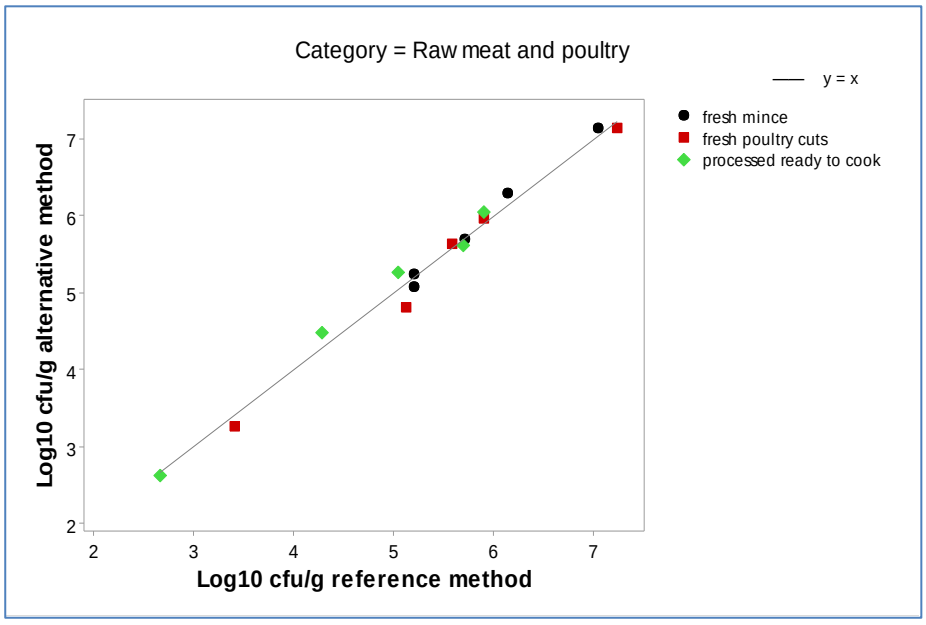


Figure 4- Scatter plot of the reference method versus alternative method results for Ready to eat foods

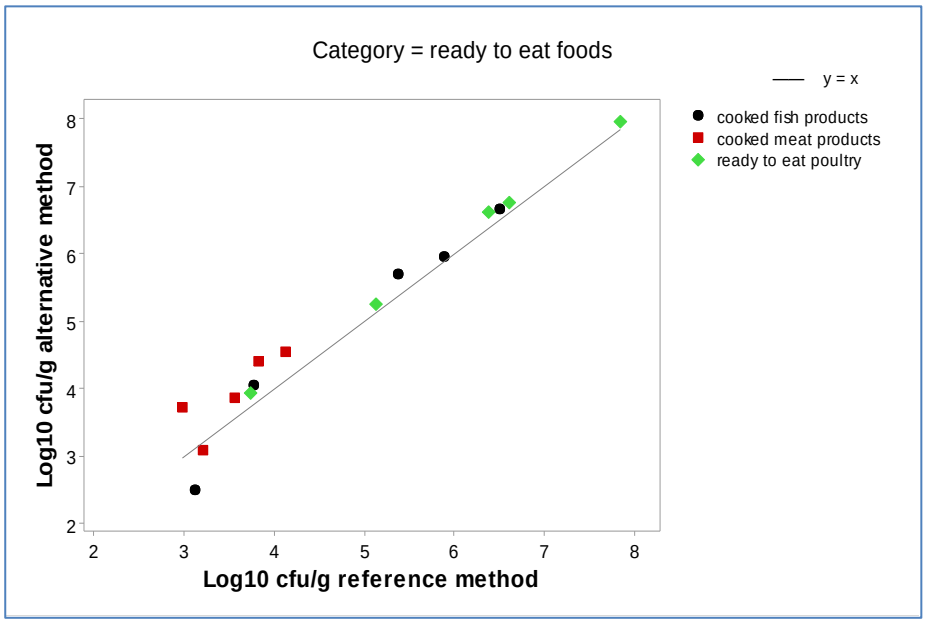


Figure 5- Scatter plot of the reference method versus alternative method results for Multi component foods

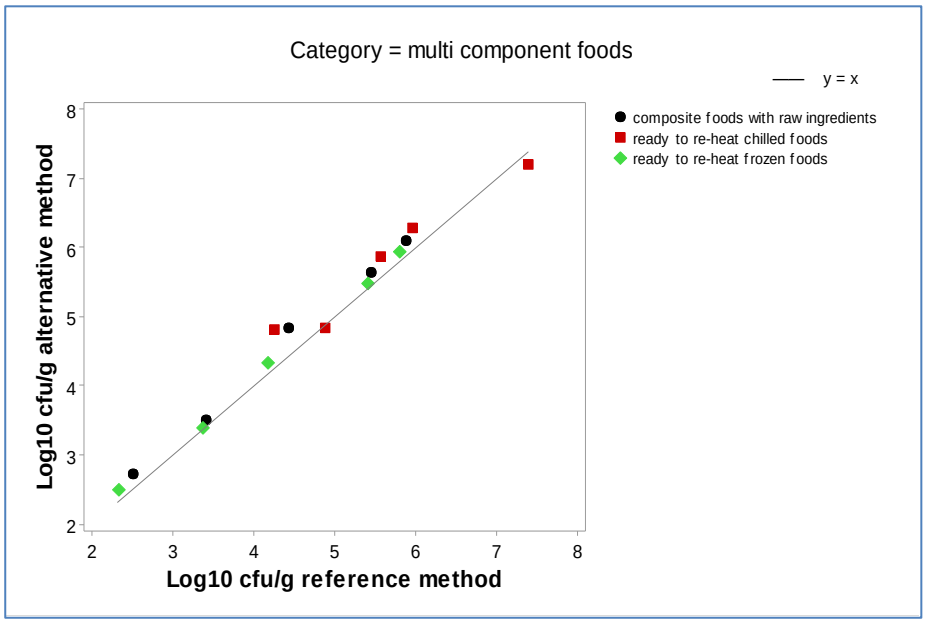
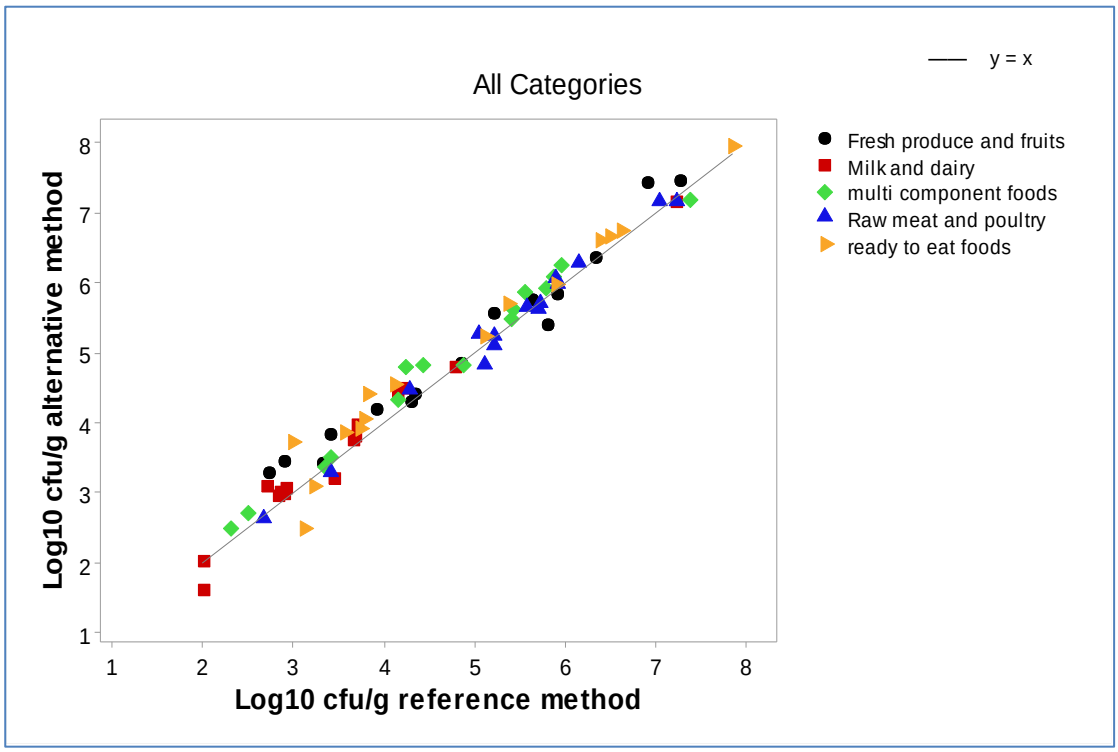


Figure 6 - Scatter plot of the reference method versus alternative method results for all categories



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.

There is a slight positive bias for the alternative method for fresh produce, ready to eat and multi component foods. This can be seen from Figures (1, 4 and 5) and from the all categories Figure (6).

A summary of the calculated values per category is provided in Table 2 and the Bland-Altman difference plot for all the samples is given Figure 7 for coliforms.

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

Category.	n	$\overline{D}$	s_D	95% Lower limit	95% Upper limit
Fresh produce and fruits	15	0.169	0.267	-0.422	0.759
Milk and dairy	15	0.077	0.209	-0.385	0.540
Multi component foods	15	0.173	0.184	-0.235	0.582
Raw meat and poultry	15	0.005	0.143	-0.313	0.322
Ready to eat foods	15	0.193	0.313	-0.501	0.887
All Categories	75	0.123	0.236	-0.350	0.597

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

Figure 7 – Bland-Altman difference plot for all the samples

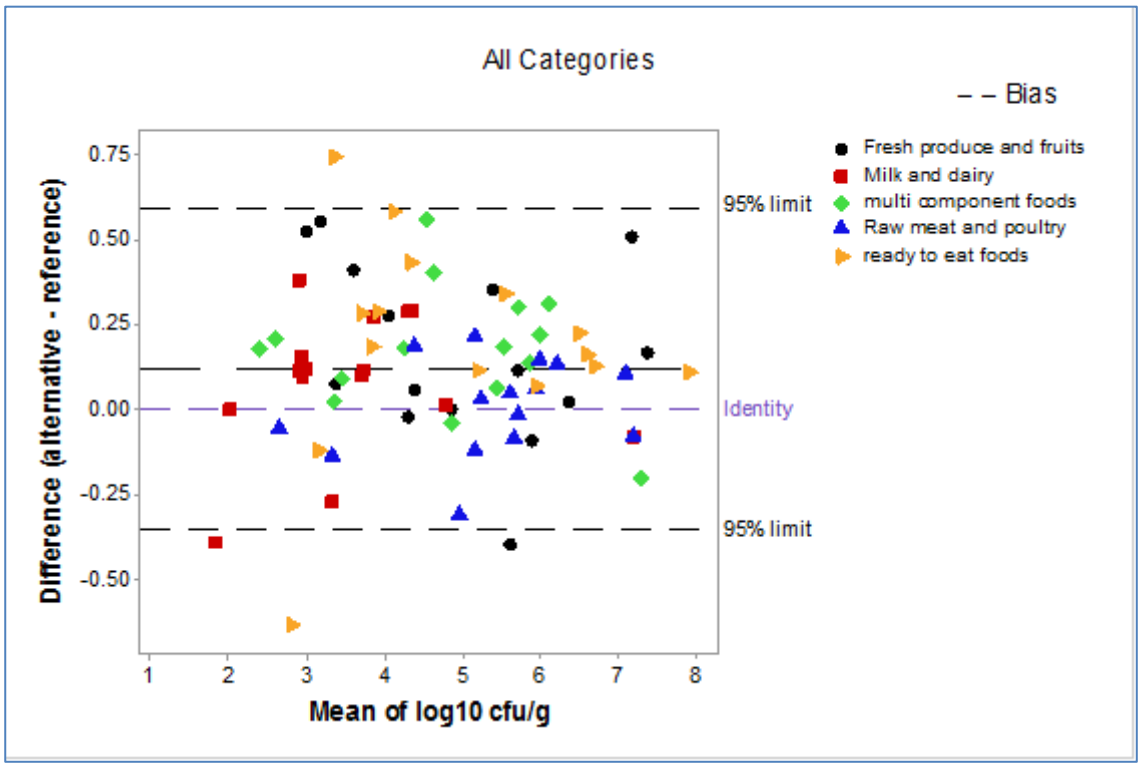


Table 3 - Data which are outside of the accepted limits - coliforms

Category	Types	Code	Food item	strain	Spiking/ seeding	Log (Ref)	Log (Alt)	Mean	Difference
Milk and dairy	dry milk products	1	Pud in a mug	*E.coli* 1476	ambient 2 weeks	2	1.602	1.801	-0.397
Ready to eat foods	cooked fish products	53	Herring Sweetcure	*E.coli* 108, *Enterobacter amingenus* NCIMB 2118	chill 2-3 days	3.113	2.477	2.795	-0.636
Ready to eat foods	cooked meat products	57	Ready to Eat Slow Cooked Shredded Ham	*E.coli* 2077, *Enterobacter gergoviae* NCIMB 13304	Heat 55oC/5 mins	2.973	3.716	3.344	0.742
Fresh produce and fruits	Leafy greens/ sprouts	28	Beansprouts	*E.coli* 6160, Natural	chill 2-3 days	5.799	5.397	5.598	-0.401

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

In this study 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.

3.1.6 Conclusion (RT study)

The relative trueness of the Alternative method 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.

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 possible to use either 2 separate batches of a single item for each food type. Or it is possibe 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.

This study was run in parallel with the study for Media Pad EC which can detect both coliforms and *E.coli*. Therefore, each sample tested was co-inoculated with an *E.coli* strain and another non-*E.coli* coliform (Table 4.

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

Category	Types	Strain for *E.coli* study	Strain for coliforms study	Item	Target Level* cfu/g	Test portions
Dairy products	Pasteurised dairy products	*E. coli* CRA 1476 from dried milk	*E. decarboxylate* CRA 5501 from skimmed milk powder	Pasteurised cream	Low 10^2	5
					Medium : 10^4	5
					High : 10^6	5
				Cream cheese	Low 10^2	5
					Medium : 10^4	5
					High : 10^6	5
					Low 10^2	5

Category	Types	Strain for *E.coli* study	Strain for coliforms study	Item	Target Level* cfu/g	Test portions
Fruits and vegetables	Fresh produce	*E.coli* CRA 3779 from frozen spinach	*Citrobacter amalonaticus* CRA 7458 from beansprouts	Ready to cook Vegetables	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	*E. coli* CRA 3384 from pork	*Escherichia fergusonii* 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	*E.coli* CRA 2003 isolated from fish	*Enterobacter amingenus* 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	*E.coli* CRA 1265 dried foods	*E.hermanii* 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

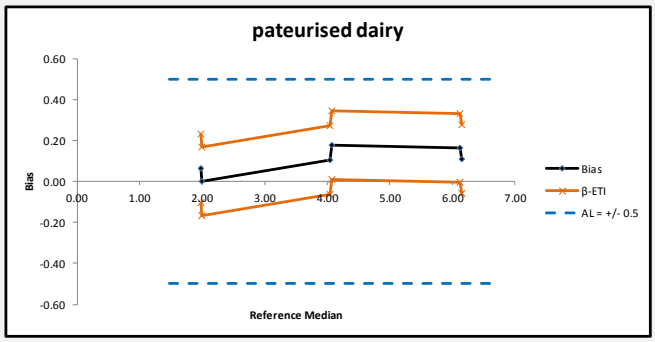
3.2.2 Calculations and interpretation of accuracy profile study

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 8 Accuracy profile for Category:Milk and dairy products (type :pasteurised)

(Food) Category	Dairy
(Food) Type	pateurised dairy

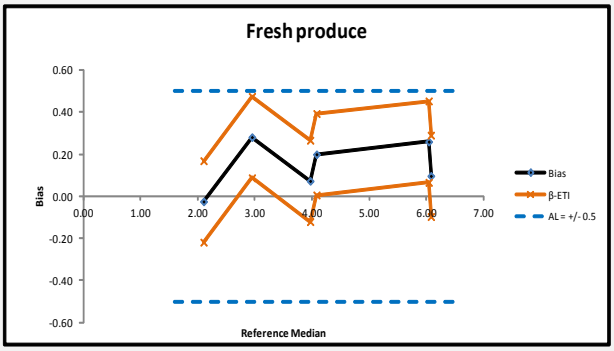


Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
1	1.98	0.064	-0.104	0.231	YES	YES
4	2.00	0.000	-0.167	0.167	YES	YES
5	4.04	0.105	-0.063	0.272	YES	YES
2	4.08	0.176	0.009	0.344	YES	YES
3	6.11	0.165	-0.003	0.332	YES	YES
6	6.15	0.109	-0.058	0.277	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.098	0.116	YES	+/- 0.500

Figure 9 Accuracy profile for Category Fresh produce and fruits (type :fresh produce)

(Food) Category	Fruit and Veg
(Food) Type	Fresh produce

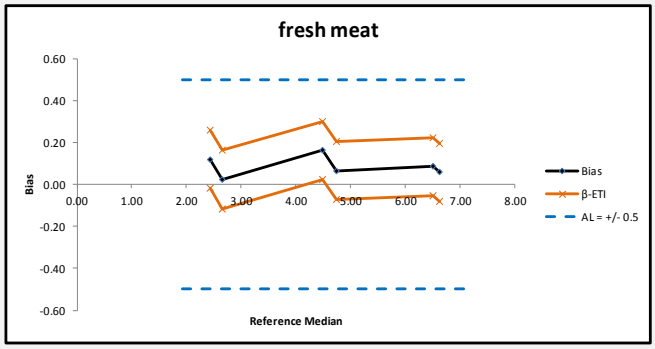


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
10	2.10	-0.025	-0.218	0.169	YES	YES
7	2.95	0.281	0.088	0.475	YES	YES
11	3.97	0.073	-0.121	0.266	YES	YES
8	4.08	0.200	0.006	0.393	YES	YES
12	6.04	0.260	0.066	0.453	YES	YES
9	6.08	0.097	-0.097	0.290	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.088	0.134	YES	+/- 0.500

Figure 10 Accuracy profile for Category Raw poultry and meats (type :fresh meat)

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

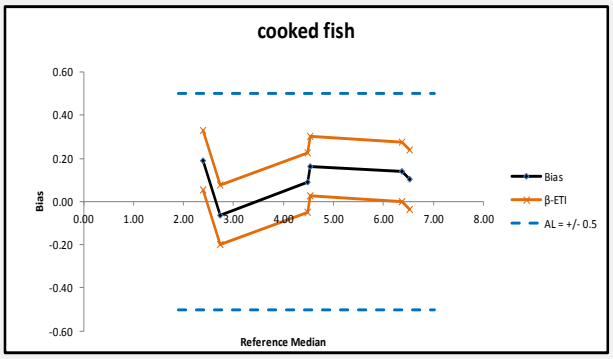


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
13	2.43	0.119	-0.020	0.257	YES	YES
16	2.66	0.023	-0.115	0.162	YES	YES
14	4.49	0.162	0.023	0.300	YES	YES
17	4.74	0.066	-0.073	0.204	YES	YES
15	6.51	0.086	-0.053	0.225	YES	YES
18	6.62	0.058	-0.081	0.197	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.097	0.096	YES	+/- 0.500

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

(Food) Category	RTE
(Food) Type	cooked fish

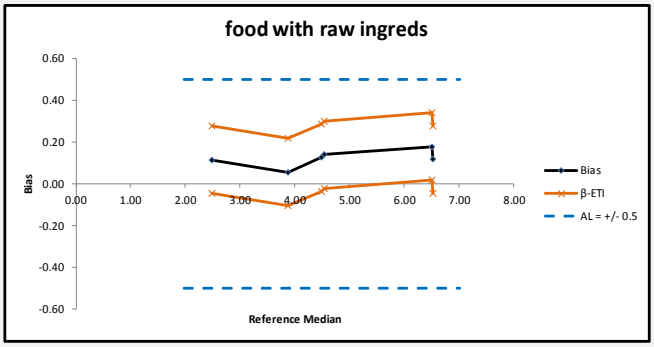


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
19	2.39	0.191	0.053	0.330	YES	YES
22	2.74	-0.062	-0.200	0.077	YES	YES
20	4.49	0.088	-0.050	0.227	YES	YES
23	4.54	0.164	0.025	0.302	YES	YES
21	6.38	0.138	0.000	0.277	YES	YES
24	6.53	0.102	-0.037	0.241	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.086	0.096	YES	+/- 0.500

Figure 11 Accuracy profile for Category Multi component foods (type :foods with raw ingredients)

(Food) Category	multi component
(Food) Type	food with raw ingreds



Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
25	2.48	0.114	-0.048	0.276	YES	YES
28	3.88	0.054	-0.107	0.216	YES	YES
29	4.48	0.125	-0.037	0.287	YES	YES
26	4.54	0.137	-0.025	0.299	YES	YES
27	6.51	0.176	0.014	0.338	YES	YES
30	6.52	0.115	-0.047	0.277	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.084	0.112	YES	+/- 0.500

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. No additional calculations were necessary. The AP graphs show a slight positive bias for fresh produce and RTE fish.

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
  Fifty strains of coliforms were grown in Nutrient Broth in at 30±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

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

All 50 inclusivity strains were detected by the reference method. Forty six strains were detected by the alternative method with four strains not detected. The not detected strains were *Enterobacter cloaceae* 1472 (from dried milk), *Escherichia alkalescens* NCTC 5183 (clinical isolate) and *Shimwellia blattae* NCTC 12127 (cockroach) and *Klebsiella rhinoscleromatis* (industrial isolate).

The identity of these strains was checked and confirmed using MALD-ToF or Rapid ID.

- Exclusivity

Of the 30 exclusivity strains tested, four were detected by the alternative method and the reference method these were *Serratia marcescens 1521, Serratia proteamaculans* NCTC 11554, *Shigella sonnei* 10352 and *Shigella sonnei* ATCC 25931. In addition, *Vibrio mimicus* NCTC 11435 was detected by the alternative method but not the reference method and *Serratia liquefaciens* 10670 and *Shigella boydii* NCTC 11321 were detected by the reference method but not by the alternative method.

The identity of these detected strains was checked and confirmed using MALD-ToF or Rapid ID.

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 can ferment lactose due to the ß-galactosidase enzyme, will also be detected on the reference medium and alternative medium. For example, some strains of Erwinia and *Serratia*, can also ferment lactose, albeit slowly, and some strains of *Citrobacter* and *Klebsiella*, show delayed or variable lactose fermentation ability.

3.3.3 Conclusion

The alternative method Media Pad CC 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 CC for enumeration of coliforms shows satisfactory results for relative trueness;
- The alternative method MC Media pad CC for enumeration of coliforms shows satisfactory results for accuracy profile;
- The alternative method MC Media pad CC for enumeration of coliforms 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 amingenus* 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 5 : 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 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 coliforms in the matrix before inoculation

In order to ensure the absence of 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 6: Levels of *E.coli* and coliforms (cfu/g) in stability samples stored at 2-8°C.

Level and time	Reference: coliforms	Alternative: coliforms
0h		
low a	3.40E+03	4.90E+03
low b	7.80E+03	6.60E+03
medium a	3.10E+05	3.70E+05
medium b	3.20E+05	3.50E+05
high a	3.80E+06	2.90E+06
high b	2.80E+06	2.20E+06
24h		
low a	5.20E+03	1.00E+04
low b	8.80E+03	6.60E+03
medium a	1.10E+06	6.80E+05
medium b	3.90E+05	5.00E+05
high a	6.90E+06	6.10E+06
high b	2.40E+06	3.50E+06
48h		
low a	1.50E+04	5.50E+03
low b	3.10E+03	5.00E+03
medium a	1.40E+05	1.80E+05
medium b	2.70E+05	3.90E+05
high a	2.50E+06	3.90E+06
high b	3.80E+06	3.40E+06
6 day		
low a	3.80E+03	3.90E+03
low b	5.40E+03	6.30E+03
medium a	2.40E+05	3.50E+03
medium b	1.70E+05	2.20E+05
high a	1.50E+06	2.90E+06
high b	2.00E+06	2.60E+06

The data showed that the levels of 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 7.

Table 7 - 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 from the water sample was >8°C for laboratories1 and 5 but the temperature from the probe showed good temperature control for these samples.

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 8.

Table 8 – Results obtained by the expert lab(cfu/g)

Level	Reference method	Alternative method
Blank	<10	<10
Low	3.30E+03	1.90E+03
Low	4.40E+03	6.30E+03
Medium	1.10E+05	6.70E+04
Medium	6.50E+04	2.20E+04
High	2.20E+06	3.30E+06
High	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 Table 9.

The accuracy profile plot is shown in Figure 13 and the statistical analysis of the data shown in Table 10.

Table 9: Summary of the results of the interlaboratory study per analyte level

		Reference method (Log cfu/g)		Alternative method (Log cfu/g)	
Collaborator/level		**Duplicate 1**	**Duplicate 2**	**Duplicate 1**	**Duplicate 2**
1	low	3.11	3.32	3.76	3.43
2	low	3.48	3.18	3.54	3.54
3	low	3.52	3.76	3.69	3.81
4	low	3.64	3.67	3.90	3.69
5	low	3.66	3.74	3.15	3.63
6	low	3.65	3.74	3.80	3.72
7	low	3.63	3.66	3.79	3.79
8	low	3.59	3.63	3.69	3.65
9	low	3.56	3.34	3.62	3.36
10	low	3.28	3.28	3.54	3.57
11	low	3.65	3.81	3.66	3.82
12	low	3.83	3.82	3.99	3.83
1	medium	4.28	4.32	4.52	4.61
2	medium	4.53	4.15	4.53	4.52
3	medium	4.78	4.58	4.80	4.63
4	medium	4.72	4.57	4.63	4.68
5	medium	4.69	4.51	4.54	4.52
6	medium	4.51	4.48	4.69	4.69
7	medium	4.57	4.68	4.64	4.68
8	medium	4.66	4.62	4.62	4.66
9	medium	4.49	4.61	4.41	4.74
10	medium	4.38	4.28	4.63	4.38
11	medium	4.69	4.74	4.96	4.92
12	medium	4.76	4.65	4.91	4.95
1	high	6.20	6.23	6.38	6.76
2	high	6.36	7.26	6.54	6.49
3	high	6.64	6.61	6.70	6.79
4	high	6.54	6.66	6.54	6.68

Collaborator/level		Reference method (Log cfu/g)		Alternative method (Log cfu/g)	
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
5	high	6.51	6.11	6.56	6.08
6	high	6.11	6.11	6.30	6.20
7	high	6.68	6.54	6.87	6.52
8	high	6.46	6.65	6.69	6.67
9	high	6.59	6.41	6.41	6.48
10	high	6.34	6.30	6.43	6.18
11	high	6.57	6.53	4.71	6.66
12	high	6.53	6.56	6.78	6.54

Figure 13. Accuracy profile of MC Media pad CC from the ILS

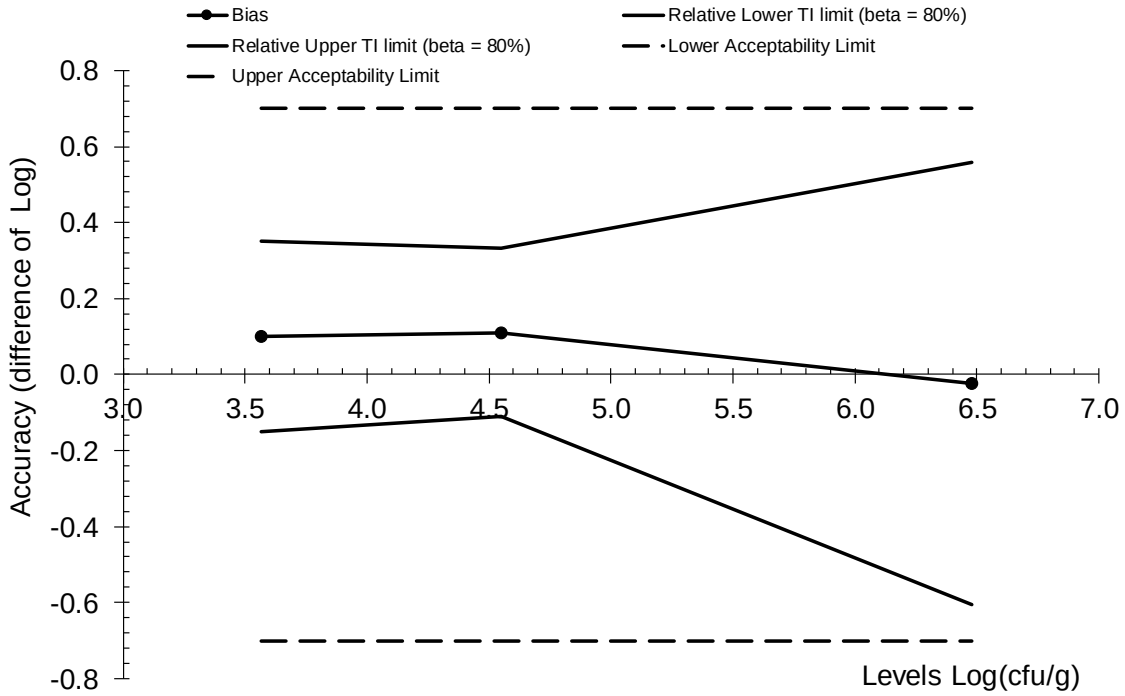


Table 12. Statistical analysis of the ILS data according to the ISO spreadsheet- coliforms

Accuracy profile	0.5		
Study Name	CC		
Date	11/04/2018		
Coordinator	Campden BRI		
Tolerance probability (beta)	80%	80%	80%
Acceptability limit in log (lambda)	0.70	0.70	0.70

TRUE

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	**3.566**	**4.552**	**6.481**			
Number of participants (K)	12	12	12	12	12	12
Average for alternative method	3.666	4.663	6.457	3.566	4.552	6.481
Repeatability standard deviation (sr)	0.148	0.095	0.432	0.108	0.112	0.210
Between-labs standard deviation (sL)	0.109	0.129	0.000	0.181	0.129	0.132
Reproducibility standard deviation (sR)	0.184	0.160	0.432	0.211	0.171	0.248
Corrected number of dof	19.921	15.521	22.957	14.288	16.675	20.757
Coverage factor	1.362	1.384	1.347			
Interpolated Student t	1.326	1.339	1.320			
Tolerance interval standard deviation	0.1888	0.1655	0.4408			
Lower TI limit	3.416	4.441	5.875			
Upper TI limit	3.916	4.884	7.039			
Bias	**0.100**	**0.111**	**-0.024**			
Relative Lower TI limit (beta = 80%)	**-0.150**	**-0.111**	**-0.606**			
Relative Upper TI limit (beta = 80%)	**0.351**	**0.332**	**0.558**			
Lower Acceptability Limit	**-0.70**	**-0.70**	**-0.70**			
Upper Acceptability Limit	**0.70**	**0.70**	**0.70**			

TRUE
TRUE

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

New acceptability limits may be based on reference method pooled variance

Pooled repro standard dev of reference	0.213

5 Overall conclusions of the validation study

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

The alternative Media pad CC™ for enumeration of coliforms shows comparable performance to the reference method ISO 4832:2006 for enumeration of coliforms in a broad range of foods

Date 28/03/2019

Signature

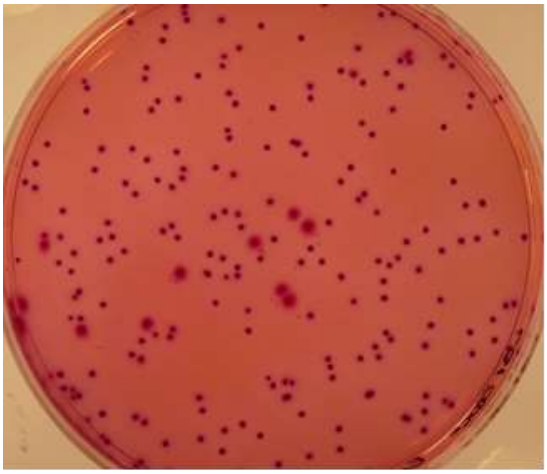
Annexes

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

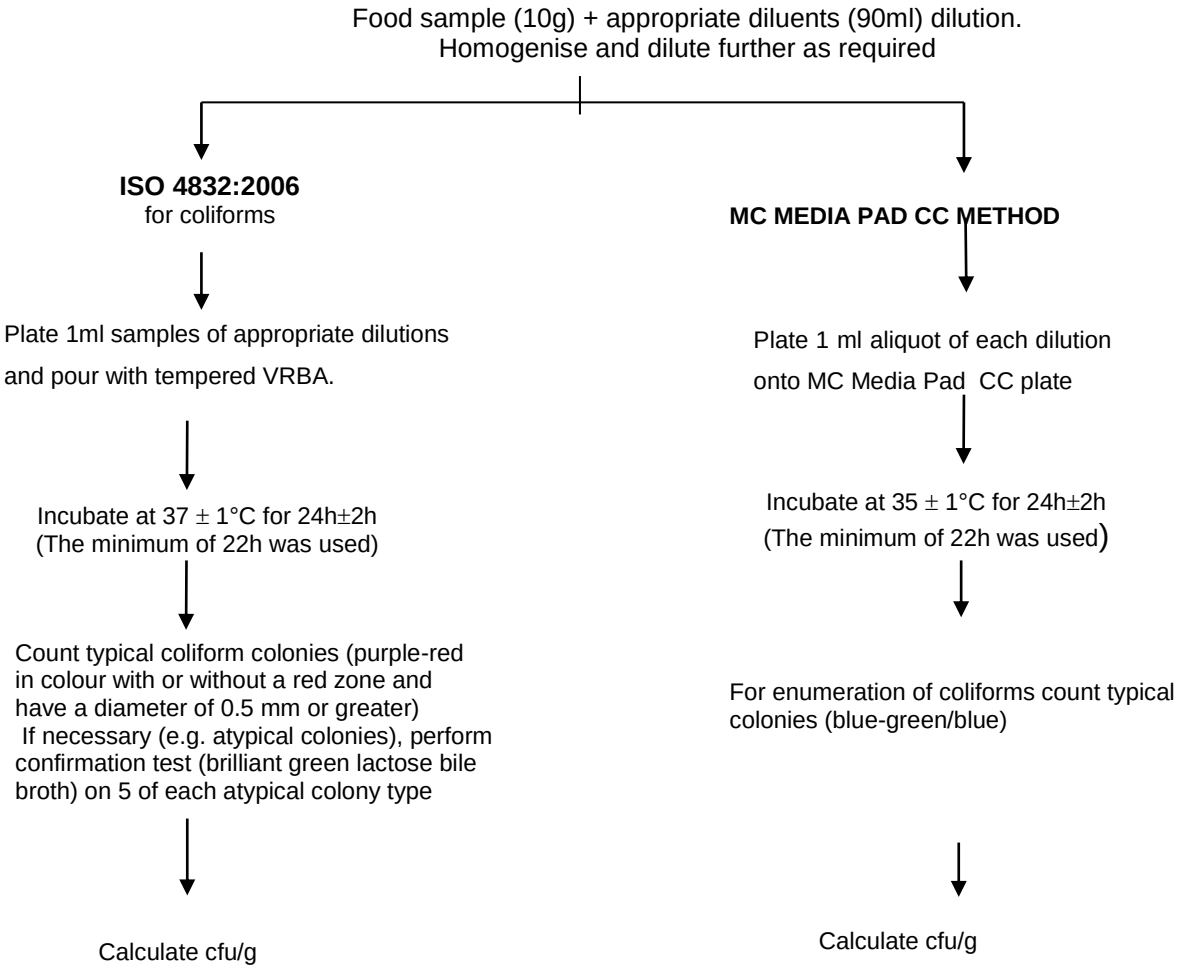
ANNEX A: Typical colony morphology and Flow diagram of the alternative method and reference methods

Picture 1: Typical colonies on MC Media Pad CC

Picture 3: Typical colonies on VRBLA



Comparison of Reference method (ISO 4832:2006) and Alternative Method: MC Media Pad CC for enumeration of coliforms



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

Product code: SK02A25 (25 plates x 40), SK02B25 (25 plates x 4), SK02A10 (10 plates x 100), SK02B10 (10 plates x 10) Creation: November 2016
Creation: November 2016 (ver. 1)Revision: S Sanita-kunTM CC " Coliform" instruction manual
Easy and accurate dry culture system for Microbial Counts

BACKGROUND

For hygiene control, it is important to determine microbial number in foodstuffs and process environment. Sanita-kun™ CC "Coliform" is intended to determine coliform number by special medium composition and specific chromogenic substrate for β-galactosidase. Sanita-kun™ pre-sterilized, ready-to-use dry culture devices simplify testing and minimize the quantity of waste. Sanita-kun™ is composed of unique adhesive sheet, a test pad coated with medium and water absorption polymer, and a transparent cover film.

TEST PRINCIPLES

Sanita-kun™ test pad is coated with selective medium and chromogenic substrate for specific detection. Once the liquid sample is inoculated onto test pad, the sample diffuses to whole pad through capillary action. The medium re-constitutes automatically. If coliform bacteria are present, they grow as blue-green/blue colored colonies on test pad.

CONTENTS and STORAGE

1000 plates; code SK02A25 (25 plates x 40), SK02A10 (10 plates x 100)
100 plates; code SK02B25 (25 plates x 4), SK02B10 (10 plates x 10)
This kit should be stored between 2-15°C. (Refrigerated)

MATERIALS REQUIRED BUT NOT PROVIDED

Incubator (35°C ± 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 stuffs

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 water, liquid food stuffs, swab test sample

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

TEST PROCEDURE

General Operation

1. Open aluminum bag and take Sanita-kun™ sheet. If necessary, write information on the cover film.
2. Lift the cover film, and drop 1mL of sample solution onto test pad.
3. Replace the cover film, and lightly press the edges of film to seal.
(It is recommended to lift the cover film diagonally for easy and sure re-sealing.)
4. Incubate test plate at **35°C±1** for **24±2** hours.

Other Application

Sanita-kun™ is also available for Stamping Technique and Falling Bacterial Test by applying a sterile diluent 30 min before use.
Sanita-kun™ website provides detailed information.
(http://www.jnc-corp.co.jp/sanita/siryou/itiran_E.htm)

INTERPRETATION

Count all colored colonies (blue-green/blue) as coliform regardless of strength of color. 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 10^4 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 sterile needle from test pad for further analysis.

PRECAUTIONS

1. The test is designed for use by quality control personnel and others familiar with testing samples potentially contaminated with coliform.
2. Read this instruction manual carefully before use.
3. After opening the aluminum bag, unused plates should be stored in the aluminum bag and sealed with tape, and kept in a cool (2-15°C) environment. After opening, use all plates within 1 month.
4. Do not expose unused plates to sunlight or ultraviolet light.
5. Do not use a discolored or damaged plate.
6. A wrinkle on test pad should not affect detection.
7. Small fragments of fabric on/ or around test pad should not affect detection.
8. Do not use the plates after the expiration date. The quality of an expired plate is not warranted.
9. The measurement range is less than 300 cfu/plate. If more than 300 cfu/plate are read, further dilution is recommended.
10. Sanita-kun™ CC "Coliform" detects coliform bacteria by existence of β-galactosidase. It is therefore certain bacteria (genus *Aeromonas* etc.) which possesses this enzyme may grow as coliform.
11. In case of applying β-galactosidase containing foods (e.g. cheese, lactic drink or liver), entirety of test pad may appear as stained.
12. The used kit must be sterilized by autoclaving or boiling, and then disposed according to local regulations for waste.

LIMITATION of WARRANTY

The Products are covered by the applicable JNC Corporation standard warranty. NO OTHER EXPRESS OR IMPLIED WARRANTY IS MADE WITH RESPECT TO THE PRODUCTS. JNC EXPRESSLY EXCLUDES THE IMPLIED WARRANTIES OF MERCHANTABILITY AND OF FITNESS FOR A PARTICULAR PURPOSE. If product is defective, JNC and JNC's authorized distributor will provide a replacement or refund at the purchase price.

CONTACT and FURTHER INFORMATION

JNC Corporation Life Chemical Launch Office
2-2-1 Otemachi,Chiyoda-ku, Tokyo 100-8105 Japan
TEL: +81-3-3243-6225, FAX: +81-3-3243-6219
E-mail: sanita-kun@jnc-corp.co.jp

Manufactured by JNC CORPORATION
2-2-1 Otemachi, Chiyoda-ku, Tokyo 100-8105 Jap