Method Comparison Study Report for the ISO 16140-2:2016 validation of MC Media pad ACplus, for the detection of total aerobic count in a broad range of foods

MicroVal study number: 2015LR52

Method/Kit name: MC Media pad ACplus

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

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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 AC plus

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 4833-1:2013 Microbiology of the food chain — Horizontal method for the enumeration of microorganisms Part 1: Colony count at 30 degrees C by the pour plate technique

Scope of validation: A broad range of foods based on categories

1. Dairy and egg products
2. Fresh produce and fruits
3. Raw poultry and meats
4. Ready to eat foods
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
-	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 total aerobic count 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 total aerobic count on MC Media pad AC, incubated at 30°C±1°C for 72 ± 3h

The reference method used was:

- ISO 4833-1:2013 Microbiology of the food chain — Horizontal method for the enumeration of microorganisms Part 1: Colony count at 30 degrees C by the pour plate technique

Categories included :

- Dairy and egg products
- Fresh produce and fruits
- Raw poultry and meats
- Ready to eat foods
- 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 AC shows comparable performance to the reference methods (ISO 4833-1:2013) for the enumeration of total aerobic count 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 AC kit insert in Annex B.

MC Media Pad AC plus: consists of a transparent cover film, an adhesive sheet, a layer of non-woven fabric and a water-soluble compound film including a culture medium formula for the detection of aerobic bacteria. The basis of the detection for is the reduction of tetrazolium salt and the production of coloured formazan resulting from growth of the bacteria. Microorganisms form red colonies after incubation for the correct conditions

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
Dairy and egg products	a	Dairy products e.g. pasteurised cream,	18	18
	b	Dry products e.g. milk powder, milk powders with probiotics, dry dessert	5	5
	c	Egg products e.g. quiche, egg custard tart	5	5
		Total	28	28
Fruits and vegetables	a	Fresh fruit/vegetable products, e.g. fresh	10	10
	b	Leafy greens/sprouts e.g. mung beans, parsley, lettuce	4	4
	c	Heat processed e.g. blanched vegetables, juices, smoothies	5	5
		Total	19	19
Raw poultry and meats	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 kebabs, seasoned chicken breasts	5	5
		Total	15	15
Ready to eat foods	a	Ready to eat poultry e.g. turkey fillet, chicken sausage, pate	5	5

Category	Types		Number of samples analyzed	Number of samples with interpretable results
(Combined category RTE/RTRH meats and poultry and fish)	b	Cooked fish products e.g. prawns, terrine, pate, smoked fish	5	5
	c	Cooked meat e.g. ham, salami, pate, corned beef	5	5
		Total	15	15
Multi component foods or meal components	a	Composite foods with raw ingredients e.g. sandwiches, pasta salads, layered salads with protein	5	5
	b	Mayonnaise based deli-salads, sandwich spreads	6	6
	c	Cooked chilled foods e.g. rice products, ready meals, chilled pizza	5	5
		Total	16	16
		TOTAL	**93**	**93**

93 samples were analysed, leading to 93 exploitable results.

3.1.2 Test sample preparation

All of the samples tested in the relative trueness study were naturally contaminated samples.

3.1.3 Protocols applied during the validation study

A single protocol was applied for the study.

Reference method plates were incubated at 30±1ºC for 72±3h

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 at least 15 interpretable results per category, and at least 5 interpretable results per tested type by the two methods.

3.1.5 Calculation and interpretation of relative trueness studys

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

Figures 1 to 5 shows the scatter plots for the individual categories and Figure 6 for all categories.

Figure 1 - Scatter plot of the reference method versus alternative method results for Dairy and eggs

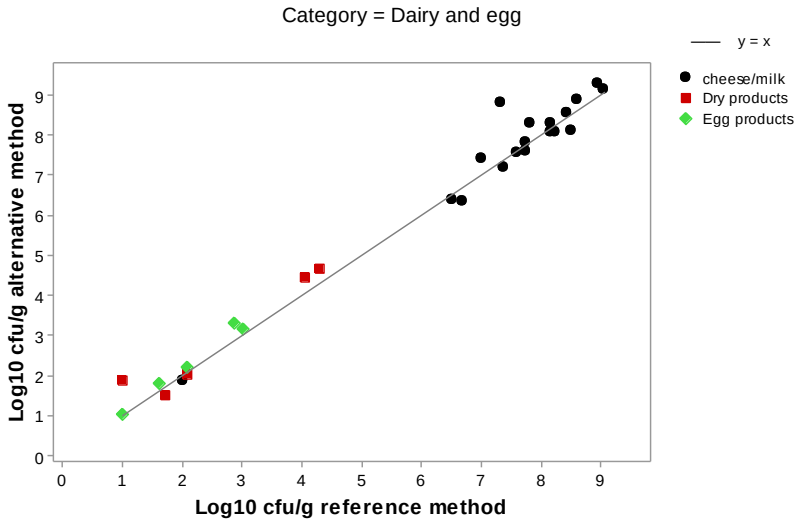


Figure 2- Scatter plot of the reference method versus alternative method results for Fruits and vegetables

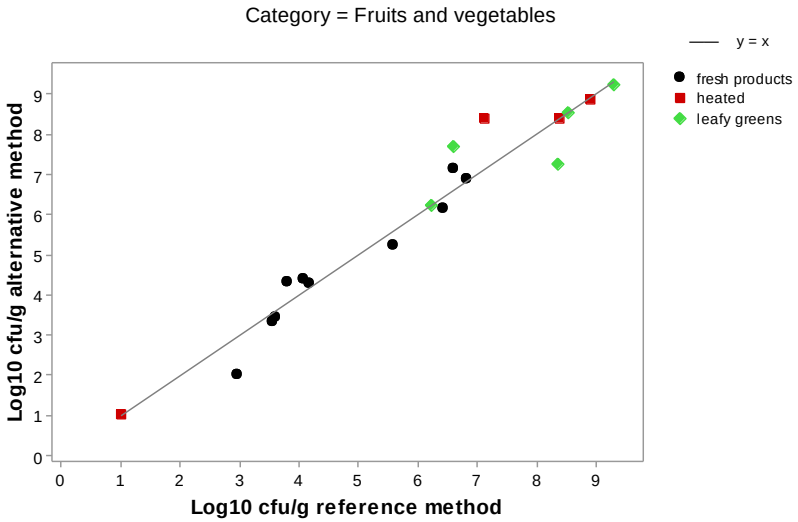


Figure 3- Scatter plot of the reference method versus alternative method results for Multi component foods

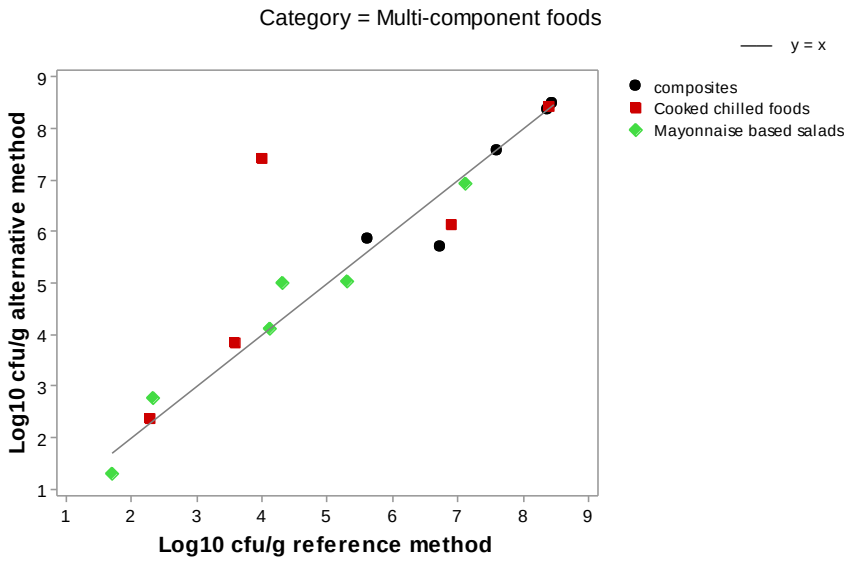


Figure 4- Scatter plot of the reference method versus alternative method results for Raw meat and poultry

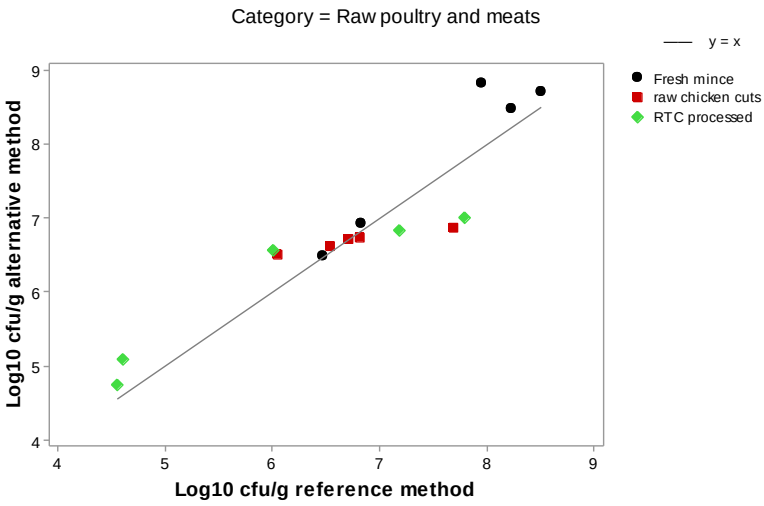


Figure 5- Scatter plot of the reference method versus alternative method results for Ready to eat foods

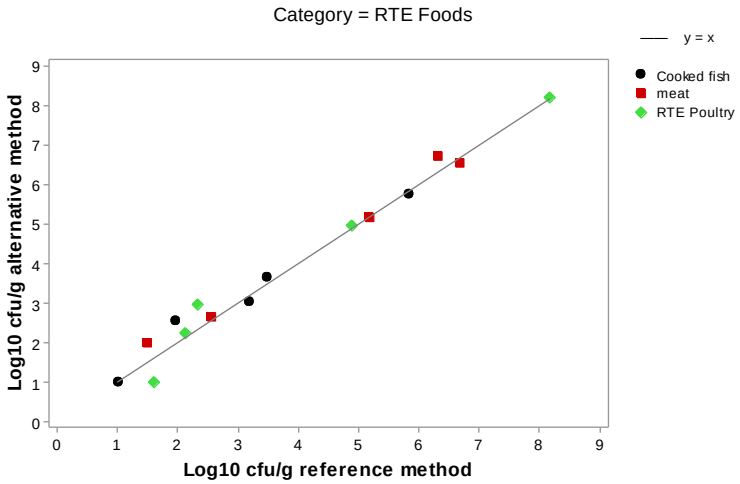
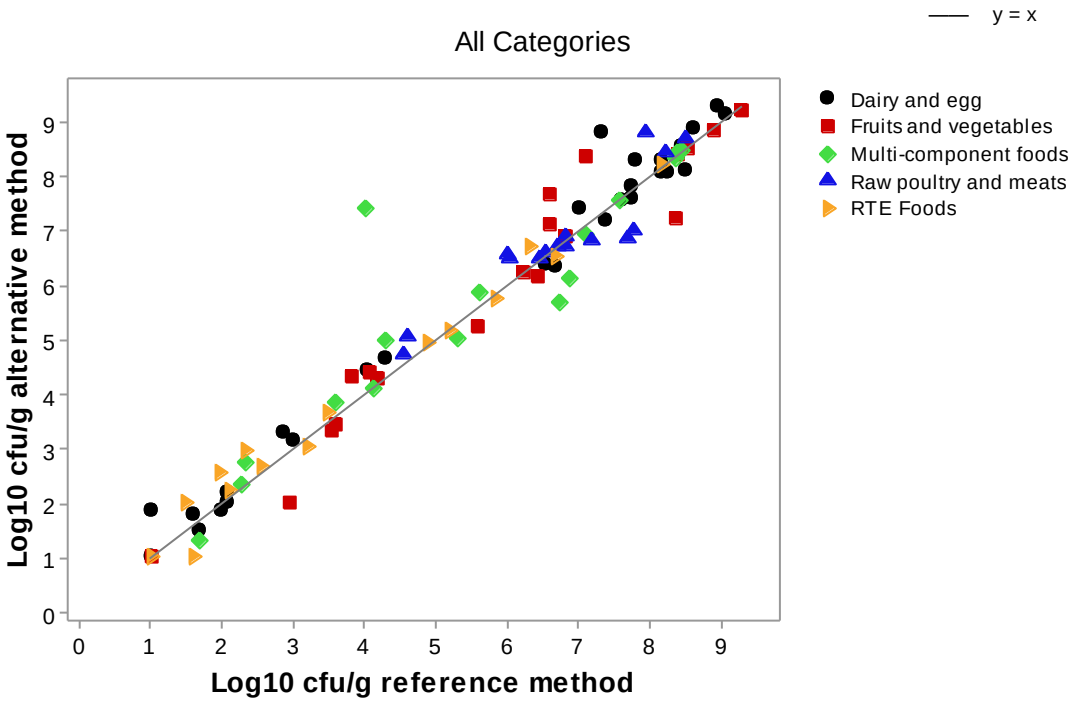


Figure 6 - Scatter plot of the reference method versus alternative method results for all categories



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 some evidence of a slight positive bias for the alternative method

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

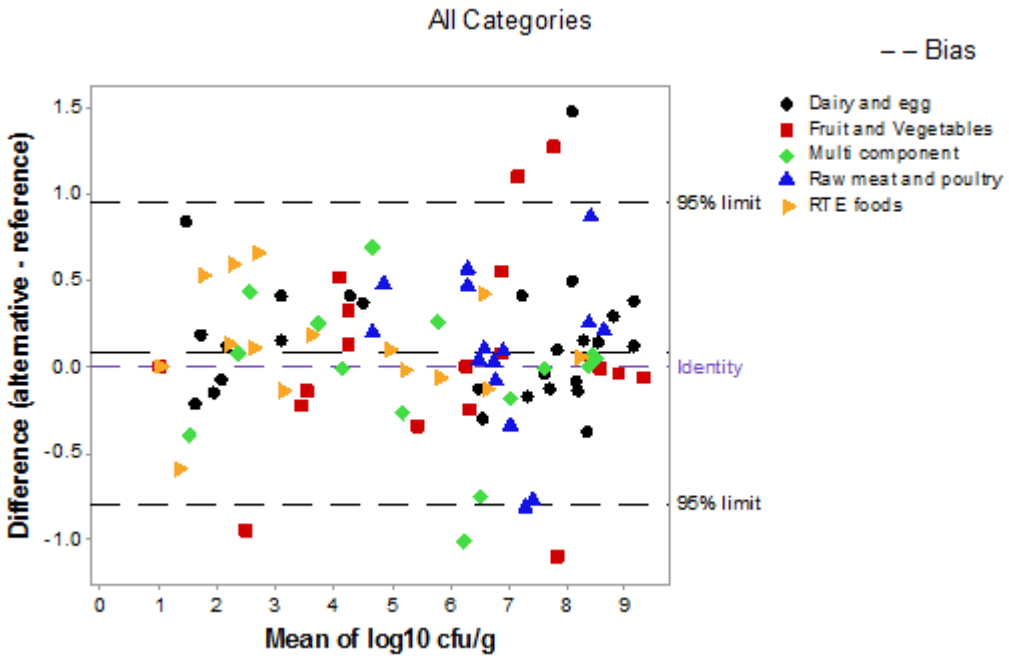
The Bland-Altman difference plot for all the samples is given Figure 7

Table 2 - Summary of the calculated values per category

Category.	n	$\overline{D}$	s_D	95% Lower limit	95% Upper limit
Dairy and egg	28	0.148	0.381	-0.648	0.944
Fruits and vegetables	19	0.043	0.571	-1.188	1.274
Multi-component	16	0.160	0.967	-1.965	2.285
Raw poultry and	15	0.078	0.461	-0.944	1.100
RTE Foods	15	0.119	0.328	-0.608	0.846
All Categories	93	0.113	0.556	-0.999	1.224

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

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



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

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

Category	Types	Code	Food item	Difference log cfu/g (alternative – reference)
Multi-component Foods	Cooked chilled foods	155	Chicken pizza	3.431*
Dairy and Egg	Cheese/milk products	122	Raw milk hard cheese	1.475
Fruits and vegetables	Heated products	136	Layered vegetables	1.276
Multi-component Foods	Composite products	56	Ham sandwich	-1.015
Fruits and vegetables	Leafy greens	66	MAP shredded lettuce	-1.103

*outlier

Comments

It is expected that not more than one in 20 data values will lie outside the CLs. Any disagreements with the expectation should be recorded.

For this data set there are 5 in 93 data values which lie outside the CLs (All categories plot). This would fit in with the expectation of not more than 1 in 20 points being outside the CL's as there are between 80 and 100 points in the data set which could theoretically have up to 5 points outside the CL's.

3.1.6 Conclusion (RT study)

The relative trueness of the Alternative method for total aerobic count 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

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.

The tested categories, types and items are provided in Table 4.

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

Category	Types	Item	Target Level* cfu/g	Test portions
Dairy products	Pasteurised dairy products	Pasteurised cream	Low 10^3	5
			Medium : 10^5	5
			High : 10^7	5
		Cream cheese	Low 10^2	5
			Medium : 10^4	5
			High : 10^7	5
Fruits and vegetables	Fresh produce	Fresh Parsley	Low 10^2	5
			Medium : 10^4	5
			High : 10^8	5
		Vegetable juice	Low 10^3	5
			Medium : 10^5	5
			High : 10^8	5
Raw poultry and meats	Fresh meat	Pork mince	Low 10^3	5
			Medium : 10^6	5
			High : 10^8	5
		Chicken fillets	Low 10^3	5
			Medium : 10^6	5
			High : 10^8	5
Ready to eat foods	Cooked fish products e.g. prawns	Fresh cooked prawns	Low 10^3	5
			Medium : 10^5	5
			High : 10^7	5

		Fish pate	Low 10^3	5
			Medium : 10^4	5
			High : 10^6	5
Multi component foods	Composite foods with raw ingredients	Sandwiches	Low 10^3	5
			Medium : 10^5	5
			High : 10^7	5
		Salad with protein	Low 10^3	5
			Medium : 10^5	5
			High : 10^7	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

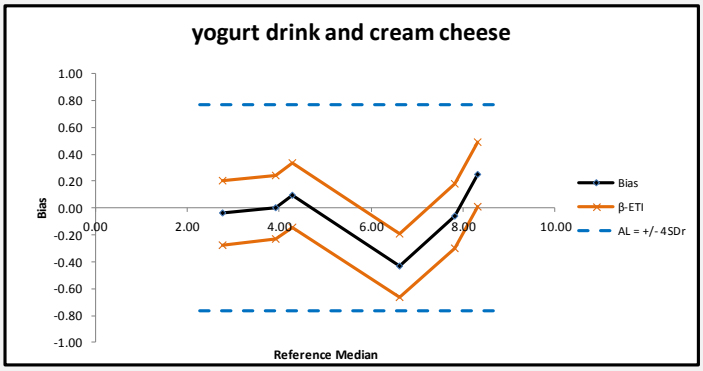
3.2.2 Calculations and interpretation of accuracy profile study

The statistical results and the accuracy profiles are provided in Figures 8 to 12.

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: Dairy and egg products (type pasteurised products)

(Food) Category	DAIRY
(Food) Type	yogurt drink and cream cheese

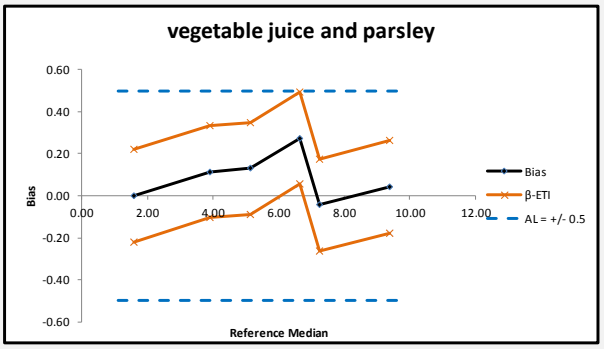


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
147a-e	2.77	-0.035	-0.273	0.203	YES	YES
88a-e	3.93	0.005	-0.234	0.243	YES	YES
160a-e	4.28	0.095	-0.143	0.333	YES	YES
84 a-e	6.62	-0.429	-0.667	-0.191	NO	YES
15 a-e	7.81	-0.058	-0.296	0.180	YES	YES
10 a-e	8.32	0.249	0.011	0.487	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.192	0.165	NO	+/- 0.768

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

(Food) Category	PRODUCE
(Food) Type	vegetable juice and parsley

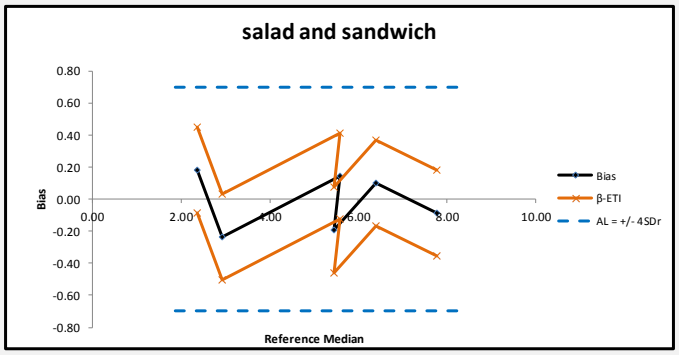


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
31 a-e	1.60	0.000	-0.219	0.219	YES	YES
25a-e	3.92	0.114	-0.105	0.334	YES	YES
85 a-e	5.13	0.131	-0.089	0.350	YES	YES
133a-e	6.64	0.274	0.055	0.493	YES	YES
190 a-e	7.25	-0.045	-0.264	0.175	YES	YES
13 a-e	9.37	0.043	-0.176	0.262	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.185	0.152	NO	+/- 0.500

Figure 10 Accuracy profile for Category: Multicomponent foods (type raw ingredients)

(Food) Category	Multicomponent
(Food) Type	salad and sandwich

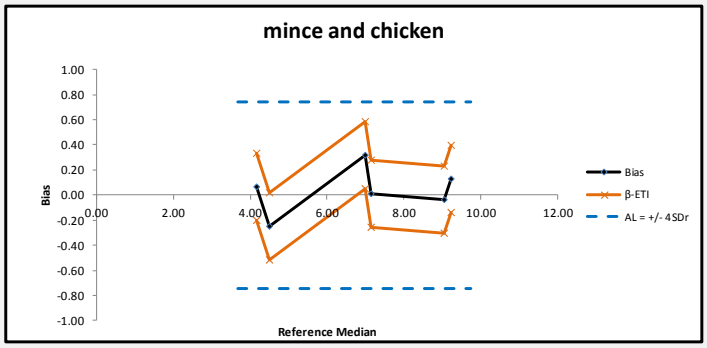


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
174 a-e	2.36	0.182	-0.085	0.449	YES	YES
6 a-e	2.93	-0.236	-0.503	0.031	NO	YES
200a-e	5.58	0.144	-0.123	0.412	YES	YES
155 a-e	5.45	-0.190	-0.457	0.077	YES	YES
180a-e	6.39	0.101	-0.166	0.368	YES	YES
79 a-e	7.78	-0.087	-0.354	0.180	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.175	0.185	NO	+/- 0.700

Figure 11 Accuracy profile for Category: Raw meats (mince and chicken)

(Food) Category	raw meat
(Food) Type	mince and chicken

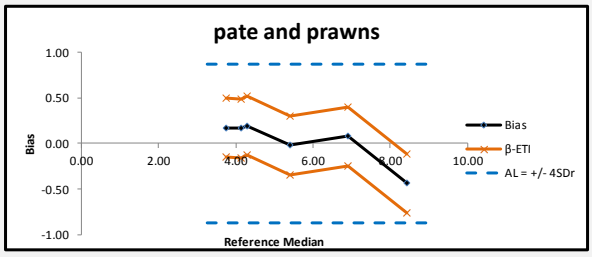


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
134a-e	4.17	0.067	-0.200	0.334	YES	YES
44 a-e	4.50	-0.247	-0.514	0.020	NO	YES
124 a-e	6.98	0.317	0.050	0.584	NO	YES
2 a-e	7.14	0.009	-0.259	0.276	YES	YES
165a-e	9.04	-0.037	-0.304	0.230	YES	YES
115 a-e	9.23	0.129	-0.138	0.396	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.186	0.185	NO	+/- 0.744

Figure 12 Accuracy profile for Category: RTE foods (fishery products)

(Food) Category	RTE Foods
(Food) Type	pate and prawns



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
197a-e	3.76	0.174	-0.150	0.497	YES	YES
186 a-e	4.13	0.166	-0.157	0.490	YES	YES
68 a-e	4.30	0.194	-0.129	0.518	NO	YES
64a-e	5.40	-0.019	-0.343	0.304	YES	YES
36 a-e	6.91	0.076	-0.247	0.399	YES	YES
23 a-e	8.44	-0.436	-0.759	-0.112	NO	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.217	0.224	NO	+/- 0.868

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

According to ISO 16140, if any of the upper or lower limits for the six samples exceeds the 0.5log Acceptability Limits (ALs) and the standard deviation, *S*ref > 0,125, then an additional evaluation procedure is followed:

New ALs are calculated as a function of the standard deviation: AL *s* = 4_ sref. If for all *i* in the accuracy profile *Ui* ≤ AL*s* and *Li* _ −AL*s* , the alternative method is accepted as being equivalent to the reference method for the given combination category and type.

- For one category (Fresh produce), the *S*ref was >0,125 but none of the upper or lower limits were exceeded so the final AL was still ± 0.5log. All data points were within these ALs.
- For the other 4 categories, the *S*ref was >0,125 AND one or more of the upper or lower limits were exceeded, therefore the new ALs calculation was done.
- For Dairy and Eggs, there were originally 2 out of 12 limits exceeded and the *S*ref was 0.192. This gave new calculated ALs of 0.768 and all data points were within these limits
- For Multicomponent foods, there were originally 1 out of 12 limits exceeded and the *S*ref was 0.175. This gave new calculated ALs of 0.700 and all data points were within these limits
- For Raw meats, there were originally 3 out of 12 limits exceeded and the *S*ref was 0.186. This gave new calculated ALs of 0.744 and all data points were within these limits
- For RTE foods, there were originally 2 out of 12 limits exceeded and the *S*ref was 0.217. This gave new calculated ALs 0.868 and all data points were within these limits

The foods tested in the accuracy profile were intended to be challenging and included foods with lactic acid bacteria; psychrotrophic species such as *Pseudomonas* and hygiene indicators such has Enterobacteriaceae. The Alternative method performed as well as the Reference method for all these food types
The accuracy of the Alternative method is satisfied as all categories met the 0.5log AL.

3.3 Inclusivity / exclusivity

The inclusivity study is a study involving pure target strains to be detected or enumerated by the alternative method. According to ISO 16140-2:2016 6.1.5, this test is not required for enumeration methods such as total counts. Therefore, it has not been done in this study.

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 ACplus for enumeration of total aerobic count shows satisfactory results for relative trueness;
- The alternative method MC Media Pad AC plus for enumeration of total aerobic count shows satisfactory results for accuracy profile;

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

4.1.2 Matrix

Chilled salmon pate was inoculated with *E.coli* CRA 1253 isolated from dry ingredients.

4.1.3 Sample preparation
Samples (10g) were inoculated with the desired level of organisms and frozen until 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 to allow thawing to occur during transportation. Each laboratory also received an additional vial containing a 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 24-72h dependent on location and speed of the International courier service. The samples sent to mainland Europe were dispatched on Friday 24th February 2017 and the samples sent to the UK collaborators were dispatched on Monday 27th February 2017. Although this is outside of the recommended 48hr transportation time, experience has shown that samples often get held up in customs from the UK to mainland Europe and it is not possible to ensure a <48hr delivery time. It is for this reason that samples are dispatched frozen and allowed to thaw during transport. The condition of the samples was recorded by each laboratory on a receipt.

4.1.5 Analysis of Samples

The analyses were started on Tuesday 28th February 2017, although some collaborators did not start until Wednesday 1st March due to receiving the samples late

4.2 Experimental parameters controls

4.2.1 Strain stability during transport

Two stability testing trials were done. A preliminary trial was done prior to the despatch of the samples using a set of samples at the medium inoculation level and a second trial was done at the same time as the ILS using set of samples at the highest inoculation level. In both trials' samples were tested immediately after inoculation, and after removal from the freezer and storage at 8±°C for 24 h, 48 h and 72h.

Table 6: Levels of total aerobic organisms (cfu/g) in stability samples stored at 2-8°C.

Time	**0h**		**24h @ 8◦C**		**96h @ 8◦C**	
Method	ACplus	Reference:	AC plus	Reference:	ACplus	Reference:
Rep a	4.50E+05	4.00E+05	4.20E+05	3.50E+05	2.80E+05	3.70E+05
Rep b	4.40E+05	3.50E+05	4.40E+05	4.70E+05	4.20E+05	4.30E+05
Rep c	5.20E+05	6.10E+05	2.90E+05	5.90E+05	3.00E+05	3.80E+05
Mean	**4.70E+05**	**4.53E+05**	**3.83E+05**	**4.70E+05**	**3.33E+05**	**3.93E+05**

The data showed that the samples were stable.

4.2.2 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	Date received	Temperature of control sample upon receipt (°C)	Average storage temperature (°C) over entire transport period
1	05/12/17	13.5	4.3
2	01/12/17	8.4	3.75
3	05/12/17	2.8	1.5
4	05/12/17	9	1.8
5	05/12/17	5.5	3.5
6	01/12/17	3.6	I-button not returned
Expert lab	05/12/17	1.8	1.0

No problem was encountered during the transport or at receipt.

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

4.3 Calculation and summary of data

4.3.1 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 8

The accuracy profile plot is shown in Figures 13 and the statistical analysis of the data is shown in Tables 9.

Table 8: Summary of the results of the interlaboratory study per analyte level

Collaborator		**Reference method (Log cfu/g)**		**Alternative method (Log cfu/g)**	
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
01	low	2.56	2.79	2.62	2.90
02	low	2.87	2.67	3.10	2.74
03	low	2.63	2.63	2.59	2.49
04	low	2.70	2.62	2.49	2.54
05	low	2.71	2.64	2.78	2.42
06	low	2.74	2.67	2.54	2.41
07	low	2.89	2.95	2.79	2.76
08	low	2.95	2.85	2.82	2.76
11	low	2.59	2.78	2.70	2.76
12	low	2.71	2.81	2.65	2.63
01	medium	4.21	4.01	4.12	4.39
02	medium	4.16	4.21	4.34	4.39
03	medium	3.76	3.89	3.94	3.78
04	medium	3.93	4.03	3.94	3.88
05	medium	4.05	3.83	3.78	3.83
06	medium	3.84	3.80	3.92	3.87
07	medium	4.06	4.04	4.09	4.06
08	medium	4.18	4.29	4.11	4.15
11	medium	3.90	4.03	4.02	4.03
12	medium	4.13	4.13	4.21	4.26
01	high	5.65	5.65	5.60	5.80
02	high	5.57	5.72	5.63	5.73
03	high	5.76	5.88	5.75	5.81
04	high	5.90	5.93	5.81	5.81
05	high	5.76	5.61	5.75	5.63
06	high	5.65	5.48	5.60	5.63
07	high	5.67	5.66	5.72	5.69
08	high	5.59	5.67	5.65	5.59
11	high	5.78	5.64	5.77	5.63
12	high	5.59	5.71	5.59	5.81
01	blank	<10		<10	
02	blank	<10		<10	
03	blank	<10		<10	
04	blank	<10		<10	
05	blank	<10		<10	
06	blank	<10		<10	
07	blank	<10		<10	
08	blank	<10		<10	
11	blank	<10		<10	
12	blank	<10		<10	

Figure 13. Accuracy profile of MC Media Pad ACplus from the ILS

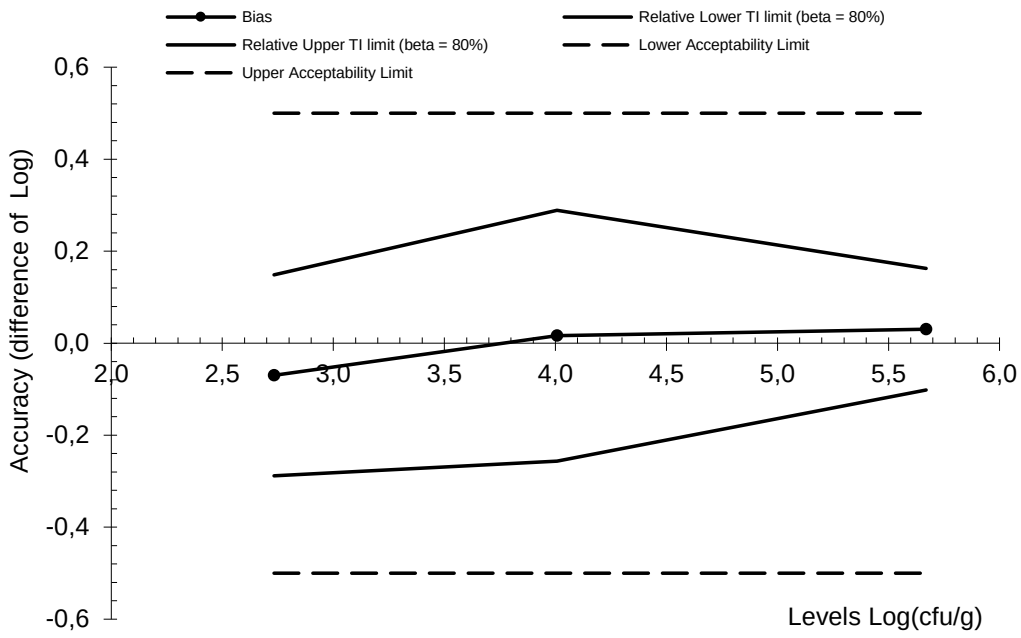


Table 9. Statistical analysis of the ILS data according to the ISO spreadsheet

Accuracy profile	0.5		
Study Name	JNC Acplus		
Date	27/03/2016		
Coordinator	Campden BRI		
Tolerance probability (beta)	80%	80%	80%
Acceptability limit in log (lambda)	0.50	0.50	0.50

FALSE

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

	Alternative method			**Reference method**		
Levels	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
Target value	2.733	4.007	5.669			
Number of participants (K)	12	12	12	12	12	12
Average for alternative method	2.663	4.023	5.700	2.733	4.007	5.669
Repeatability standard deviation (sr)	0.127	0.072	0.083	0.086	0.116	0.092
Between-labs standard deviation (sL)	0.098	0.181	0.050	0.072	0.108	0.098
Reproducibility standard deviation (sR)	0.160	0.194	0.097	0.112	0.159	0.135
Corrected number of dof	19.597	12.611	20.994	19.010	18.235	17.240
Coverage factor	1.364	1.404	1.358			
Interpolated Student t	1.326	1.353	1.323			
Tolerance interval standard deviation	0.1647	0.2017	0.0999			
Lower TI limit	2.445	3.750	5.568			
Upper TI limit	2.882	4.295	5.832			
Bias	**-0.070**	**0.016**	**0.030**			
Relative Lower TI limit (beta = 80%)	**-0.288**	**-0.257**	**-0.102**	FALSE		
Relative Upper TI limit (beta = 80%)	**0.149**	**0.289**	**0.163**	FALSE		
Lower Acceptability Limit	**-0.50**	**-0.50**	**-0.50**			
Upper Acceptability Limit	**0.50**	**0.50**	**0.50**			

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	0.137

5 Overall conclusions of the validation study

- The alternative method Media pad AC plus™ for enumeration of total aerobic count shows satisfactory results for relative trueness;
- The alternative Media pad ACplus™ for enumeration of total aerobic count shows satisfactory results for accuracy profile;
- The alternative Media pad ACplus™ for enumeration of total aerobic count is selective and specific.
- The alternative Media pad ACplus™ for enumeration of total aerobic count shows satisfactory performance in the ILS

The alternative Media pad ACplus™ for enumeration of total aerobic count shows comparable performance to the reference method ISO 4833-1:2013 for enumeration of total aerobic count in a broad range of foods

Date : 28/03/2019

Signature: 

Annexes

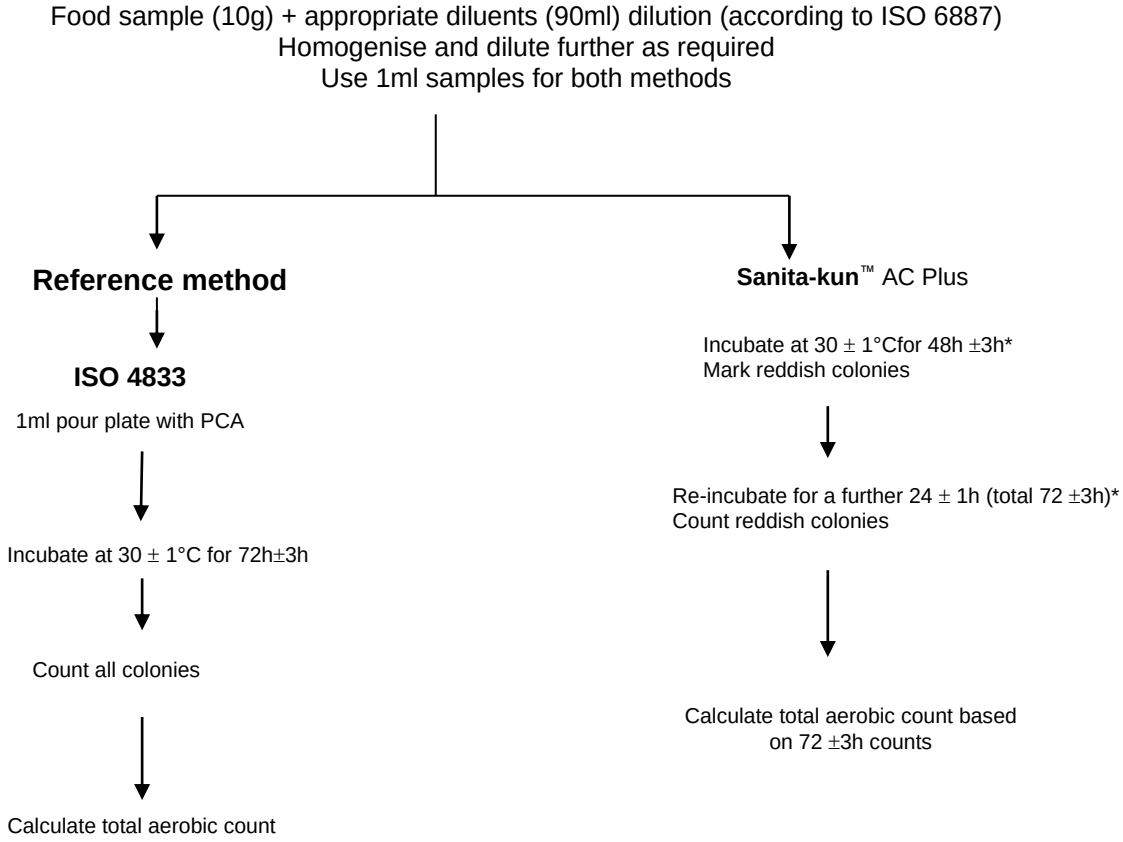
A. Flow diagram of the reference and alternative method
B. Test kit insert

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

Picture 1: Typical colonies on Media Pad ACplus

Picture 2: Typical colonies on PCA

Comparison of Reference method (ISO 4833) and Alternative Method: Sanita-kun™: "Aerobic count plus" enumeration of Aerobic plate count



ANNEX B: Kit insert

Product code: SK01A25 (25 plates×40), SK01B25 (25 plates×4), SK01A10 (10 plates×100), SK01B10 (10 plates×10)

Creation: March 2017 (ver. 1)
Revision:

MC-Media Pad™ "ACplus" instruction manual

Easy and accurate dry culture system for Microbial Counts

◇BACKGROUND

For hygiene control, it is important to determine the microbial count in foodstuffs and the process environment, MC-Media Pad "ACplus" is intended to determine the total aerobic count using a special medium composition and unique redox indicator dyes for not only standard but also rapid enumeration. 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. MC-Media Pad is made by ISO 9001 certified factory.

◇TEST PRINCIPLES

MC-Media Pad "ACplus" is coated with a growth medium and a redox indicator for detection. Once the liquid sample is inoculated onto the test pad, the sample diffuses through the whole pad by capillary action. The medium re-constitutes automatically. If target organisms are present, they grow as red colored colonies on the test pad.

◇CONTENTS and STORAGE

- ●1000 plates・・・・code SK01A25 (25 plates×40) SK01A10 (10 plates×100)
- ●100 plates・・・・・code SK01B25 (25 plates×4) SK01B10 (10 plates×10)

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

◇MATERIALS REQUIRED BUT NOT PROVIDED

- ●Incubator (30 or 35 ± 1°C)
- ●Stomacher or Blender
- ●Sampling bag(Recommended for Stomacher; bag with filter to eliminate food debris)
- ●Pipette or Pipettor and pipette tips
- ●Maximum Recovery Diluent (MRD)
- ●Phosphate Buffered Saline, Saline or appropriate diluents according to EN ISO 6887

◇SAMPLE PREPARATION

●For solid food stuffs

Homogenize a 10-g test portion in 90 mL of MRD, Phosphate Buffered Saline, Saline or appropriate diluents with a stomacher. If necessary, make a 10-fold serial dilution.

●For water, liquid food stuffs, swab test sample

Sample can be applied directly or diluted with MRD or appropriate diluents as for solid foodstuffs. If necessary, pH of sample should be adjusted to neutral (pH 7.0 ± 0.2).

◇TEST PROCEDURE

●General Operation

1. Open the aluminum bag, and remove the MC-Media Pad. If necessary, write information on the cover film.
2. Lift the cover film and drop 1.0 mL 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. For standard usage, incubate test plate at 35 ± 1°C for 48 ± 2 hours (acc. FDA-BAM) or 30 ± 1°C for 72 ± 3 hours (acc. EN ISO 4833). For rapid usage, incubate test plate at 35 ± 1°C for 24 ± 2 hours or 30 ± 1°C for 48 ± 2 hours.

The standard usage is applicable for all food stuffs. For food stuffs which contain large amounts of lactic acid bacteria (e.g. *Lactobacillus* sp.) and psychrophilic bacteria (e.g. *Pseudomonas* sp.) rapid usage may not be applicable.

●Other Application

MC-Media Pad is also available for Wiping/Stamping technique, Membrane filter method, and Airborne falling bacteria test. MC-Media Pad website provides detailed information.
(http://www.jnc-corp.co.jp/MC-MP/)

◇INTERPRETATION

Count all reddish colored colonies. Certain bacteria (in particular *Bacillus* species strains) may form diffuse and fuzzy round shapes. In that case, dark colored points should be counted as colonies. For large numbers of colonies, colony counts can be estimated by counting colonies in one grid square and multiplying by 20. If more than 10^4 microbes are grown, the entire 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, a target colony can be picked up with a sterile needle from the 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 aerobic microbes.
2. Read this instruction manual carefully before use.
3. After opening the aluminum bag, unused plates should be stored in the aluminum bag 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 the test pad should not affect detection.
7. Small fragments of fabric on or around the 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 counted, further dilution is recommended.
10. The rapid mode test is not suitable for all foods. Therefore, suitability should be verified using your own samples before applying.
11. The nature (high viscosity food or food dye) of food may affect test usage or results. In that case, the causes need to be eliminated by dilution or other means.
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

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Manufactured by **JNC CORPORATION**

"Sanita-kun" is reborn as "MC-Media Pad" for the future.