Method Comparison Study Report for the ISO 16140-2:2016 Validation of Compact Dry TC for the enumeration of total viable organisms in a broad range of foods

MicroVal study number: 2007LR01

Method/Kit name: Compact Dry TC

Report version:Summary report 25/09/2019

MicroVal Expert Laboratory:Campden BRI, 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: Nissui Pharmaceutical Co. Ltd

Expert Laboratory: Campden BRI

Method/Kit name: Compact Dry TC

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

Scope of validation: 7 categories A broad range of foods (five categories) , pet food and environmental samples:

- Dairy products
- Fishery products
- Fresh and procesed produce
- Raw meat and poultry products
- RTE meat and poultry products
- Pet foods and animal feeds
- Environmental samples

Certification organisation: Lloyd's Register

List of abbreviations

- AL Acceptability Limit
- AP Accuracy Profile
- Art. Cont. Artificial contamination
- CFU Colony Forming Units
- CL confidence limit (usually 95%)
- EL Expert Laboratory
- $\bar{D}$ Average difference
- g Gram
- h Hour
- ILS Interlaboratory Study
- Inc/Ex Inclusivity and Exclusivity
- LOQ Level of Quantification
- MCS Method Comparison Study
- min minute
- ml Millilitre
- MR (MicroVal) Method Reviewer
- MRD Maximum Recovery Diluent
- 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
- PCA Plate count Agar
- RT Relative Trueness
- RTE Ready to Eat
- RTC Ready to Cook
- RTRH Ready to Re-heat
- SD standard deviation of differences
- 10^{-1} dilution 10-fold dilution of original food
- 10^{-2} dilution 100-fold dilution of original food

Contents

1 Introduction

In this project a MicroVal validation study, based on ISO 16140-2:2016, of an alternative method for the enumeration of total count in 7 different categories was carried out. This study was based on 5 food categories (i.e. a broad range of foods), pet food/animal feed and environmental samples. The study was carried out by Campden BRI as the MicroVal Expert Laboratory.

This was a renewal of a method that has already been validated for a broad range of foods according to the superseded ISO16140:2003 standard for enumeration of total count in a broad range of foods.

Five levels of contamination were used for each product category, covering a minimum, a central and a maximum level plus two intermediary levels. Quintuplet test portions were examined for each sample tested giving a total of 125 data points (5 categories x 5 levels x 5 replicates).

All the points within each category were obtained from a single food item

Category/Item	Contamination levels (cfu/g)	Replicates per level
Raw ground beef	10^1-10^2, 10^2-10^3, 10^3-10^4, 10^4 -10^5, 10^5-10^6	5
Cooked chicken	10^1-10^2, 10^2-10^3, 10^3-10^4, 10^4 -10^5, 10^5-10^6	5
Lettuce	10^1-10^2, 10^2-10^3, 10^3-10^4, 10^4 -10^5, 10^5-10^6	5
Milk powder	10^1-10^2, 10^2-10^3, 10^3-10^4, 10^4 -10^5, 10^5-10^6	5
Frozen fish	10^1-10^2, 10^2-10^3, 10^3-10^4, 10^4 -10^5, 10^5-10^6	5

Relevant sets of the available data were used for the AP part of the renewal study. According to the agreed protocol, a low, medium and high level from each of the 5 sets of data available were used for half of the required AP samples per category. A second set of low, medium and high samples was obtained in this renewal study renewal study to complete the required number of samples (see Table 2). All of the data for the Pet foods/animal feeds and Environmental samples AP study was obtained in the renewal study as no data exists for these categories (see Table 1).

In addition, all the RT data was obtained in the renewal study as there were no relevant data available from the original study for this part.

The alternative method used was: Compact Dry TC. The method is summarised below.

- *Dilution of 10g portions of food in appropriate diluent*. Stomach 1 minute.*
- *Make further serial dilutions as required*
- *Enumeration appropriate dilutions on Compact Dry TC by pour plate (1ml)*
- *Incubation at 30±1°C for 48h±3h (45h will be used)*

**according to ISO 6887*

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

Scope of the validation study is a broad range of foods, plus pet food/animal feed and environmental samples

Categories included:

- Dairy products
- Fishery products
- Fresh and procesed produce
- Raw meat and poultry products
- RTE meat and poultry products
- Pet foods and animal feeds
- Environmental samples

Criteria to be evaluated during the study:

- Method Comparison Study (MCS)
  - Relative Trueness study
  - Accuracy profile study
  - Limit of Quantification study (LOQ)[1]
  - Inclusivity and exclusivity study
- Interlaboratory Study (ILS)[2]

The final conclusion on the Method Comparison study is summarized below:

The alternative method Compact Dry TC shows comparable performance to the reference method (ISO 4833-1: 2013) for the enumeration of colony count at 30°C in a broad range of foods, pet foods/animal feed and environmental samples

[1] LOQ is only needed for instrumental methods. It does not apply to methods based on counting visible colonies

[2] Note: depending on the type study, the ILS may only be partly needed, eg in extension or renewal studies. In this study the data was already avaialble but was re-nalysed with the ISO16140-2 :2016 statistical approach

2 Method protocols

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

According to ISO 16140-2 the reference method and alternative methods were performed with, as far as possible, exactly the same sample.

2.1 Reference method

ISO 4883-1:2013. Microbiology of food and animal feeding stuffs- Horizontal method for the enumeration of microorganisms. Part 1: Colony count at 30 degrees C by the pour plate technique

See the flow diagram in Annex A. In summary:

- 1ml samples of appropriate dilutions were pour plated with PCA and incubated under aerobic conditions at 30±1°C for 72±3h

Sample preparations used in the reference method and the alternative 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. Single plates of successive dilutions were tested as a minimum. In order to increase the reliability, duplicate plates were done where considered necessary based on the expected contamination level and dilution pated. If only 1 dilution was plated, then duplicate plates were used.

2.2 Alternative method

See the flow diagram in Annex A[3] . In summary

- 1ml samples of appropriate dilutions were plated into the centre of the Compact Dry TC plates. The lids were placed on the plates and the plates inverted and incubated at 30 ± 1°C for 48 ± 3h. (45h was used)
- Following incubation, red and otherwise coloured colonies were counted as stipulated by the manufacturer's instructions, and the CFU/g was calculated for each sample.

See the kit insert in Annex B[4].

The alternative method principle is based on enumeration on a rehydratable media plate containing nutrietns and an indicator dye to detect microbial growth. Compact Dry TC are ready-to-use dry media comprising culture medium and a cold-soluble gelling agent, rehydrated by inoculating 1ml diluted sample into the centre of the self-diffusible medium. The Compact Dry TC (Total Count) method contains the redox indicator

[3] *Note: Or in a separate Annex B if needed*
[4] *Note: Additionally, the test kit insert must be provided separately with the protocol and reports.*

tetrazolium salt and is an alternative method to the standard plate count, enabling determination of aerobic colony counts in foods after 48h incubation.

Target organisms grow as red coloured colonies on a clear background. A picture is provided in Figure 1.

Figure 1: Compact Dry TC

2.3 Study design

Samples of product containing the target organism were diluted 1 in 10 with an appropriate diluent according to ISO 6887 (parts 1, 2, 3, 4 and 5) and homonegised in a stomacher (Table 1)

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 contaminated samples. Different categories, types and items were tested for this.

A total of 7 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 analysed

Category	Types	Preparation (ISO)	No of samples analysed	No of samples with interpretable results
Dairy products (combined category; raw milk and heat processed)	Dry	6887-5	5	5
	Pasteurised dairy products	6887-5	5	5
	Pasteurised milk	6887-5	6	6
Fishery products Combined category: raw, RTE, RTRH, RTC	Raw	6887-3	5	5
	RTE fish	6887-3	5	5
	Acidified and marinated	6887-3	5	5
Produce and fruits (combined category fresh and processed)	Cut RTE	6887-4	5	5
	Heat processed	6887-4	5	5
	Vegetable and fruit juices	6887-4	5	5
Raw and RTC meat and poultry (Combined category)	Cuts unprocessed	6887-2	5	5
	Mince unprocessed	6887-2	5	5
	RTC	6887-2	5	5
RTE and RTRH meat and poultry (Combined category)	RTE cooked	6887-2	5	5
	Fermented or dried	6887-2	5	5
	Cured smoked	6887-2	5	5
Pet food and animal feed	Dry Food	6887-4	5	5
	Wet food (raw and canned)	6887-2	5	5
	Animal feeds (poultry and fish)	6887-4	5	5
Environmental samples (food or feed production)	Surfaces (wipes, swabs)	6887-1 ISO 18593:2018	5	5
	Process water	6887-1	5	5
	Dusts	6887-1 ISO 18593:2018	5	5

106 samples were analysed, leading to 106 exploitable results.

3.1.2 Test sample preparation

All samples tested were naturally contaminated. No artificial contamination was needed for this part.

3.1.3 Protocols applied during the validation study

Incubation time

All samples for the alternative method were incubated for 45h as this is the shortest incubation time in the range 48±3h.

Confirmations if required for the alternative method

No confirmations were required for this 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 incubation protocol, and 5 interpretable results per tested type.

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).

Figure 2 shows the scatter plot for the Dairy Category

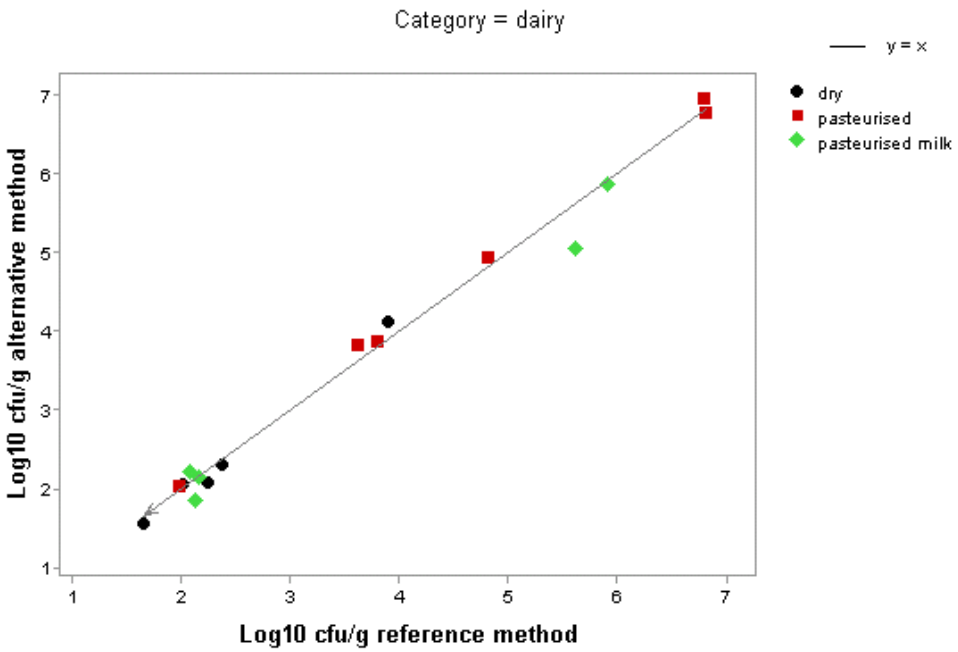


Figure 3 shows the scatter plot for the Fishery Products Category

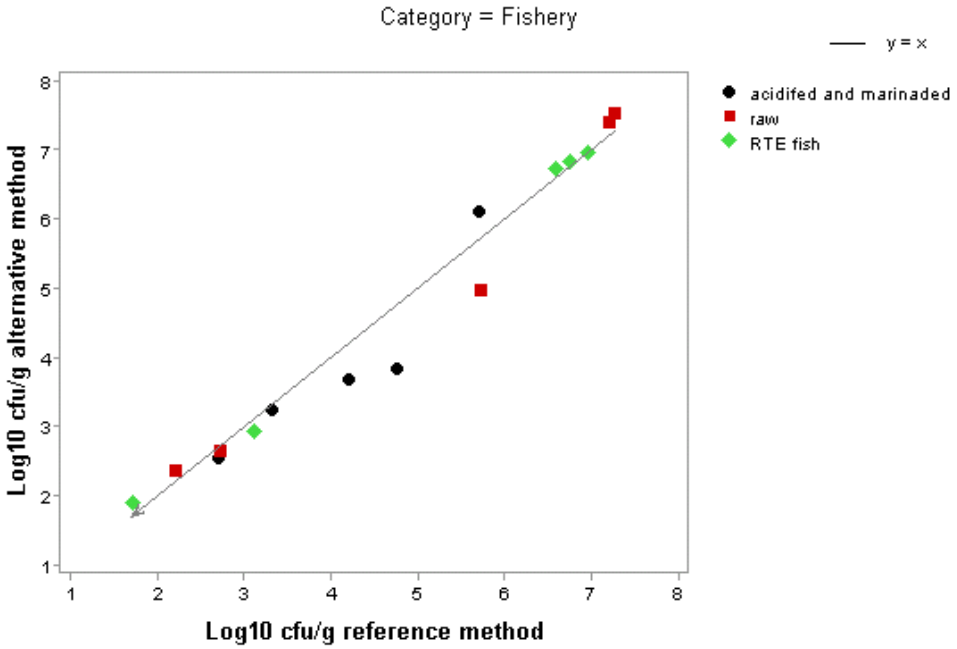


Figure 4 shows the scatter plot for the Fresh Produce

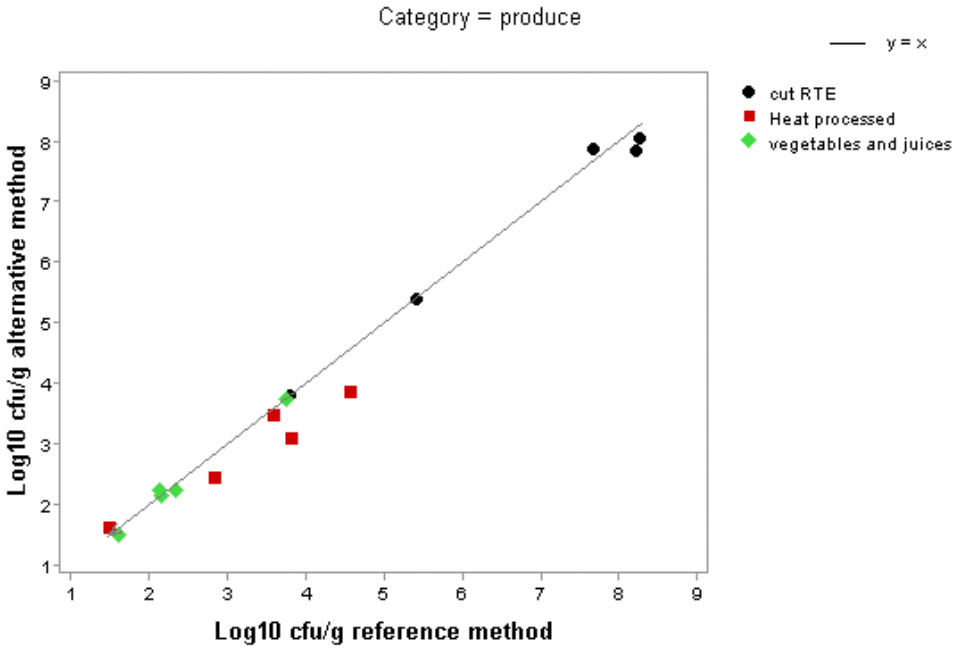


Figure 5 shows the scatter plot for the Raw and RTC Meat & Poultry Products

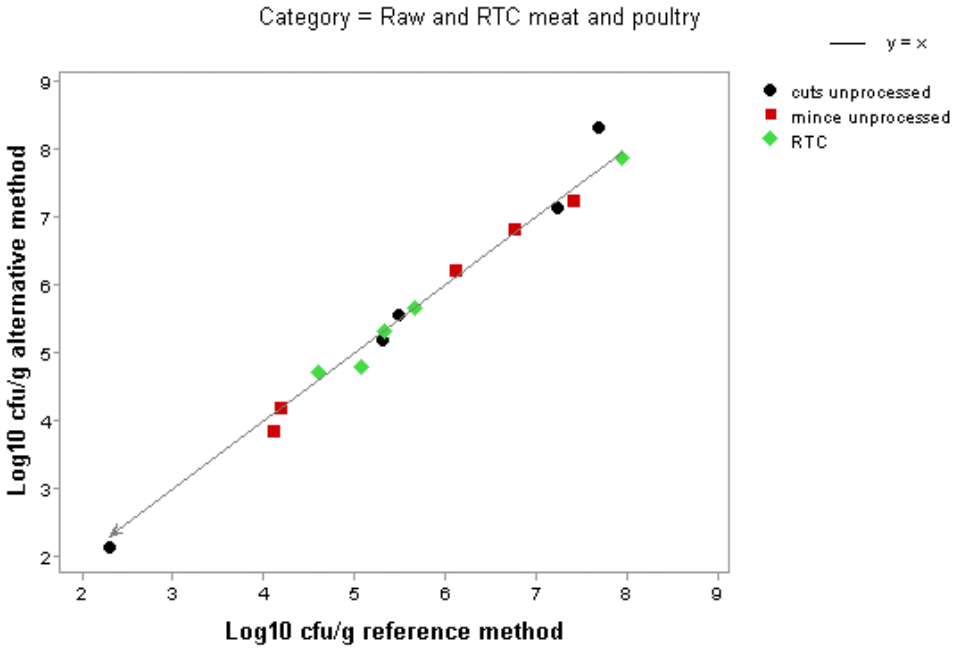


Figure 6 shows the scatter plot for the RTE & RTRH Meat & Poultry Products

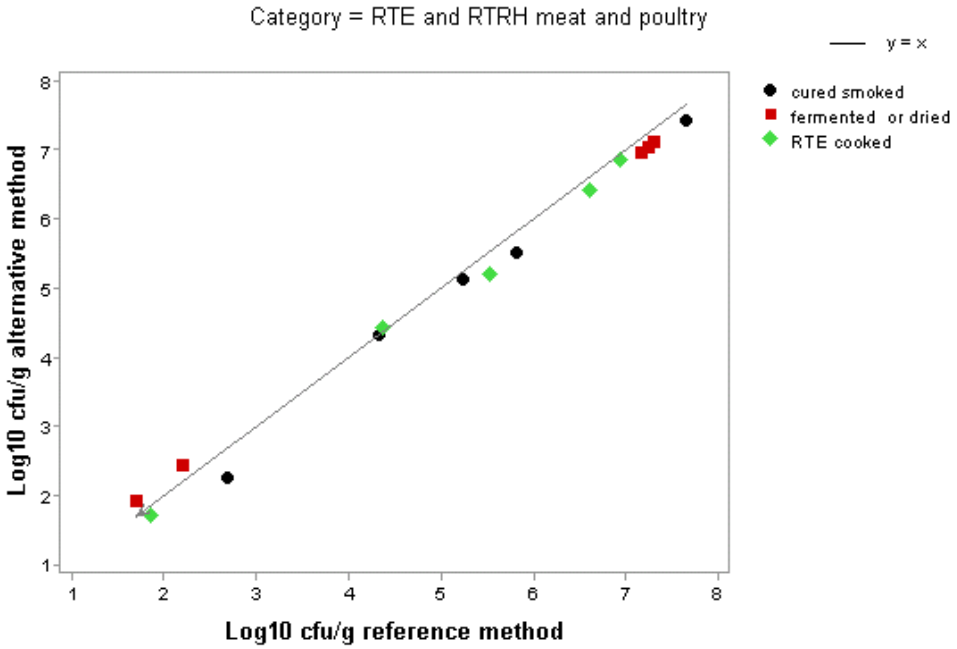


Figure 7 shows the scatter plot for the Pet foods and Animal Feeds Category

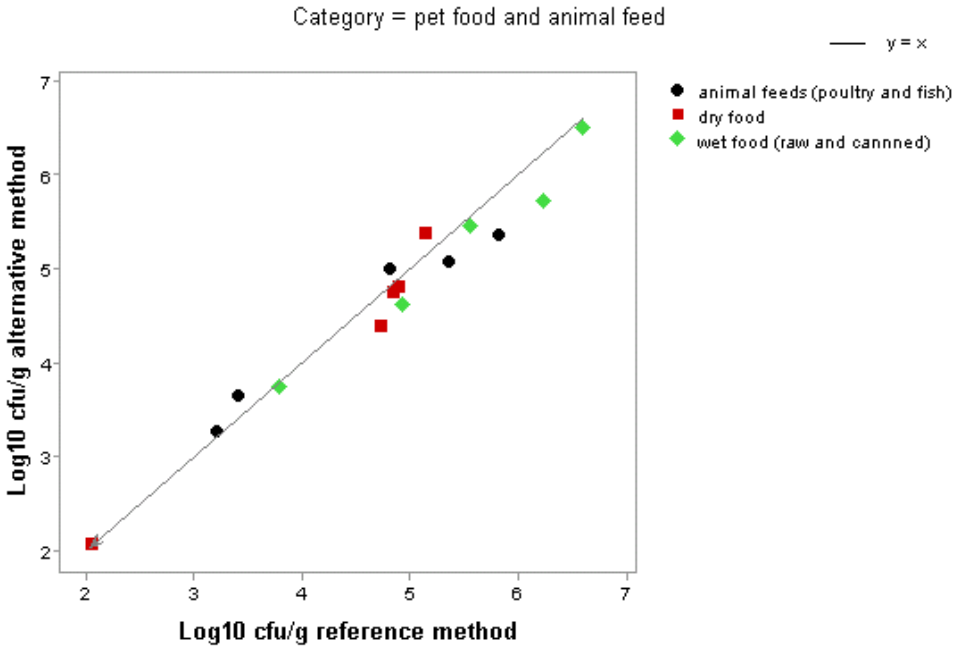


Figure 8 shows the scatter plot for the Environmental Category

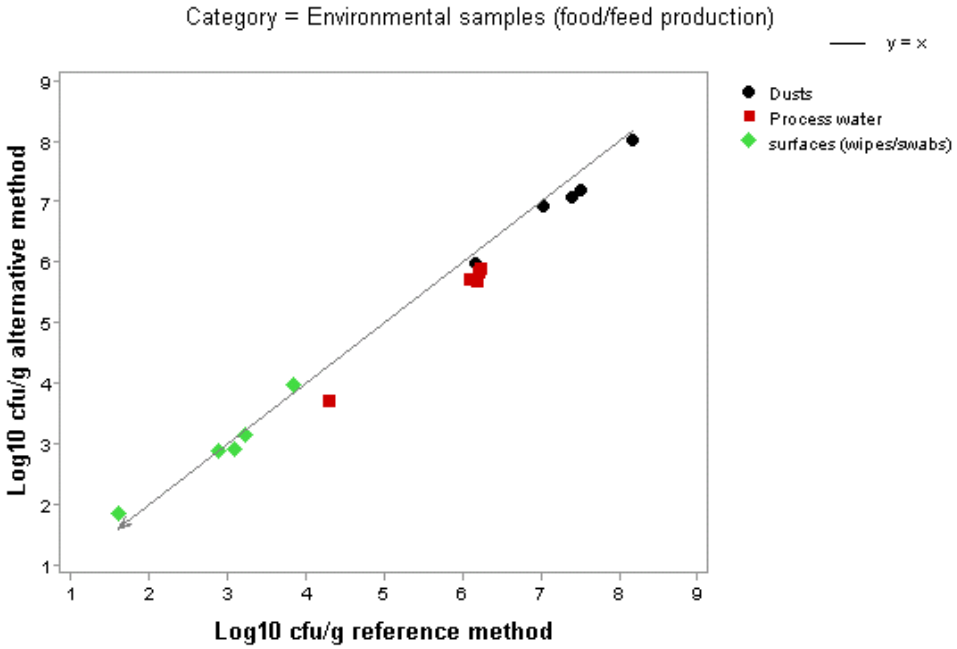
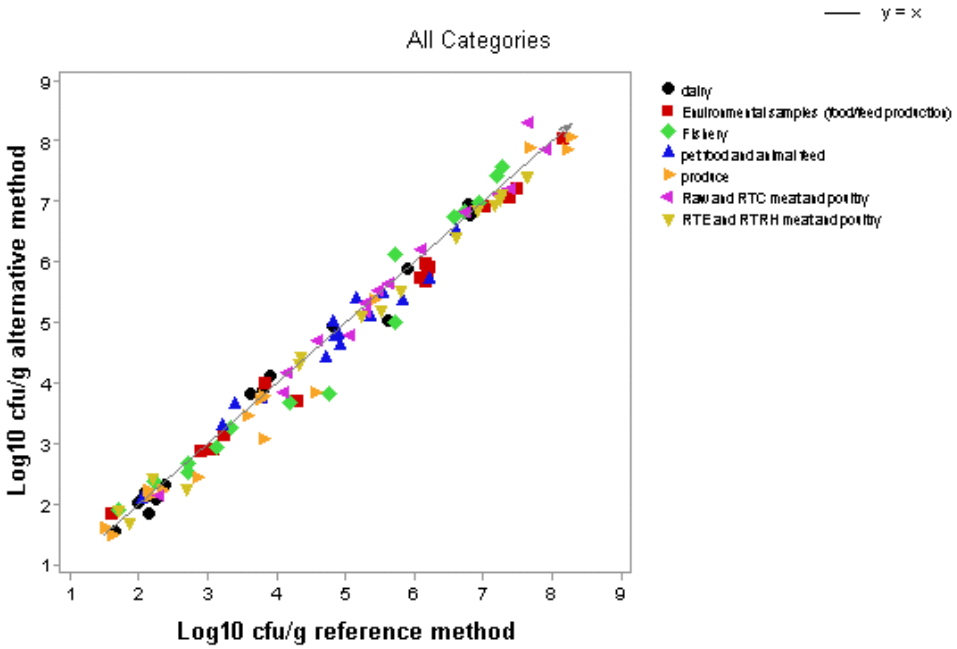


Figure 9 shows the scatter plot for all the categories.



According to ISO16140-2:2016 6.1.2.3, the results of the scatter plot are interpreted on the visual observation of the amount of bias and extreme results.

The data in the scatter plots show no obvious disagreement across all the samples. There are some signs of negative bias for some samples of the processed fresh produce and acidified fishery products. These samples may contain lactic acid bacteria or stressed cells which may be enumerated better on the reference method due to the longer incubation time. This situation was only observed for a few samples within these types as shown in Table 3.

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

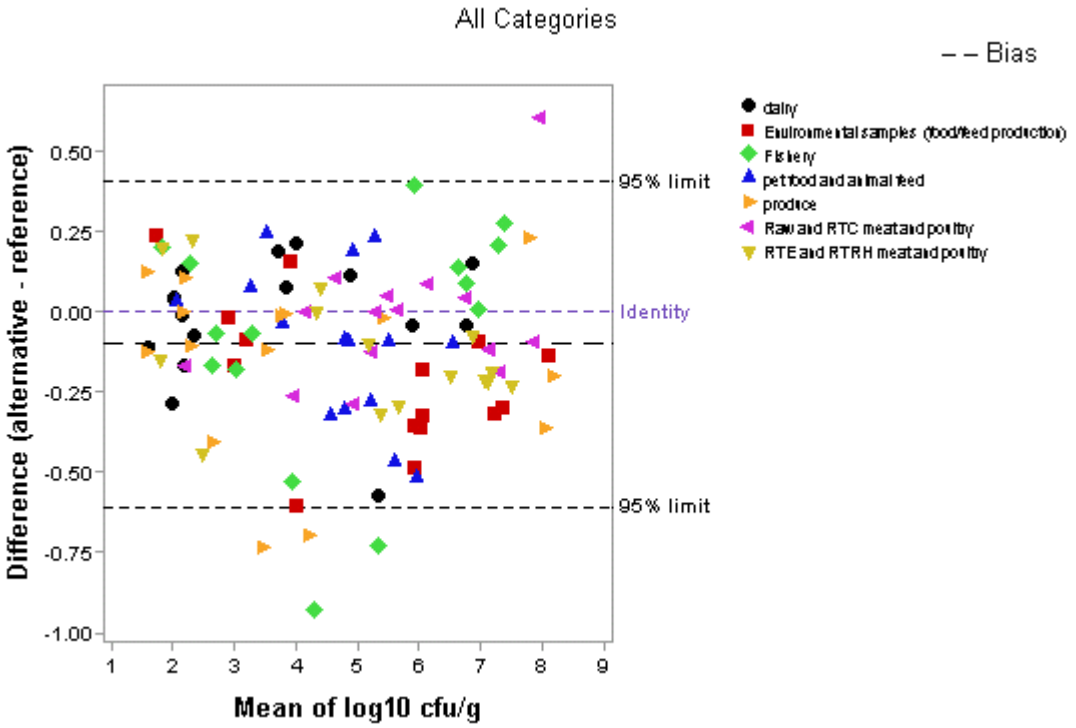
Table 2 - Summary of the calculated values per category

Category	n	$\bar{D}$	SD	95 % low limit	95 % upper limit
Dairy	16	-0.021	0.199	-0.458	0.416
Environmental samples	15	-0.202	0.227	-0.705	0.301
Fishery	15	-0.079	0.381	-0.924	0.765
Pet food and animal feed	15	-0.102	0.239	-0.630	0.427
Fresh produce	15	-0.154	0.284	-0.783	0.474
Raw and RTC meat and poultry	15	-0.021	0.214	-0.495	0.453
RTE and RTRH meat and poultry	15	-0.127	0.188	-0.544	0.290
All Categories	106	-0.100	0.255	-0.608	0.408

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

The Bland-Altman difference plot for all the samples is given Figure 10.

Figure10 – Bland-Altman difference plot for all the samples



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

Table 3 - Data which are outside of the accepted limits

Category	**Type**	**Code**	**Reference method Log cfu/g**	**Alternative method Log cfu/g**	**Mean Log cfu/g**	**Difference Alternative – reference)**	**Lower / Upper limits**
Raw and RTC meat and poultry	cuts unprocessed	47	7.69	8.30	7.99	0.611	0.407
Fishery	raw	16	5.72	4.99	5.35	-0.733	-0.608
Fishery	acidified and marinated	30	4.76	3.83	4.29	-0.931	-0.608
produce	Heat processed	37	3.81	3.07	3.44	-0.734	-0.608
produce	Heat processed	40	4.55	3.85	4.20	-0.699	-0.608

Comments

It is expected that not more than one in 20 data values will lie outside the CLs. In this study there were 5 data points from a total of 106 data points which were outside of the accepted limits. This meets the expectation

The five data points outside the limit were from 3 different categories and 4 different food types

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 and the scatter plot shows good agreement between the reference and alternate method. There is a very slight negative bias for the alternative method.

3.2 Accuracy profile study

The accuracy profile study is a comparative study between the results obtained by the reference and the results of the alternative method. This study is conducted using artificially contaminated samples, using one type per category.

3.2.1 Categories, sample types and strains

In this study seven food, feed and environmental sample categories were tested with a single batch of two different 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 category. The following food type/strain pairs were studied (See Table 4):

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

As this is a renewal study, some data used for the accuracy profile analysis was retained from the original study. These combinations are highlighted in grey in Table 4. It should be noted that the data from the original study was not artificially contaminated (except for milk powder) but contained naturally present organisms. Whilst all AP studies should be artificially contaminated according to ISO16140-2:2016, it was agreed with the MicroVal Technical Committee to keep the original data sets for this renewal.

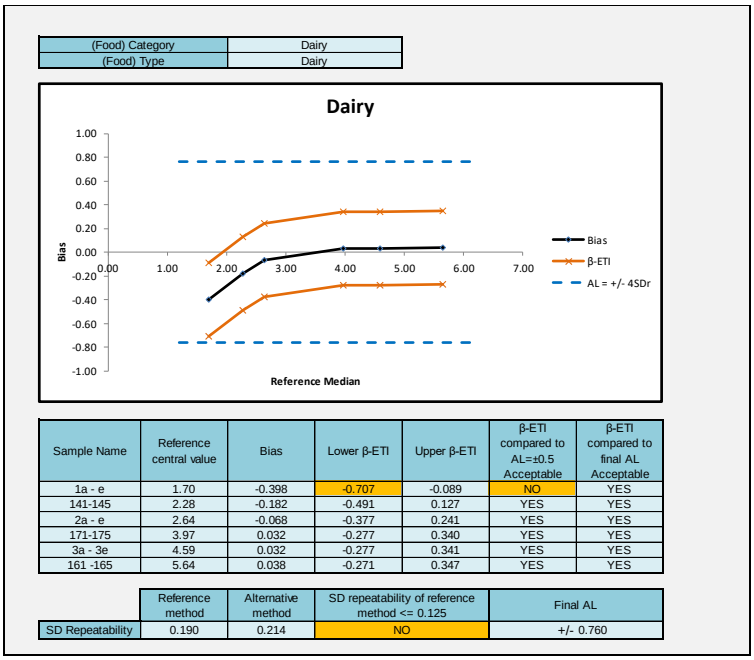
Table 4 - Categories, types, items, strains and inoculation levels for accuracy profile study

Category	Types	Strain	Item	Level
Dairy products (combined category; raw milk and heat processed)	Dry dairy products	*E.faecalis* NCIMB 1993	Milk powder	10^2 cfu /g
				10^3 cfu/g
				10^5 cfu /g
		Bacillus cereus CRA 1724 Dried milk	Dessert powder	10^2 cfu /g
				10^3 cfu/g
				10^4 cfu/g
Fishery products Combined category: raw, RTE, RTRH, RTC	RTC	natural	Frozen white fish	10^3 cfu /g
				10^4 cfu/g
				10^6cfu /g
		Pseudomonas fragi CRA7222 spoiled fish	Chilled tuna steak	10^2 cfu /g
				10^3 cfu/g
				10^5 cfu/g
Produce and fruits (combined category fresh and processed)	Cut ready to eat	natural	Lettuce	10^2 cfu /g
				10^3 cfu/g
				10^5cfu /g
		E.coli CRA3379 Spinach	Spinach	10^2 cfu /g
				10^3 cfu/g
				10^4 cfu/g
Raw and RTC meat and poultry (Combined category)	Fresh meats	natural	Raw ground beef	10^3 cfu /g
				10^6 cfu/g
				10^7cfu /g
		Citrobacter freundii CRA403 chicken	Chicken breast fillets	10^3 cfu /g
				10^5 cfu/g
				10^6 cfu/g
RTE and RTRH meat and poultry (Combined category)	Cooked products	natural	Cooked chicken	10^3 cfu /g
				10^3 cfu/g
				10^5cfu /g
		Hafnia alvei CRA7417 (from pate)	Pork liver pate	10^2 cfu /g
				10^3 cfu/g
				10^5 cfu/g
Pet food and animal feed	Wet food (cooked)	*Staph aureus* CRA 1246 (from pork sausage)	Dog pate	10^2 cfu /g
				10^3 cfu/g
				10^5cfu /g
			Cat pate	10^2 cfu /g
				10^3 cfu/g
				10^4 cfu/g
Environmental samples	Process water	*Pseudomonas fluorescens CRA 7774* (from wash house)	Wash water	10^2 cfu /g
				10^3 cfu/g
				10^5 cfu /g
			Cooling water	10^2 cfu /g
				10^3 cfu/g
				10^5 cfu/g

3.2.2 Calculations and interpretation of accuracy profile study

The statistical results and the accuracy profiles are provided Figures 11 to 17.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 11 – Accuracy profile for Dairy products

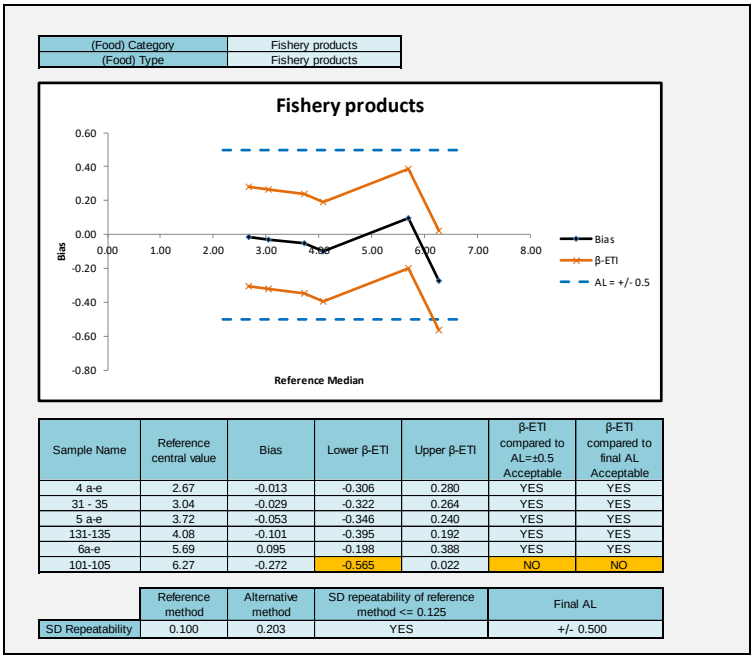


(Food) Category	Dairy
(Food) Type	Dairy

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
1a - e	1.70	-0.398	-0.707	-0.089	NO	YES
141-145	2.28	-0.182	-0.491	0.127	YES	YES
2a - e	2.64	-0.068	-0.377	0.241	YES	YES
171-175	3.97	0.032	-0.277	0.340	YES	YES
3a - 3e	4.59	0.032	-0.277	0.341	YES	YES
161 -165	5.64	0.038	-0.271	0.347	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.190	0.214	NO	+/- 0.760

Figure 12 – Accuracy profile for Fishery products

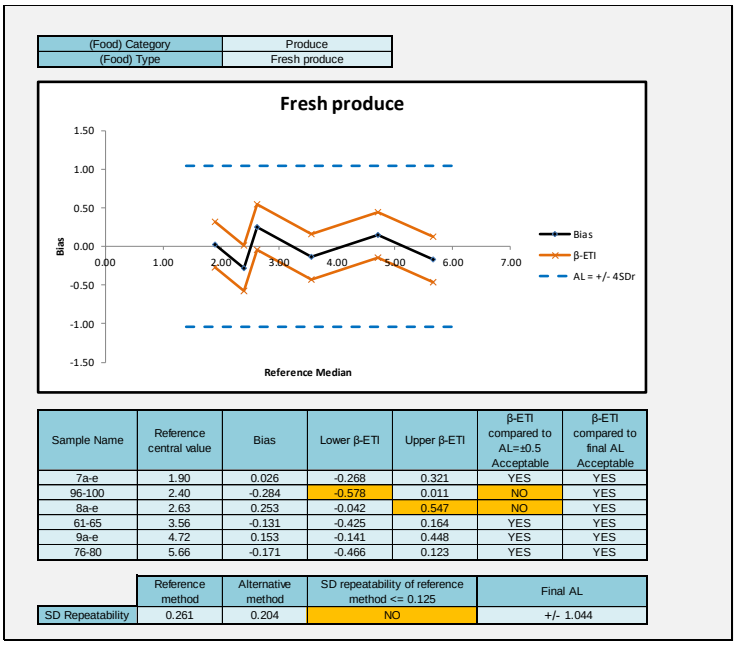


(Food) Category	Fishery products
(Food) Type	Fishery products

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
4 a-e	2.67	-0.013	-0.306	0.280	YES	YES
31 - 35	3.04	-0.029	-0.322	0.264	YES	YES
5 a-e	3.72	-0.053	-0.346	0.240	YES	YES
131-135	4.08	-0.101	-0.395	0.192	YES	YES
6a-e	5.69	0.095	-0.198	0.388	YES	YES
101-105	6.27	-0.272	-0.565	0.022	NO	NO

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.100	0.203	YES	+/- 0.500

Figure 13 – Accuracy profile for Fresh produce

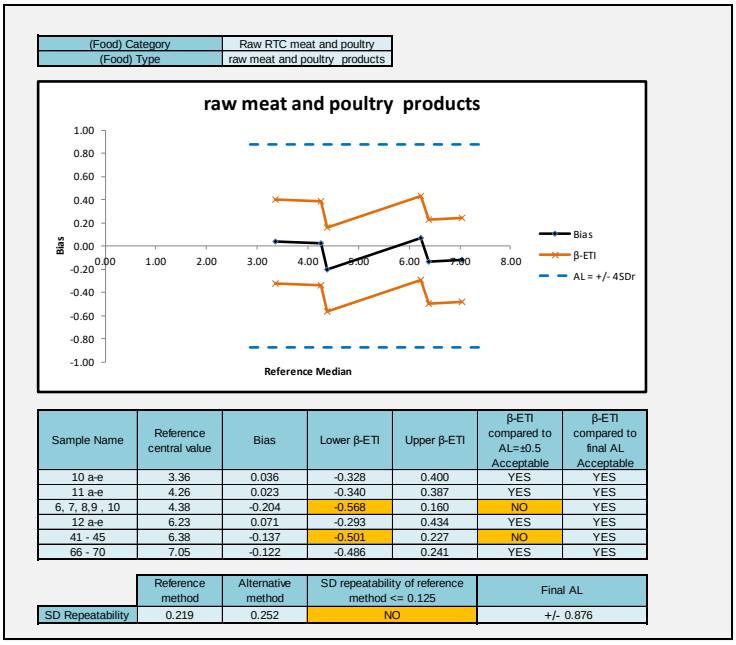


(Food) Category	Produce
(Food) Type	Fresh produce

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
7a-e	1.90	0.026	-0.268	0.321	YES	YES
96-100	2.40	-0.284	-0.578	0.011	NO	YES
8a-e	2.63	0.253	-0.042	0.547	NO	YES
61-65	3.56	-0.131	-0.425	0.164	YES	YES
9a-e	4.72	0.153	-0.141	0.448	YES	YES
76-80	5.66	-0.171	-0.466	0.123	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.261	0.204	NO	+/- 1.044

Figure 14 – Accuracy profile for Raw and RTC Meat and Poultry products



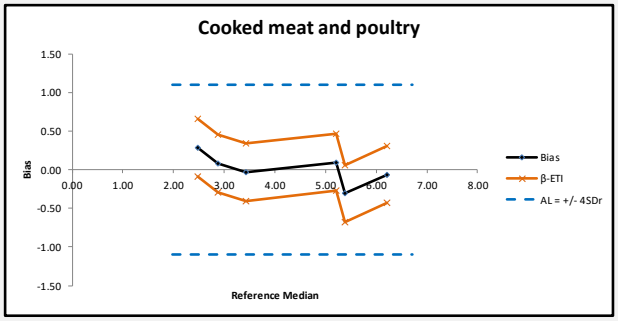
(Food) Category	Raw RTC meat and poultry
(Food) Type	raw meat and poultry products

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 a-e	3.36	0.036	-0.328	0.400	YES	YES
11 a-e	4.26	0.023	-0.340	0.387	YES	YES
6, 7, 8,9 , 10	4.38	-0.204	-0.568	0.160	NO	YES
12 a-e	6.23	0.071	-0.293	0.434	YES	YES
41 - 45	6.38	-0.137	-0.501	0.227	NO	YES
66 - 70	7.05	-0.122	-0.486	0.241	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.219	0.252	NO	+/- 0.876

Figure 15 – Accuracy profile for RTE and RTRH Meat and Poultry products

(Food) Category	RTE and RTRH meat and
(Food) Type	Cooked meat and poultry

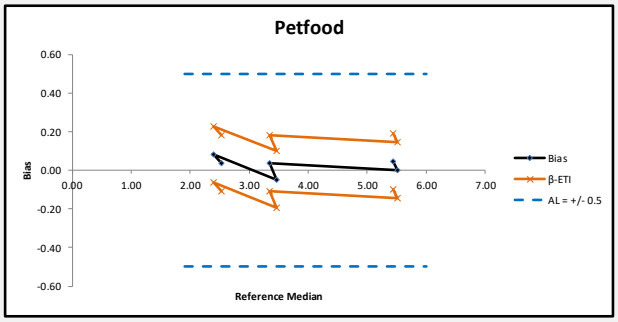


Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
13a -13e	2.48	0.287	-0.086	0.659	NO	YES
14a - 14e	2.87	0.082	-0.290	0.455	YES	YES
41 - 50	3.43	-0.033	-0.406	0.339	YES	YES
15a - e	5.20	0.097	-0.276	0.469	YES	YES
151 - 155	5.37	-0.310	-0.683	0.062	NO	YES
106 110	6.22	-0.062	-0.434	0.311	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.275	0.258	NO	+/- 1.100

Figure 16 – Accuracy profile for Pet food and animal feed

(Food) Category	Petfood
(Food) Type	Petfood

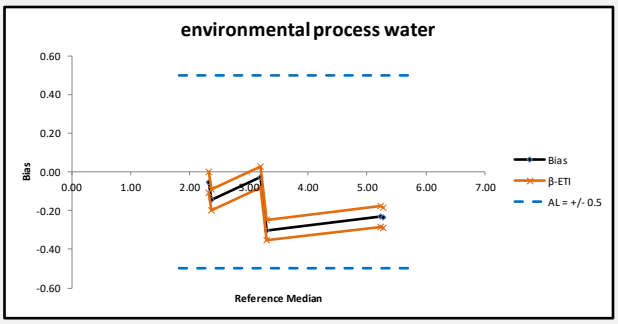


Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
19 a - e	2.52	0.036	-0.110	0.182	YES	YES
16 a - e	2.40	0.083	-0.063	0.229	YES	YES
20 a - e	3.46	-0.047	-0.193	0.098	YES	YES
17 a- e	3.34	0.038	-0.108	0.184	YES	YES
21 a- e	5.51	0.000	-0.146	0.146	YES	YES
18 a - e	5.43	0.046	-0.100	0.192	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.143	0.101	NO	+/- 0.500

Figure 17 – Accuracy profile for Environmental samples

(Food) Category	environmental process water
(Food) Type	environmental process water



Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
25 a - e	2.32	-0.055	-0.108	-0.002	YES	YES
22 a - e	2.36	-0.145	-0.198	-0.092	YES	YES
26 a - e	3.20	-0.028	-0.081	0.025	YES	YES
23 a - e	3.30	-0.301	-0.354	-0.248	YES	YES
24 a- e	5.23	-0.230	-0.284	-0.177	YES	YES
27 a-e	5.28	-0.237	-0.291	-0.184	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.088	0.037	YES	+/- 0.500

Comments

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 were followed, as described in ISO 16140-2:2016 and the new acceptability limits were calculated
In this study the following two categories met the AL of 0.5log.

- Pet food and animal feed
- Environmental samples

In this study, the following categories required the new AL to be calculated. These are shown below.

- Dairy products (AL ± 0.76)
- Fresh produce (AL ± 1.044)
- Meat products (AL ± 0.90)
- Poultry products (AL ± 0.772)

All of these categories met the new recalculated AL values.

For one category, Fishery Products, a new AL was not needed to be calculated but it should be noted that one item (high level for white fish) was just outside the AL ± 0.50. This level was based on the original data set with naturally contaminated product.

It is interesting to note that the two new categories validated for this method (pet food/animal feeds and environmental samples) showed good agreement between the methods and met the AL of ± 0.50. For these categories , all samples were artificially inoculated with target organisms.

For the other five categories tested in the original study, the AL limits are wider between 0.76 and 1.044. For these categories the samples from the original study were naturally contaminated which added to the wider AL observed. These AL values are typical for total microbial counts where a diverse range of microorganisms is present.

The accuracy of the Alternative method is satisfied as the all categories met the 0.5log AL or the re-calculated AL . There was only one case which was just outside the ±0.5 and this was for naturally contaminated fish samples.

3.3 Inclusivity / exclusivity

As this method is not selective and is a general counting method, an inclusivity / exclusivity study is not required.

3.4 Limit of quantification (LOQ)

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

The alternate method is based on visible colonies.

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

3.5 Conclusion (MCS)

Overall, the conclusions for the Method Comparison are:

- The alternative method Compact Dry TC for enumeration of total viable organisms shows satisfactory results for relative trueness
- The alternative Compact Dry TC for enumeration of total viable organisms 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.

The data used for the ILS calculations was generated in the original validation report. It has been recalculated using the appropriate calculations and all the relevant details from the oringal study are included here for information.

4.1 Study organisation

4.1.1 Collaborators
Samples were sent to 13 laboratories in 5 different countries

4.1.2 Matrix and strain used
Pasteurised milk samples were inoculated with *Escherichia coli* (CCFRA code 11017, NCTC 12241). Samples were individually inoculated with the relevant dilution of the *E.coli* strain.

4.1.3 Sample preparation
Samples were prepared and inoculated as described below:

For each laboratory, 8 x 25ml samples of milk were dispensed into sterile 30ml plastic universals (Sterilin, 128B). Two samples remained uninoculated, whereas the other six samples were used for the three contamination levels (low, medium and high). Appropriate dilutions of the *E. coli* culture were used to individually inoculate 2 x 25ml samples at the low (10^2 CFU/ml), medium (10^3 CFU/ml) and high (10^4 CFU/ml) contamination levels.

The samples were blind-coded (as shown in Table 5) and stored at 2-8°C prior to despatch.

A set of samples was also prepared for the EL although the data from these was not used in the data analysis.

The target levels and codes are shown below

Table 5 : Contamination levels

Contamination level	***Sample code***
Uninoculated	M2
Uninoculated	M5
Low (100 cfu/g)	M4
Low (100 cfu/g)	M6
Medium (1000 cfu/g)	M1
Medium (1000 cfu/g)	M7
High (10,000 cfu/g)	M3
High (10,000 cfu/g)	M8

4.1.4 Labelling and shipping
Prior to despatch, each set of milk samples were packed into plastic containers (DGP (UK) Limited PP001). These plastic containers were then placed inside a thermal control unit (Air-Sea Containers Limited, TC-2 code 289) with cool packs (Air-Sea Containers Limited, CP-15 code 406). 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. Postage was arranged so that each laboratory would receive and commence testing of their

samples on Monday 20th November 2006. Any delay with postage or setting up samples was recorded by the Expert Laboratory which tested a complete set of samples on the appropriate testing days.

4.1.5 Analysis of Samples

A total of 13 collaborative laboratories received and tested their samples on 20/11/06 as requested by the Expert Laboratory. Two collaborative laboratories (Lab 12 and Lab 13) failed to receive their samples on the stipulated date and one laboratory (Lab 11) had to defer testing until the following day. These three laboratories performed testing of the samples one day later (21/11/06) than the rest of the collaborative laboratories. As a consequence, the Expert Laboratory analysed two sets of samples, one on each of the two dates (20/11/06 and 21/11/06) to establish if there was any effect of this delay on the samples and outcome of the study. However, the temperature measurements obtained from each of the collaborative laboratories upon receipt of the samples (see Table 6) were all within the acceptable limit stated in the study protocol (≤8°C upon receipt).
The data provided by laboratory 4 was omitted from the statistical analysis because of unacceptably high counts (10^2 cfu/g) in the negative control samples tested by both Compact Dry TC and reference method.

4.2 Experimental parameters controls

4.2.1 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 6.

Table 6 - Sample temperatures at receipt

Laboratory	Date received	Temperature of control sample upon receipt (°C)
1	20/11/06	4.0
2	20/11/06	1.4
3	20/11/06	0.5
4	20/11/06	2.2
5	20/11/06	0.6
6	20/11/06	1.6
7	20/11/06	8.0
8	20/11/06	2.5
9	20/11/06	2.1
10	20/11/06	3.0
11	21/11/06	2.7
12	21/11/06	6.5
13	21/11/06	2.7

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 7.

Table 7a – Results (log_{10} cfu/g) obtained by the expert lab (set 1 analysed on 20/11/06)

Level	**Reference method**	**Alternative method**
Blank	<10	<10
Low	2.83	2.71
Low	2.81	2.70
Medium	3.77	3.75
Medium	3.78	3.72
High	4.73	4.70
High	4.92	4.94

Table 7b – Results (log_{10} cfu/g obtained by the expert lab (set 2 analysed on 21/11/06)

Level	**Reference method**	**Alternative method**
Blank	<10	<10
Low	2.73	2.82
Low	2.76	2.60
Medium	3.85	3.77
Medium	3.81	3.78
High	4.75	4.77
High	4.65	4.87

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 8.

The accuracy profile plot is shown in Figure 18 and the statistical analysis of the data shown in Table 9

Table 8 : Summary (log_{10} cfu/g) of the results of the interlaboratory study per analyte level (k)

Laboratory	Level	Reference method (BP)		Alternative method (BS)	
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
1	Low	2.70	2.68	2.53	2.54
2	Low	2.89	2.78	2.74	2.55
3	Low	2.82	2.83	2.69	2.71
5	Low	2.78	2.74	2.64	2.58

Laboratory	Level	Reference method (BP)		Alternative method (BS)	
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
6	Low	2.54	2.84	2.83	2.82
7	Low	2.81	2.89	2.75	2.78
8	Low	2.84	2.73	2.62	2.61
9	Low	2.76	2.86	2.74	2.77
10	Low	2.77	2.81	2.65	2.64
11	Low	2.78	2.71	2.66	2.77
12	Low	2.73	2.76	2.77	2.80
13	Low	2.79	2.85	2.73	2.83
1	Medium	3.69	3.73	3.47	3.56
2	Medium	3.84	3.79	3.34	3.31
3	Medium	3.97	3.97	3.78	3.80
5	Medium	3.82	3.83	3.73	3.72
6	Medium	3.75	3.79	3.75	3.78
7	Medium	3.93	3.97	3.80	3.85
8	Medium	3.86	3.90	3.58	3.69
9	Medium	3.86	3.91	3.82	3.75
10	Medium	3.70	3.76	3.68	3.70
11	Medium	3.87	3.74	3.71	3.74
12	Medium	3.86	3.86	3.78	3.83
13	Medium	3.85	3.93	3.82	3.92
1	High	4.79	4.67	4.67	4.73
2	High	4.84	4.87	4.29	4.31
3	High	4.99	4.91	4.89	4.76
5	High	4.88	4.78	4.77	4.74
6	High	4.78	4.88	4.75	4.85
7	High	4.93	4.95	4.81	4.84
8	High	4.88	4.76	4.77	4.73
9	High	4.93	5.00	4.83	4.99
10	High	4.60	4.62	4.73	4.69
11	High	4.89	4.82	4.74	4.86
12	High	4.95	4.98	5.00	4.98
13	High	4.87	4.86	4.86	4.85

Figure 18. Accuracy profile of Compact Dry TC from the ILS

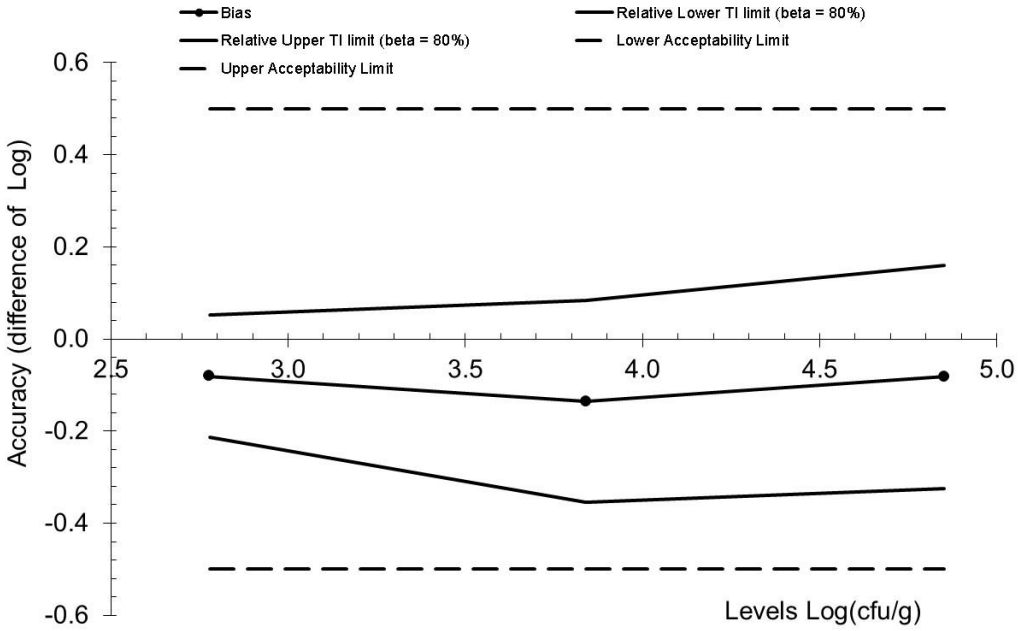

Bias
Relative Lower TI limit (beta = 80%)
Relative Upper TI limit (beta = 80%)
Lower Acceptability Limit
Upper Acceptability Limit
Accuracy (difference of Log)
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
2.5
3.0
3.5
4.0
4.5
5.0
Levels Log(cfu/g)

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

Accuracy profile	0.5		
Study Name	Compact Dry TC		
Date	recalculated 30/07/2019		
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	2.698	3.841	4.851			
Number of participants (K)	12	12	12	12	12	12
Average for alternative method	2.779	3.705	4.769	2.698	3.841	4.851
Repeatability standard deviation (sr)	0.077	0.042	0.055	0.052	0.039	0.053
Between-labs standard deviation (sL)	0.000	0.150	0.163	0.080	0.076	0.097
Reproducibility standard deviation (sR)	0.077	0.155	0.172	0.096	0.086	0.110
Corrected number of dof	22.957	11.843	12.175	14.750	13.569	13.838
Coverage factor	1.347	1.411	1.408			
Interpolated Student t	1.320	1.357	1.355			
Tolerance interval standard deviation	0.0783	0.1615	0.1790			
Lower TI limit	2.675	3.486	4.527			
Upper TI limit	2.882	3.924	5.012			
Bias	**0.081**	**-0.135**	**-0.082**			
Relative Lower TI limit (beta = 80%)	**-0.022**	**-0.355**	**-0.325**	FALSE		
Relative Upper TI limit (beta = 80%)	**0.184**	**0.084**	**0.160**	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"

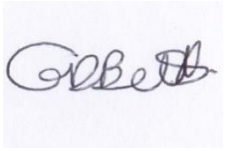
New acceptability limits may be based on reference method pooled variance	
Pooled repro standard dev of reference	0.098

5 Overall conclusions of the validation study

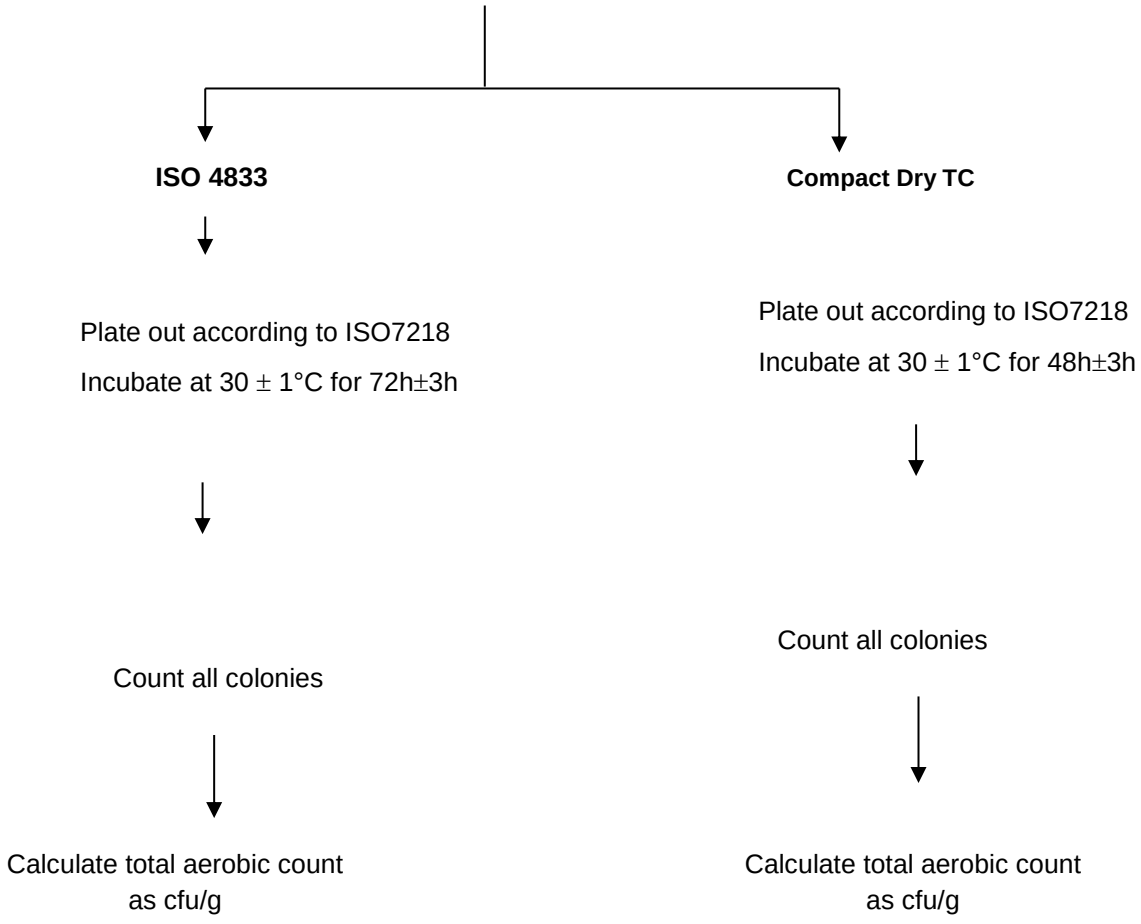
- The alternative method Compact Dry TC for enumeration of total viable organisms shows satisfactory results for relative trueness;
- The alternative method Compact Dry TC for enumeration of total viable organisms shows satisfactory results for accuracy profile;
- The alternative method Compact Dry TC for enumeration of total viable organisms shows satisfactory performance in the ILS
- The alternative method Compact Dry TC for enumeration of total viable organisms shows comparable performance to the reference method ISO 4883-1:2013. Microbiology of food and animal feeding stuffs- Horizontal method for the enumeration of microorganisms. Part 1: Colony count at 30 degrees C by the pour plate technique

Date: 26/08/2019

Signature: Dr Gail Betts



ANNEX A: flow diagram of the reference and alternative method



ANNEX B: Kit insert

HyServe

Compact Dry TC medium for total count/Gesamtkeimzahl/milieu pour nombre total de germes/medio para recuento total/ medio per conta totale /meio para contagem total de germes

40 plates/Platten/plaques/placas/lastre/placas	ID-No. 1 000 166
240 plates /Platten/plaques/placas/lastre/placas	ID-No. 1 000 167
920 plates/Platten/plaques/placas/lastre/placas	ID-No. 1 002 877

English

Compact Dry TC is a ready to use, chromogenic plate for the enumeration of total count

Sample pretreatment

Viable count in water or liquid foodstuff
Drop 1 ml of specimen (dilute if necessary) on the middle of the Compact Dry plate.

Viable count in solid foodstuff
Add buffer solution to the sample and homogenize by stomacher®. Drop 1 ml of specimen (dilute if necessary) on the middle of the dry sheet of the Compact Dry plate.

Viable count in swab test specimen
Use the swab to wipe the surface, put into the device with wiping solution. Drop 1 ml of wiping solution (dilute if necessary) on the middle of the Compact Dry plate. It is recommended to use "Swab for Compact Dry" offered by HyServe Id-No. 1 002 952/3 (40/240 pieces).

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

Incubation time 48 ± 3 hours (or 72 ± 3 hours)
Incubation temperature 30 ± 1 °C (tested by NordVal and MicroVal against against 4833:2003) 35 ± 1 °C tested by AOAC against AOAC Official Method 966.23)

Interpretation of the results
Colonies grown are almost all red. Red and otherwise colored colonies together are the total count.

Storage and shelf life
Keep at room temperature (+ 1 to +30 °C).Total shelf life 18 months after manufacturing.

Notes
- some colonies might not be clearly red colored.
- high concentrations on places will cause the entire growth area to become red/ pink. In this case dilute the sample.
- after use please follow the current disposal regulations.
- the growth area is 30 cm². the back of the plate has a grid carved of 1 cm x 1 cm to make the colony counting easier. In case of any difficulties to count colonies due to large number of colonies grown, total viable count can be obtained by multiplying 30 by an average number of colonies per grid counted from several grids.
- Compact dry plates are produced at an ISO 9001/ISO 13485: 2003 certified site
- AOAC approval No. 010604
- MicroVal approval No.0703-001LR/ISO 4833 (2003)
- ISO EN 16140:2003
- NordVal certificate No 033 for food

Deutsch

Compact Dry TC ist eine gebrauchsfertige, chromogene Platte zum Nachweis der Gesamtkeimzahl

Probenvorbereitung

Lebendkeimzahl in Wasser oder flüssigen Lebensmitteln
1 ml der Probe (evtl. verdünnen) in der Mitte der Compact Dry Platte aufbringen.

Lebendkeimzahl in festen Lebensmitteln
Zugabe von Pufferlösung und Homogenisierung der Lebensmittelprobe im Stomacher® ist erforderlich. 1 ml der Probe (evtl. verdünnen) in der Mitte der Compact Dry Platte aufbringen.

Lebendkeimzahl aus Tupfer-Proben
Mit den sterilen, feuchten Nattetupfer kann z.B. die Oberfläche gewischt werden. Der Tupfer wird zurück in die Aufnahmeflüssigkeit überführt. Nach Schütteln wird die gesamte Lösung (1 ml) in der Mitte der Compact Dry Platte aufgebracht. Es wird empfohlen den Swab für Compact Dry von HyServe, Id-No. 1 002 952/3 (40/240 Stück) zu verwenden.

Testanweisung
1. Öffnen des Deckels und Auftropfen von 1 ml Probenmaterial in die Mitte der Compact Dry Platte.
2. Das Probenmaterial diffundiert automatisch und gleichmäßig in die Nährsubstanz und rehydriert das Gewebe innerhalb von Sekunden zu einem Gel.
3. Platte mit Deckel verschließen und beschriftbare Fläche zur Kennzeichnung verwenden.
4. Geschlossene Platte umdrehen und in einen Brutschrank legen.
5. Nach Inkubation die Anzahl der farbigen Kolonien von der Rückseite der Platte her zählen. Ein weißes Papier als Unterlage erleichtert den Zählvorgang.

Inkubationszeit 48 ± 3 Stunden (72 ± 3 Stunden)
Inkubationstemperatur 30 ± 1 °C (tested by NordVal and MicroVal against against 4833:2003) 35 ± 1 °C tested by AOAC against AOAC Official Method 966.23)

Interpretation des Ergebnisses
Nahezu alle Kolonien nehmen rote Farbe an. Rote und andersfarbige Kolonien zusammengezählt ergeben die gesamte Lebendkeimzahl der Lebensmittelprobe.

Lagerung und Haltbarkeit
Bei Raumtemperatur aufbewahren (+ 1 bis +30 °C). Haltbarkeit bis 18 Monate nach Herstellung.

Bemerkungen
- nicht alle Kolonien zeigen möglicherweise eine eindeutige rotfärbung.
- nach Gebrauch entsprechend der gültigen Abfallregelung die platten entsorgen.
- die plattenfläche beträgt 30 cm². Auf der plattenrückseite ist ein raster mit 1cm x 1cm eingraviert, um die koloniezählung zu erleichtern. Sollte es problematisch sein auf Grund hoher koloniedichte eine ganze platte auszuzählen, sind einzelne Quadrate auszuzählen und der mittelwert mit 30 zu multiplizieren.
- Compact dry platten können bis zu 300 kolonien pro platte nachweisen. daher ist es erst nötig kontaminationen, die diese zahlenkeimzahl überschreiten, zu verdünnen und die verdünnungen auf die platte aufzubringen.
- Compact dry platten werden in einem ISO 9001/ISO 13485: 2003 zertifiziertem betrieb gefertigt.
- AOAC approval No. 010604
- MicroVal approval No.0703-001LR/ ISO 4833(2003)
- ISO EN 16140:2003
- NordVal certificate No 033 for food

Français

Compact Dry TC est une plaque chromogène prête à l'utilisation pour détecter le nombre total de germes

Traitement préliminaire de l'échantillon

Nombre de germes revivifiables dans l'eau ou dans des aliments liquides
Appliquer 1 ml de l'échantillon (le diluer si nécessaire) au centre de la plaque Compact Dry.

Nombre de germes revivifiables dans des aliments solides
Il est nécessaire d'ajouter une solution tampon à l'échantillon et de l'homogénéiser par Stomacher®. Appliquer 1 ml de l'échantillon (le diluer si nécessaire) au centre de la plaque Compact Dry.

Nombre de germes revivifiables dans des échantillons prélevés
Utiliser le tampon pour essuyer la surface,le placer dans l'unité avec la solution d'essuyage. Appliquer 1 ml de la solution d'essuyage (la diluer si nécessaire) au centre de la plaque Compact Dry. Il est recommandé d'utiliser le tampon 'Swab for Compact Dry' distribué par la société HyServe Id-No. 1002952/3 (40/240 pièces)

Instructions pour le test
1. Ouvrir le couvercle et appliquer 1 ml de l'échantillon sur la plaque Compact Dry.
2. L'échantillon se répand automatiquement et uniformément sur la feuille et en l'espace de quelques secondes, il transforme la feuille sèche en un gel.
3. Refermer le couvercle de la plaque et inscrire les informations nécessaires dans la partie correspondante.
4. Retourner la plaque fermée et la placer dans l'incubateur.
5. Après le temps d'incubation, compter le nombre de colonies de couleur au dos de la plaque. Les colonies peuvent être comptées plus simplement en plaçant du papier blanc sous la plaque.

Temps d'incubation 48 ± 3 heures; (72 ± 3 heures)
Température d'incubation 30 ± 1 °C (tested by NordVal and MicroVal against against 4833:2003) 35 ± 1 °C tested by AOAC against AOAC Official Method 966.23)

Interprétation des résultats
Pratiquement toutes les colonies se colorent en rouge. Les colonies rouges et les colonies d'autres couleurs constituent le nombre total de germes revivifiables.

Stockage et durée de conservation
Stockage à température ambiante (+ 1 à +30 °C). Durée totale de conservation 18 mois après fabrication.

Remarques
- Quelques colonies risquent de ne pas se colorer nettement en rouge.
- des concentrations élevées sur les plaques entraînent une coloration rouge/rose de toute la surface. dans un tel cas, il faut diluer l'échantillon.
- Après l'utilisation, éliminer les plaques en respectant les réglements correspondants en vigueur.
- la surface de la plaque est de 30 cm². une grille de 1 cm x 1 cm est taillée dans le dos de la plaque afin de faciliter le calcul des colonies. S'il est toutefois difficile de compter le nombre de colonies, suite à un grand nombre de colonies, il est possible de déterminer le nombre total de germes revivifiables dans certains carrés de la grille et d'en multiplier par 30 la valeur moyenne obtenue.
- Les plaques Compact Dry sont fabriquées dans une usine certifiée conforme à ISO 9001/ ISO 13485: 2003
- AOAC approval No. 010604
- MicroVal approval No.0703-001LR / ISO 4833(2003)
- ISO EN 16140:2003
- NordVal certificate No 033 for food