ISO 16140-2:2016 validation of Brilliance™ CampyCount Agar for the enumeration of Thermotolerant *Campylobacter* species particularly *C. jejuni* and *C. coli* in raw and ready to cook poultry products.

MicroVal study number: 2008LR12 renewal

Method/Kit name: Brilliance™ *CampyCount* Agar

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Standardized report - Quantitative methods
Method Comparison Study and ILS Report
2008LR12 renewal Brilliance™ *CampyCount*
Agar Summary report

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: Thermo Fisher Scientific
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Expert Laboratory: Campden BRI

Method/Kit name: Brilliance™ CampyCount Agar

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/TS 10272-2 :2006 - Microbiology of food and animal feeding stuffs -- Horizontal method for detection and enumeration of *Campylobacter* spp. -- Part 2: Colony-count technique – *used for original study*

ISO 10272-2: 2017 - Microbiology of the food chain - Horizontal method for detection and enumeration of *Campylobacter* spp. - Part 2: Colony-count technique *used for renewal study*

Scope of validation: Raw poultry and ready to cook poultry products

Certification organization: Lloyd's Register

List of abbreviations

- AL Acceptability Limit
- AP Accuracy Profile
- Art. Cont. Artificial contamination
- CFU Colony Forming Units
- CL confidence limit (usually 95%)
- EL Expert Laboratory
- $\overline{D}$ Average difference
- g Gram
- h Hour
- ILS Interlaboratory Study
- Incl/Excl Inclusivity and Exclusivity
- LOQ Level of Quantification
- MCS Method Comparison Study
- min minute
- ml Millilitre
- MR (MicroVal) Method Reviewer
- MVTC MicroVal Technical Committee
- 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
- Sp Spread plate- not possible to count
- 10^{-1} dilution 10-fold dilution of original food
- 10^{-2} dilution 100-fold dilution of original food

And, in *Campylobacter* studies,

- PSD Peptone Salt Diluent
- BCCA Brilliance™ CampyCount Agar
- mCCDA modified Charcoal Cefoperazone Deoxycholate Agar
- CBA Columbian blood agar
- Latex kit Thermo Scientific *Campylobacter* Test
- OBIS campy Oxoid Biological Identification System for *Campylobacter*
- MHB Muller Hinton Broth

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Contents

1	**Introduction**	**5**
2	**Method protocols**	**6**
2.1	Reference method	6
2.2	Alternative method	6
2.3	Study design	7
3	**Method comparison study**	**7**
3.1	Relative trueness study	7
	3.1.1 Number of samples	7
	3.1.2 Test sample preparation	8
	3.1.3 Protocols applied during the validation study	8
	3.1.4 Test results	9
	3.1.5 Calculation and interpretation of relative trueness study	9
	3.1.6 Conclusion (RT study)	13
3.2	Accuracy profile study	13
	3.2.1 Categories, sample types and strains	13
	3.2.2 Calculations and interpretation of accuracy profile study	13
3.3	Inclusivity / exclusivity	14
	3.3.1 Protocols	14
	3.3.2 Results	15
	3.3.3 Conclusion	15
3.4	Limit of quantification (LOQ)	15
3.5	Conclusion (MCS)	16
4	**Interlaboratory study**	**16**
4.1	Study organisation	16
	4.1.1 Collaborators	16
	4.1.2 Matrix and strain used	16
	4.1.3 Sample preparation	16
	4.1.4 Labelling and shipping	17
	4.1.5 Analysis of Samples	17
4.2	Experimental parameters controls	17
	4.2.1 Detection of Thermotolerant *Campylobacter* in the matrix before inoculation	17
	4.2.2 Strain stability during transport	17
	4.2.3 Logistic conditions	18
4.3	Calculation and summary of data	18
	4.3.1 MicroVal Expert laboratory results	18
	4.3.2 Results obtained by the collaborative laboratories	19
5	**Overall conclusions of the validation study**	**22**
ANNEX A: Flow diagram of the alternative method and reference methods		**233**
ANNEX B : Kit Insert		**24**

1 Introduction

In this project a MicroVal validation study, based on ISO 16140-2:2016, of an alternative method for the enumeration of *Campylobacter* spp in a single food category (raw and ready to cook poultry) was carried out by Campden BRI as the MicroVal Expert Laboratory.

This is a renewal of a method that has already been validated according to the superseded ISO16140:2003 standard for enumeration of *Campylobacter* species in poultry products. The original study was done by RIKILT/RIVM.

Five levels of contamination were used for the original study, covering a minimum, a central and a maximum level plus two intermediary levels. Duplicate test portions were examined for each sample tested and this data has been used for the RT part of the renewal study as it covers all three of the required food types within this category. Only one additional RT data point was required to complete the raw and ready to cook poultry category (Table 1).

There was no data available in the original study design to do the AP analysis as this part requires the testing of five replicate test portions of 6 samples per category, and therefore all new data was required for this part (Table 4).

There was sufficient Incl/Excl data available from the original study to cover the requirements of ISO16140-2:2016 but some additional strains have also been tested here.

It is worth noting that the original study was done using ISO/TS 10272-2:2006 and the current renewal study has been done with ISO 10272-2:2017. Changes made in the revision of the reference method were considered as minor and unlikely to have any impact on the alternative method validation. The changes are a minor revision to the reference method confirmation procedures, i.e. the use of 'absence of aerobic growth at 25°C' in the new reference method instead of 'absence of microaerobic growth at 25°C' and 'absence of aerobic growth at 41.5°C' in the old reference method

In addition, it should also be noted that the original study was done with the old latex kit, Dryspot Campylobacter Test (Oxoid DR 0150M) rather than the current latex kit: Thermofisher Scientific Campylobacter Test (DR 0155M). Some additional studies were done by RIVM which demonstrated that the new kit gave a comparative performance to the reference confirmation tests. The Thermofisher Scientific Campylobacter Test DR 0155M kit was used in this renewal study.

The alternative method used was: Brilliance™ CampyCount Agar.

The reference method used was: ISO/TS 10272-2 :2006 - Microbiology of food and animal feeding stuffs -- Horizontal method for detection and enumeration of *Campylobacter* spp. -- Part 2: Colony-count technique – *used for original study*

ISO 10272-2: 2017 - Microbiology of the food chain - Horizontal method for detection and enumeration of *Campylobacter* spp. - Part 2: Colony-count technique *used for renewal study*

Categories included:

- Raw poultry and ready to cook poultry products.

Criteria evaluated during the study have been:

- Relative trueness study;
- Accuracy profiles
- Inclusivity and exclusivity
- Interlaboratory Study

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

The alternative method Brilliance™ CampyCount Agar shows comparable performance to the reference methods (ISO 10272-2:2017, ISO 10272-2:2006) method for the enumeration of Thermotolerant Campylobacter species in a raw poultry and ready to cook poultry products.

2 Method protocols

The Method Comparison Study was carried out using 10gram 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. In summary:

- 0.1ml samples of appropriate dilutions were spread plated on mCCDA and incubated under microaerophilic conditions at 41.5±1°C for 44±4h
- Five typical colonies per plate used in the final count were confirmed using 3 methods
  - ISO 10272-2: Oxidase, morphology and motility, aerobic growth at 25°C
  - OBIS Campy
  - Thermofisher Latex kit

Sample preparations used in the reference method and the alternative method were carried out according to ISO 6887-series part 1 and part 2 for meat and meat products.

2.2 Alternative method

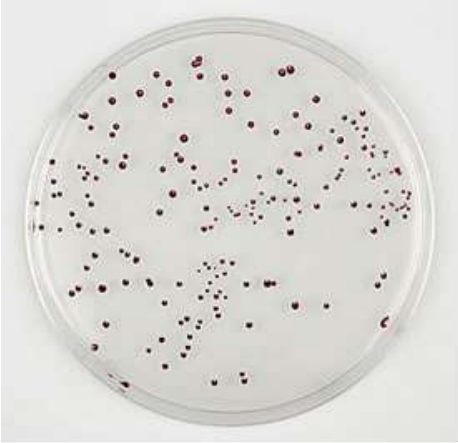
See the flow diagram of the alternative method in Annex A.

See the kit insert in Annex B.

The alternative method principle is based on chromogenic media. It is a spread plate method intended to enumerate Thermotolerant Campylobacter species particularly C.jejuni and C.coli. The agar is a transparent medium which is highly selective and contains an indicator that is metabolised by target organism resulting in dark red colonies.

A picture is provided in Figure 1:

Figure 1: BCCA



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 and artificially contaminated samples. One category with different types and items were tested.

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.

The 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	Inoculated	Inoculation by mixing	Natural	Total
Raw poultry and ready to cook poultry (RTC) products	Carcass, meats and cuts e.g., Chicken livers and chicken skin	10	8	46	64
	Minced chicken meat	12	2	2	16
	Processed RTC e.g. Seasoned poultry	0	4	0	4
Total		22	14	48	84

In total 84 data points were used in the analysis with 64 of these being allowable points. This is considerably more than the 15 interpretable results needed.

3.1.2 Test sample preparation
It is preferable to test naturally contaminated samples. In the original RT study there were 48 naturally positive and 14 contaminated by mixture wth naturally positive sample.

For the single point needed in the renewal study artificial contamination was used.

Artificial contaminations were obtained by:

- Seeding with appropriate strains
    - and storing chilled for minimum 48h at <5°C;

3.1.3 Protocols applied during the validation study

A single protocol was applied for the study.

Reference method plates were incubated at 41.5±1°C for 44±4h, under microaerophillic conditions.

Alternative method plates were incubated at 41.5±1°C for 48±1h, under microaerophillic conditions (the minimum time of 47h was used)

Method confirmation

- Five typical colonies from two plates used in the calculations were confirmed using 3 methods
    - ISO 10272-2: Oxidase, morphology and motility, aerobic growth at 25°C
    - OBIS Campy
    - Thermofisher Latex kit (DR 0155M)

3.1.4 Test results

3.1.5 Calculation and interpretation of relative trueness study

During the original study a significant bias between the methods was observed for naturally contaminated chicken thigh skin, (air packed) with the alternative method giving lower results than the reference method. In the orignal study it was agreed to analyse the data without these samples and to state the following limitation on the final certificate:

"Note; It may be that in chicken thighs BCCA plates give a lower yield than mCCDA plates."

In order to be consistent with the initial study,during the reanalysis of the data for this renewal study, the chicken thigh data have been included in Figures 2, 4 and Table 2, and have been excluded in Figures 3, 5 and Table 2.

The bias in the data observed in the original has also been observed in this renewal stdy. Therefore further analysis of the data and any conclusions made from the data were based on the reduced data set, excluding the airpacked thigh samples.

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

Figures 2 and 3 shows the scatter plots for the individual categories and all categories with and without the air-packed chicken thighs.

Figure 2 - Scatter plot of the reference method versus alternative method results all data circles show air-packed data

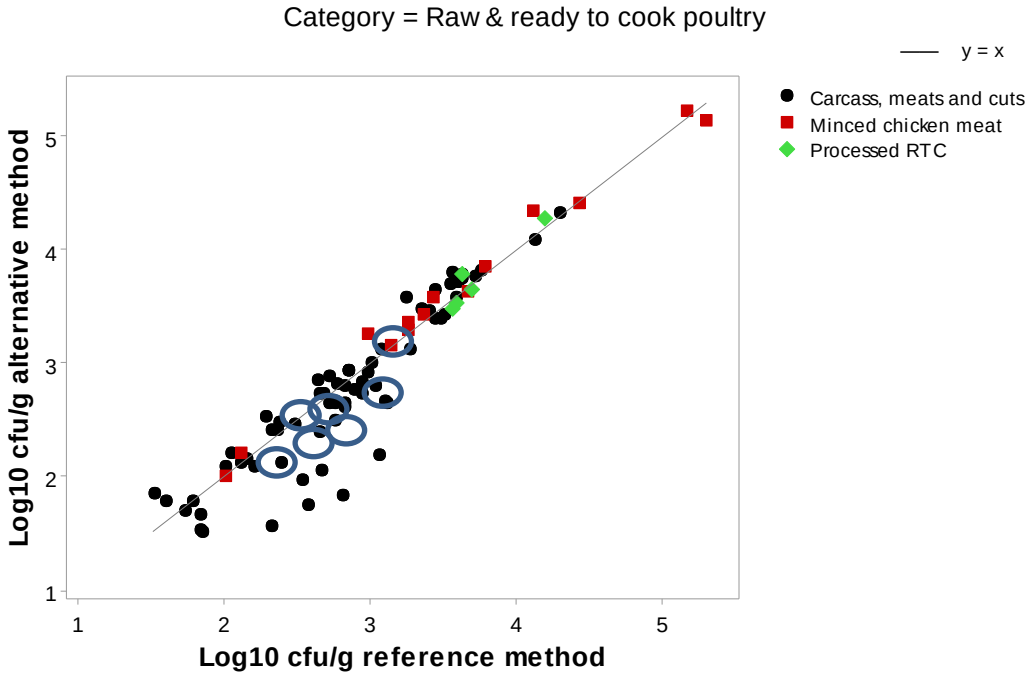
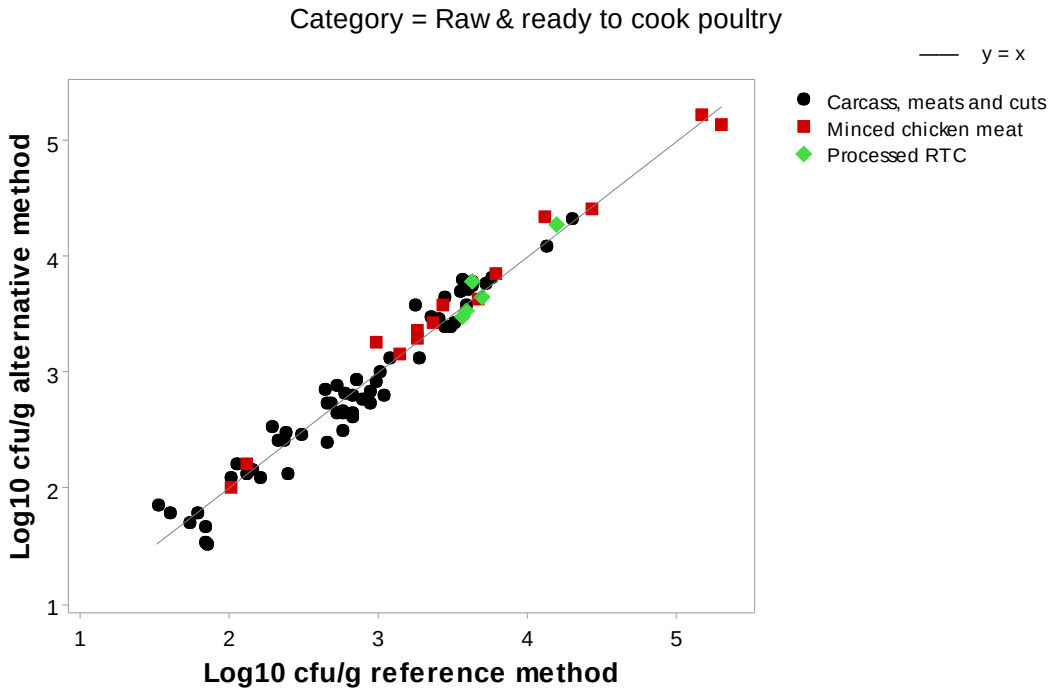


Figure 3 - Scatter plot of the reference method versus alternative method results reduced data set (excluding air-packed samples)



According to ISO 16140-2:2016, the results of the scatter plot are interpreted based on a visual observation on the amount of bias and extreme results.

There was extremely good agreement between the two methods with almost no positive or negative bias.

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 Figures 4 and 5.

Table 2 - Summary of the calculated values per category – all and reduced data

Category.	**n**	$\overline{D}$	S_D	**95% Lower limit**	**95% Upper limit**
Raw & ready to cook poultry - all data	84	-0.061	0.257	-0.575	0.453
Raw & ready to cook poultry - reduced data	77	0.005	0.148	-0.291	0.302

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

Figure 4 – Bland-Altman difference plot for all the samples

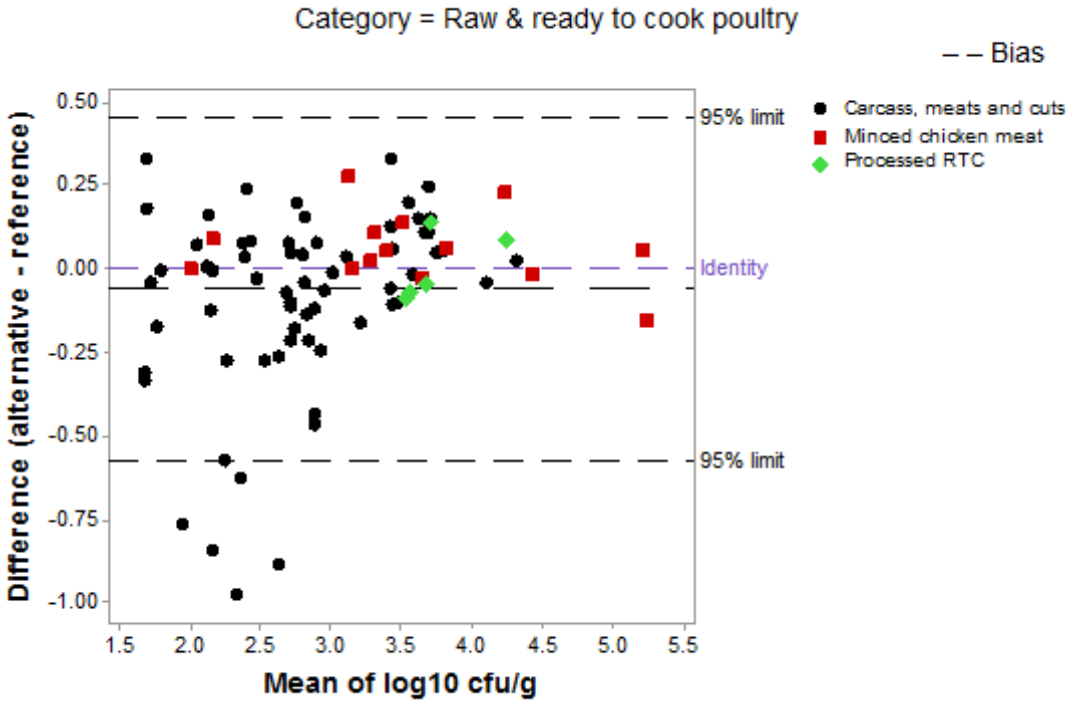


Figure 5 – Bland-Altman difference plot for the reduced number samples

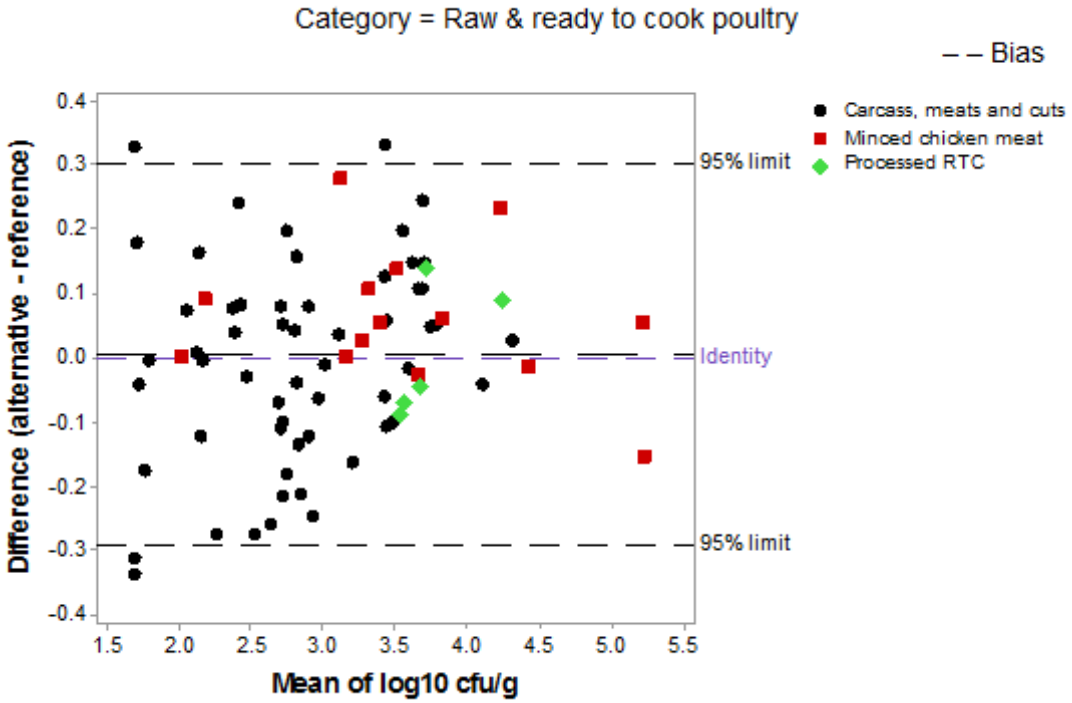


Table 3 - Data which are outside of the accepted limits (reduced data)

Category	Types	Code	Food item	strain	Spiking/ seeding	Log (Ref)	Log (Alt)	Mean	Difference
Raw & ready to cook Poultry	Carcass, meats & cuts	43	Chicken liver	Naturally contaminated		3.250	3.580	3.415	0.329
		511	Chicken skin	*C.coli* C161	0.1ml into 10g	1.519	1.845	1.682	0.327
		88a	Chicken fillet pieces	Naturally contaminated		1.833	1.519	1.676	-0.314
		189a	Chicken skin leg	Naturally contaminated		1.845	1.505	1.675	-0.340

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 77 data points which were outside of the accepted limits, 2 with a slight positive bias and 2 with a slight negative bias. This meets the expectation. The data points which were outside the accepted limits were all the same type however this type was by far the most tested with 54 of the 77 samples tested belonging this category. Also, the CL are very narrow at < 0.3 with these four data points falling just outside. There is no overall bias to the data.

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.

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
It is possible to run this study in two different ways. 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.

In this study one food category was tested with a 2 separate batches of a single food type using 6 samples per type. The two batches were contaminated at a low, intermediate and a high level. For each sample, 5 replicates (5 different test portions) were tested. A total of 30 samples were analysed per food type. The conditions tested are shown in Table 4

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

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

Category	Types	Strain	Item	Level
Raw poultry and ready to cook poultry products	RTC poultry products	*C. jejuni* CRA5562 from chicken	Seasoned raw turkey batch 1	Level 1x5: 1000 cfu /g
				Level 2x5: 25,000 cfu/g
				Level 3x5 500,000 cfu/g
		C. coli CRA329 from pork	Seasoned raw turkey batch 2	Level 1x5: 1000 cfu /g
				Level 2x5: 25,000 cfu/g
				Level 3x5 500,000 cfu/g

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

Total number of samples tested= 60

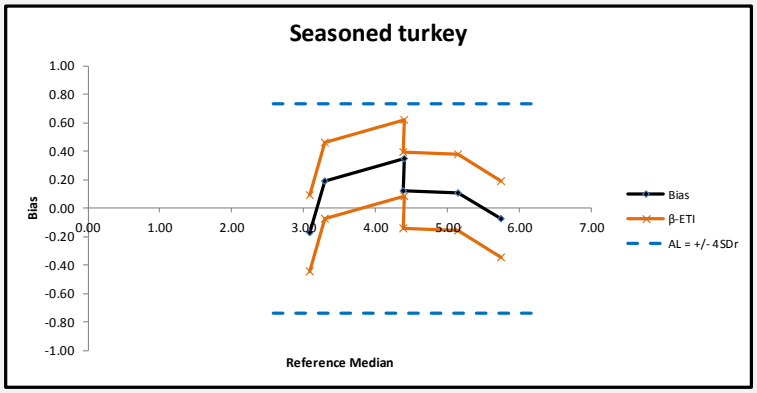
3.2.2 Calculations and interpretation of accuracy profile study

The statistical results and the accuracy profiles are provided in Figure 6.

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 5 Accuracy profile

(Food) Category	Raw poultry
(Food) Type	Seasoned turkey



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	3.08	-0.176	-0.445	0.092	YES	YES
34	3.30	0.190	-0.078	0.459	YES	YES
30	4.40	0.350	0.082	0.619	NO	YES
33	4.38	0.125	-0.144	0.393	YES	YES
35	5.15	0.109	-0.159	0.378	YES	YES
32	5.74	-0.078	-0.346	0.191	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.184	0.186	NO	+/- 0.736

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 one level did not meet the AL of 0.5log. However, the standard deviation of the reference method was >0.125, the additional calculations were carried out and the reference method met the newly calculated AL of ± 0.736.

The accuracy of the Alternative method is satisfied as all samples met the 0.5log AL or the re-calculated 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
  A minimum of fifty strains of the target organism needed to be tested to meet the requirements of ISO16140-2:2016
  Fifty-five strains of *Campylobacter* species were evaluated in the original study and extension study by RIVM. In addition, a further eleven cultures were tested here making a total of 66 strains tested. Each strain was grown in MHB broth at 37±1°C for 48h 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
  A minimum of thirty strains *of* non target organisms needed to be tested to meet the requirements of ISO16140-2:2016. Thirty two strains of non-*Campylobacter* spp. were evaluated in the original study. In addition, a further twelve cultures were tested here making a total of forty four strains tested. All strains 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

All raw data are given in Annex H.

- Inclusivity

Of the 66 inclusivity strains tested, all *C. jejuni subsp. jejuni* strains and all *C. coli* strains gave a positive result (typical growth) using the alternative method and the reference method.

The *C. jejuni subsp. doylei* strain did not grow on either of the plates, as could be expected because of the incubation temperature of 41,5°C. Two *C. upsaliensis* strains and one *C. hyointestinalis* strain tested, did not grow on mCCDA or BCCA. One of the *C. lari* strains failed to grow on BCCA but did grow on mCCDA. The remaining two *C. lari* strains did grow on both mCCDA and BCCA.

BCCA like the reference method, is more specific for enumeration of *C. jejuni* and *C. coli*. Hence it is not surprising that other *Campylobacter* strains are less easily detected on both methods

- Exclusivity

Of the 44 exclusivity strains tested, thirty-nine of the non-*Campylobacter* strains gave a negative result (no growth at all) using the alternative method and reference method.

Acinetobacter baumanii , *Acinetobacter calcoaceticus,* two *Pseudomonas aeruginosa strains* and one of the four *E. coli* strains did show growth on both mCCDA and BCCA plates. On mCCDA this growth was atypical (white versus grey colonies), on BCCA plates the growth was typical (red colonies). However, confirmation tests (microscopy, Dryspot, O.B.I.S Thermofisher latex kit) readily indicated these strains not to be *Campylobacter*.

3.3.3 Conclusion

The alternative method Brilliance™ CampyCount Agar for enumeration of Thermotolerant Campylobacter species in raw and ready to cook poultry products 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 Brilliance™ CampyCount Agar for enumeration of Thermotolerant *Campylobacter* shows satisfactory results for relative trueness;
- The alternative method Brilliance™ CampyCount Agar for enumeration of Thermotolerant Campylobacter shows satisfactory results for accuracy profile;
- The alternative method Brilliance™ CampyCount Agar for enumeration of Thermotolerant *Campylobacter* is selective and specific.

Restriction of use: The method comparison study revealed that it may be that in air-packed chicken thighs Brilliance CampyCount Agar plates give a lower yield than mCCDA plates.

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.

This section desribes the analysis of the original study data according to the ISO16140-2:2016 statisitcs.

4.1 Study organisation

4.1.1 Collaborators
Samples were sent to 17 laboratories in eight different countries.

4.1.2 Matrix and strain used
Samples of minced chicken meat were inoculated with *C. jejuni* strain Camp145 isolated from poultry.
Samples were individually inoculated with the relevant dilution of the *C. jejuni* strain.

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

Each laboratory received eight samples of 10 g, i.e. two samples per inoculation level L1, L2, and L3 and 2 samples of the blank L0. All samples were blind-coded.

Each sample was individually inoculated with 1 ml of the appropriate inoculation suspension L1, L2, or L3 to achieve the following target contamination levels:

Level 0 < 100 cfu/g (Blank)
Level 1: 2,500 cfu/g
Level 2: 50,000 cfu/g
Level 3: 1000,000cfu/g

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

4.1.4 Labelling and shipping
Eight samples (two samples per contamination level) were transported to each of the collaborative laboratories on Monday 31 May 2010. Each laboratory also received a "Temp Control" vial containing 10 ml of water, for a temperature measurement at actual time of receipt.

Transport of samples was under refrigerating conditions (isolating boxes with ice packs), monitored by means of a temperature probe. The arrival time, the temperature of the water vial and the condition of the samples was recorded by each laboratory.

In addition, the organising laboratory tested a set of samples at the same time as the collaborating laboratories to confirm the presence of the target organisms and the contamination levels. The expert laboratory data was not used in the calculations.

4.1.5 Analysis of Samples
The majority of the collaborative laboratories and the expert laboratory carried out the analyses on June 1st, 2010 with the alternative and reference methods. Two laboratories (C and E) received and tested the samples on June 2nd 2010 and a further 2 laboratories (D and N) received and tested the samples on June 3rd 2010. A third laboratory (Q) also received their samples on June 3rd but were not able to test the samples due to other commitments.

4.2 Experimental parameters controls

4.2.1 Detection of Thermotolerant Campylobacter in the matrix before inoculation

To ensure the absence of Thermotolerant *Campylobacter* in the food matrix, the reference method was performed on 10 portions (10 g) before the inoculation. All the results were negative.

4.2.2 Strain stability during transport
Ten samples of each of L1, L2 and L3 were tested at the EL for stability after storage at <8°C for 0, 24 and 48h on both the alternative and reference method.

Table 5: Levels of Thermotolerant Campylobacter (Log_{10} *cfu/g) in stability samples*

The data	L1			L2			L3		
day	**0**	**1**	**2**	**1**	**2**	**3**	**1**	**2**	**3**
1	3.80	3.97	3.65	4.84	4.99	4.19	5.53	6.15	5.56
2	3.77	3.95	*	4.95	4.99	4.29	5.75	6.16	5.41
3	3.87	3.90	3.27	4.95	4.83	4.09	5.79	6.12	5.02
4	3.90	3.88	3.06	4.97	4.93	4.2	5.74	6.16	5.56
5	3.91	3.99	3.28	5.03	4.90	4.08	*	6.16	5.34
6	3.89	3.94	2.85	4.96	4.91	*	5.81	6.20	5.48
7	3.92	3.89	3.27	4.95	5.02	4.09	5.77	6.20	5.28
8	3.96	3.83	2.93	4.86	4.80	4.12	*	6.13	5.73
9	3.73	4.01	2.98	4.98	4.98	4.26	5.61	6.18	5.96
10	3.83	3.87	*	4.91	4.97	*	5.66	6.27	*
average	**3.86**	**3.92**	**3.16**	**4.94**	**4.93**	**4.17**	**5.71**	**6.17**	**5.48**

*missing data

4.2.3 Logistic conditions

The date and time of arrival of the samples, as well as the temperature of the water vial and the iButton upon arrival are given in Table 6. The temperature of the samples during transport, as recorded by the iButtons, all stayed below 8°C, except for labs D and N due to the delay in delivery. Labs J and M reported a temperature of the water vial of > 8°C, but this was not confirmed by the iButton measurements. These higher water vial temperatures most likely were caused by a prolonged handling in the laboratory.

Table 6 - Sample temperatures at receipt

Organising laboratory	Maximum Temperature measured by iButton (°C)	Temperature of water Vial measured at receipt (°C)	Receipt and analysis date
A	2.0	2.1	1-06-2010
B	2.0	5.0	1-06-2010
C	1.5	3.8	2-06-2010
D	14.0	15.2	3-06-2010
E	1.0	3.3	2-06-2010
F	8.0	4.9	1-06-2010
G	3.5	3.6	1-06-2010
H	7.0	7.7	1-06-2010
I	7.0	5.0	1-06-2010
J	1.5	10.0	1-06-2010
K	7.0	8.0	1-06-2010
L	1.5	2.2	1-06-2010
M	7.0	19.1	1-06-2010
N	9.5	12.1	3-06-2010
O	7.0	8.4	1-06-2010
P	2.5	4.4	1-06-2010
Q	nt	nt	3-06-2010
EL	2.0	5.0	1-06-2010

4.3 Calculation and summary of data

From the original 17 labs, three were excluded from the calculations. Lab Q, which did not participate in the study due to late arrival of the samples and Labs D and J which did not have countable results for both methods for all samples.

4.3.1 MicroVal Expert laboratory results
The results obtained by the expert laboratory are given in Table 7.

Table 7 – Results obtained by the expert lab (Log_{10} *cfu/g)*

Level	Reference method	Alternative method
Blank	<10	<10
Low	3.16	3.34
Low	3.50	3.82
Medium	4.29	4.37
Medium	4.20	4.74
High	5.77	6.02
High	5.23	5.92

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.

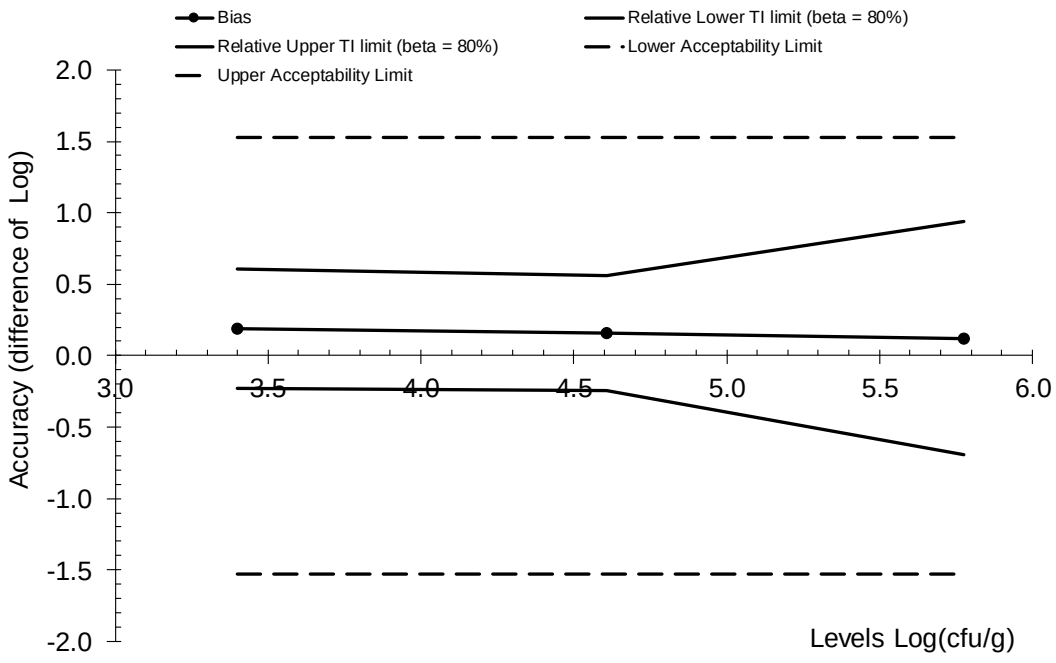
The accuracy profile plot is shown in Figure 7 and the statistical analysis of the data shown in Table 9.

Table 8: Summary of the results of the interlaboratory study(Log_{10} *cfu/g) per analyte level (*Data in grey shaded cells not used)

		Reference method (Log cfu/g)		Alternative method (Log cfu/g)	
Collaborator/level		**Duplicate 1**	**Duplicate 2**	**Duplicate 1**	**Duplicate 2**
A	low	3.65	3.74	3.64	3.86
B	low	3.18	3.19	3.21	3.71
C	low	3.78	3.48	3.75	3.62
D	low	2.5	<2.0	2.86	2.61
E	low	3.53	3.72	3.73	3.59
F	low	3.73	3.76	3.68	3.88
G	low	3.46	3.65	3.78	3.48
H	low	3.56	3.42	3.78	3.64
I	low	2.74	2.66	3.3	2.86
J	low	<2.0	2.50	2.36	2.89
K	low	2.48	3.06	2.88	3
L	low	3.88	3.81	3.88	3.89
M	low	3.6	3.72	3.73	3.9
N	low	2.56	3.21	3.3	3.63
O	low	3.64	3.70	3.84	3.77
P	low	3.62	2.66	3.7	3.42
A	medium	4.75	4.92	4.88	4.85
B	medium	4	4.31	4.49	4.85
C	medium	4.62	4.84	4.66	4.80
D	medium	4.46	4.54	4.62	4.51
E	medium	4.89	4.84	4.92	4.83
F	medium	4.81	4.85	4.94	4.96
G	medium	4.76	4.87	4.82	4.83
H	medium	4.57	4.66	4.79	4.75
I	medium	4.48	4.63	4.97	4.94
J	medium	2.96	3.8	2.66	3.13
K	medium	4.81	3.1	5.04	3.56
L	medium	4.92	4.91	4.91	5
M	medium	4.68	4.24	4.87	4.83
N	medium	4.7	4.65	4.74	4.72
O	medium	4.79	4.61	4.89	4.6
P	medium	4.66	4.15	4.35	4.65
A	high	6.13	6.04	6.14	6.13

Collaborator/level		Reference method (Log cfu/g)		Alternative method (Log cfu/g)	
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
B	high	5.06	5.54	5.15	5.79
C	high	5.75	6.03	6.08	6.07
D	high	5.88	5.92	5.96	5.93
E	high	6.16	6.14	6.15	6.08
F	high	6.13	6.22	6.19	6.23
G	high	6.07	6.16	6.28	6.27
H	high	5.82	5.86	6.15	6.11
I	high	6.26	5.79	6.11	5.71
J	high	5.23	4.3	4.98	4.81
K	high	3.73	4.73	3.36	5.3
L	high	5.98	5.83	6.05	6.26
M	high	6.07	5.71	6.25	6.07
N	high	6.04	6.04	6.22	6.04
O	high	5.92	5.94	6.09	6
P	high	4.88	5.67	5.79	5.02

Figure 7. Accuracy profile of Brilliance™*CampyCount* Agar from the ILS



Standardized report - Quantitative methods
Method Comparison Study and ILS Report
2008LR12 renewal Brilliance™ *CampyCount*
Agar Summary report

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

Accuracy profile	0.5						
Study Name	Campycount Brilliance						
Date	calculated 09/01/2018 from existing data						
Coordinator	Campden BRI						
Tolerance probability (beta)	80%	80%	80%				
Acceptability limit in log (lambda)	1.53	1.53	1.53				
	Alternative method				**Reference method**		
Levels	**Low**	**Medium**	**High**		**Low**	**Medium**	**High**
Target value	**3.400**	**4.608**	**5.775**				
Number of participants (K)	14	14	14		14	14	14
Average for alternative method	3.588	4.766	5.896		3.400	4.608	5.775
Repeatability standard deviation (sr)	0.181	0.301	0.425		0.263	0.360	0.289
Between-labs standard deviation (sL)	0.245	0.000	0.425		0.339	0.120	0.483
Reproducibility standard deviation (sR)	0.304	0.301	0.601		0.429	0.380	0.563
Corrected number of dof	18.376	26.963	20.940		18.791	26.506	16.872
Coverage factor	1.368	1.337	1.358				
Interpolated Student t	1.329	1.314	1.323				
Tolerance interval standard deviation	0.3131	0.3063	0.6169				
Lower TI limit	3.171	4.363	5.080				
Upper TI limit	4.004	5.168	6.712				
Bias	**0.187**	**0.158**	**0.121**				
Relative Lower TI limit (beta = 80%)	**-0.229**	**-0.244**	**-0.695**	TRUE			
Relative Upper TI limit (beta = 80%)	**0.603**	**0.560**	**0.937**	TRUE			
Lower Acceptability Limit	**-1.53**	**-1.53**	**-1.53**				
Upper Acceptability Limit	**1.53**	**1.53**	**1.53**				
New acceptability limits may be based on reference method pooled variance							

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.

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

5 Overall conclusions of the validation study

- The alternative method Brilliance™ CampyCount Agar for enumeration of Thermotolerant *Campylobacter* shows satisfactory results for relative trueness;
- The alternative method Brilliance™ CampyCount Agar for enumeration of Thermotolerant *Campylobacter* shows satisfactory results for accuracy profile;
- The alternative method Brilliance™ CampyCount Agar for enumeration of Thermotolerant *Campylobacter* is selective and specific.
- The alternative method Brilliance™ CampyCount Agar for enumeration of Thermotolerant *Campylobacter* shows satisfactory performance in the ILS

Restriction of use: The method comparison study revealed that it may be that in air-packed chicken thighs Brilliance CampyCount Agar plates give a lower yield than mCCDA plates.

The alternative method Brilliance™ CampyCount Agar for enumeration of Thermotolerant *Campylobacter* in raw and ready to eat poultry shows comparable performance to the reference method.

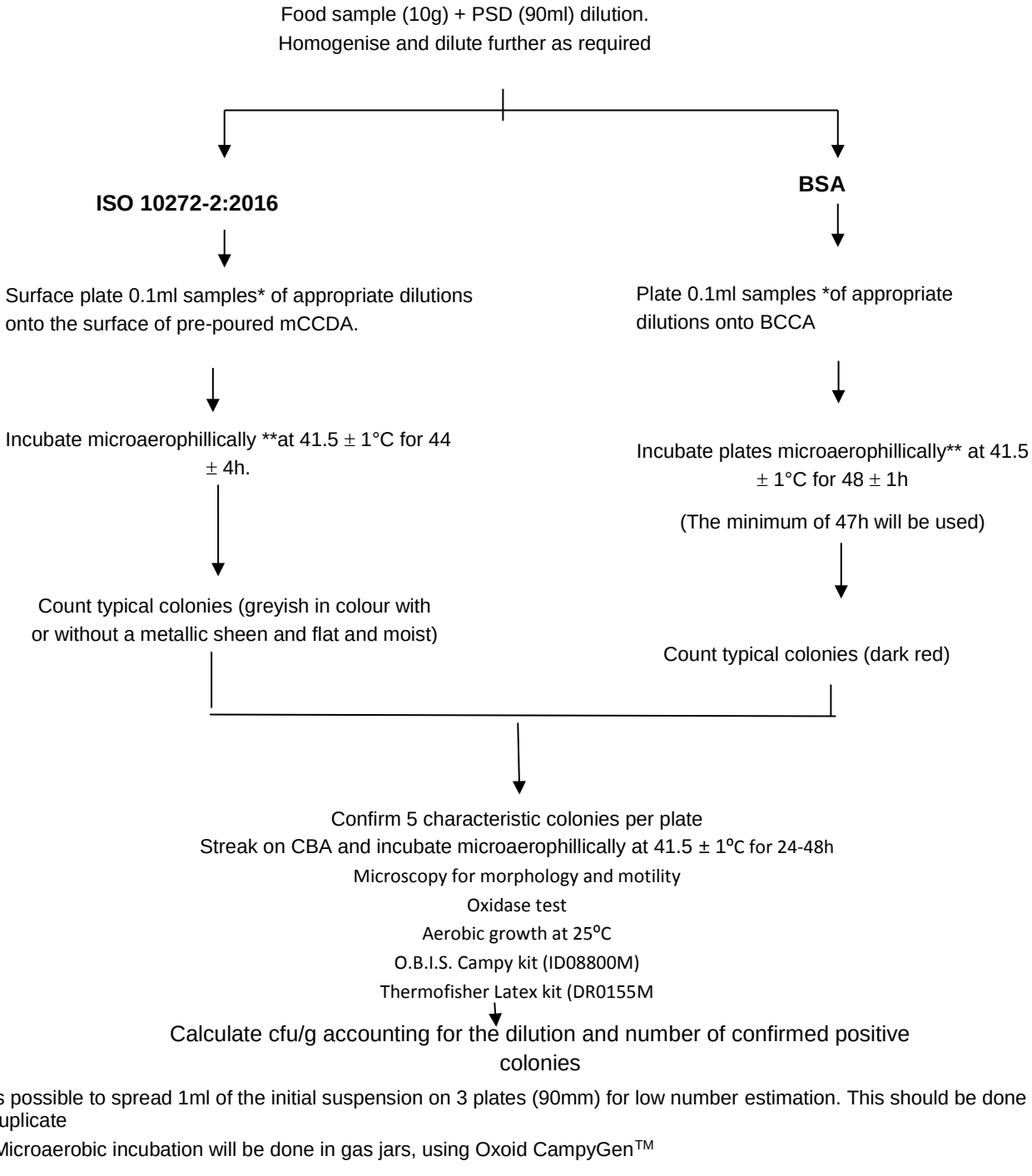
Date 28/03/2019

Signature

Annexes

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

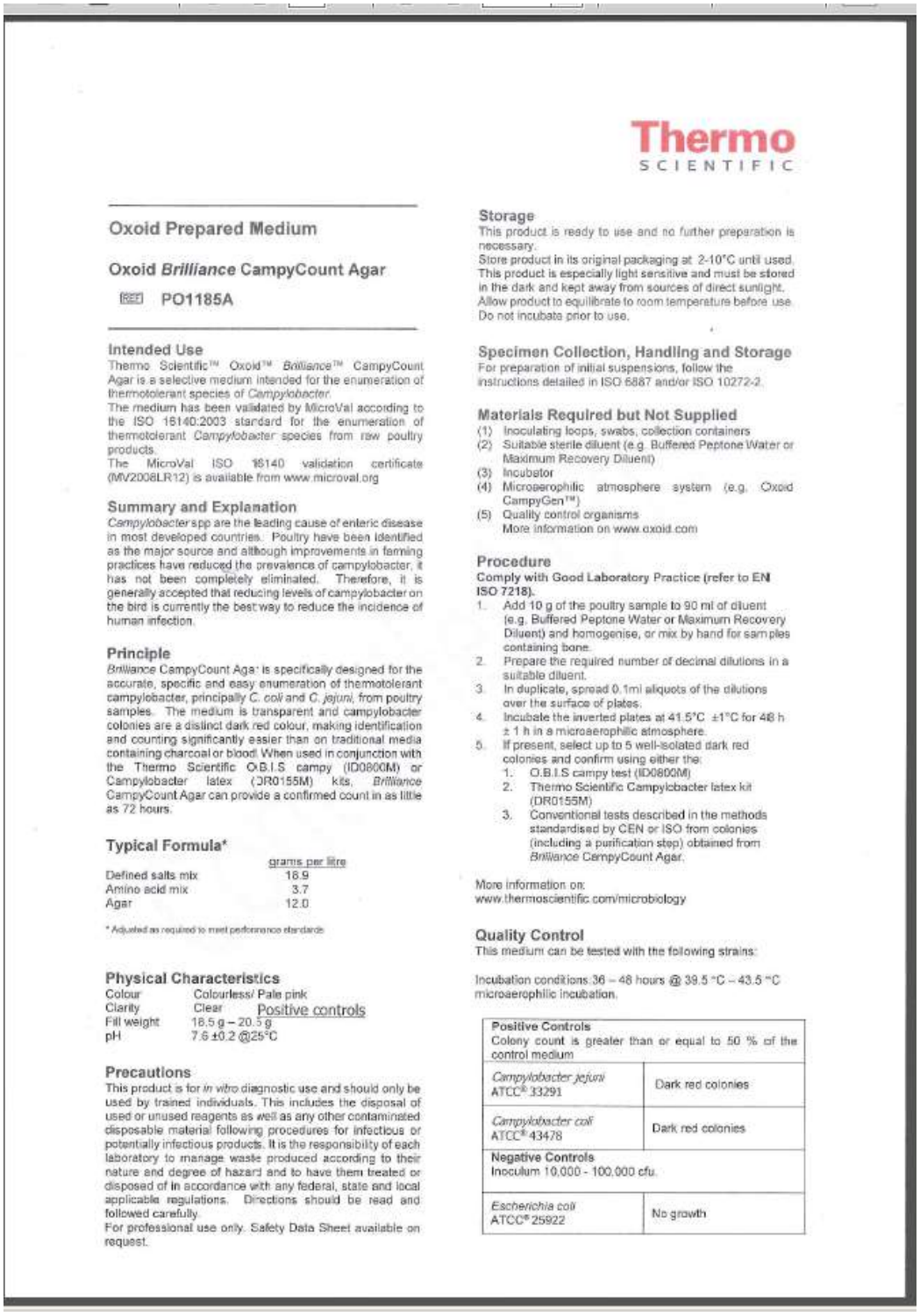
ANNEX A: Flow diagram of the alternative method and reference methods



*It is possible to spread 1ml of the initial suspension on 3 plates (90mm) for low number estimation. This should be done in duplicate

** Microaerobic incubation will be done in gas jars, using Oxoid CampyGen™

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



Thermo SCIENTIFIC

Oxoid Prepared Medium

Oxoid *Brilliance* CampyCount Agar

REF PO1185A

Intended Use
Thermo Scientific™ Oxoid™ *Brilliance*™ CampyCount Agar is a selective medium intended for the enumeration of thermotolerant species of *Campylobacter*.
The medium has been validated by MicroVal according to the ISO 16140:2003 standard for the enumeration of thermotolerant *Campylobacter* species from raw poultry products.
The MicroVal ISO 16140 validation certificate (MV2008LR12) is available from www.microval.org

Summary and Explanation
Campylobacter spp are the leading cause of enteric disease in most developed countries. Poultry have been identified as the major source and although improvements in farming practices have reduced the prevalence of campylobacter, it has not been completely eliminated. Therefore, it is generally accepted that reducing levels of campylobacter on the bird is currently the best way to reduce the incidence of human infection.

Principle
Brilliance CampyCount Agar is specifically designed for the accurate, specific and easy enumeration of thermotolerant campylobacter, principally *C. coli* and *C. jejuni*, from poultry samples. The medium is transparent and campylobacter colonies are a distinct dark red colour, making identification and counting significantly easier than on traditional media containing charcoal or blood. When used in conjunction with the Thermo Scientific O.B.I.S campy (ID0800M) or Campylobacter latex (DR0155M) kits, *Brilliance* CampyCount Agar can provide a confirmed count in as little as 72 hours.

Typical Formula*

	grams per litre
Defined salts mix	16.9
Amino acid mix	3.7
Agar	12.0

* Adjusted as required to meet performance standards

Physical Characteristics

Colour	Colourless/ Pale pink
Clarity	Clear
Fill weight	18.5 g – 20.5 g
pH	7.6 ±0.2 @25°C

Positive controls

Precautions
This product is for *in vitro* diagnostic use and should only be used by trained individuals. This includes the disposal of used or unused reagents as well as any other contaminated disposable material following procedures for infectious or potentially infectious products. It is the responsibility of each laboratory to manage waste produced according to their nature and degree of hazard and to have them treated or disposed of in accordance with any federal, state and local applicable regulations. Directions should be read and followed carefully.
For professional use only. Safety Data Sheet available on request.

Storage
This product is ready to use and no further preparation is necessary.
Store product in its original packaging at 2-10°C until used.
This product is especially light sensitive and must be stored in the dark and kept away from sources of direct sunlight.
Allow product to equilibrate to room temperature before use.
Do not incubate prior to use.

Specimen Collection, Handling and Storage
For preparation of initial suspensions, follow the instructions detailed in ISO 6887 and/or ISO 10272-2.

Materials Required but Not Supplied
(1) Inoculating loops, swabs, collection containers
(2) Suitable sterile diluent (e.g. Buffered Peptone Water or Maximum Recovery Diluent)
(3) Incubator
(4) Microaerophilic atmosphere system (e.g. Oxoid CampyGen™)
(5) Quality control organisms
More information on www.oxoid.com

Procedure
Comply with Good Laboratory Practice (refer to EN ISO 7218).
1. Add 10 g of the poultry sample to 90 ml of diluent (e.g. Buffered Peptone Water or Maximum Recovery Diluent) and homogenise, or mix by hand for samples containing bone.
2. Prepare the required number of decimal dilutions in a suitable diluent.
3. In duplicate, spread 0.1ml aliquots of the dilutions over the surface of plates.
4. Incubate the inverted plates at 41.5°C ±1°C for 48 h ± 1 h in a microaerophilic atmosphere.
5. If present, select up to 5 well-isolated dark red colonies and confirm using either the:
   1. O.B.I.S campy test (ID0800M)
   2. Thermo Scientific Campylobacter latex kit (DR0155M)
   3. Conventional tests described in the methods standardised by CEN or ISO from colonies (including a purification step) obtained from *Brilliance* CampyCount Agar.

More information on:
www.thermoscientific.com/microbiology

Quality Control
This medium can be tested with the following strains:

Incubation conditions:36 – 48 hours @ 39.5 °C – 43.5 °C microaerophilic incubation.

Positive Controls Colony count is greater than or equal to 50 % of the control medium	
Campylobacter jejuni ATCC® 33291	Dark red colonies
Campylobacter coli ATCC® 43478	Dark red colonies
Negative Controls Inoculum 10,000 - 100,000 cfu.	
Escherichia coli ATCC® 25922	No growth

Staphylococcus aureus ATCC® 25923	No growth
Candida albicans ATCC® 10231	No growth

Note:

It is the responsibility of the user to perform Quality Control testing taking into account the intended use of the medium, and in accordance with any local applicable regulations (frequency, number of strains, incubation temperature etc.).

The product should not be used if:
(1) The product is contaminated
(2) The colour has changed
(3) The expiration date has passed
(4) There are other signs of deterioration

Performance

MicroVal ISO 16140 Study

Linearity

Six samples of minced chicken meat and five samples of chicken thighs with skin were artificially contaminated with *C. jejuni* and *C. coli* respectively. Each food type was contaminated within the ranges of: 50 to 2.5x10² CFU/g, 2.5 to 7.5x10² CFU/g, 1x10³ to 2x10³ CFU/g, 2x10³ to 5x10³ CFU/g, 1x10⁴ to 2.5x10⁴ CFU/g, 1x10⁵ to 2.5x10⁵ CFU/g and two samples were analysed by both the ISO reference method (ISO 10272-2) and the *Brilliance* CampyCount Agar method.

Food Category	Food Product	Regression line
Poultry Products	Minced chicken meat/*C. jejuni* strain C145	y=0.165x + 0.971
	Chicken thigh (with skin)/*C. coli* strain C161	y= -0.119x + 1.074

Relative accuracy study

103 poultry products were analysed in duplicate by both the ISO reference method (ISO 10272-2) and the *Brilliance* CampyCount Agar plate.
Valid count data was obtained from 24 naturally contaminated poultry meat samples and 7 artificially contaminated samples.

The naturally contaminated levels ranged between 1.7 to 4.3 $\log_{10}$ CFU/g.

The equations of the regression line between the *Brilliance* CampyCount Agar plate and the ISO reference method for all categories combined and for each of the confirmation methods are:

Confirmatory test	Regression line	r
ISO 10272-2:2006	y=1.05x-0.14	0.99
O.B.I.S Campy	y=1.05x-0.14	0.99
Thermo Scientific Campylobacter latex kit	y=1.05x-0.14	0.99

There was no significant bias between the reference and *Brilliance* CampyCount Agar methods. The relationship of the methods does not deviate significantly from linearity.

Selectivity (inclusivity/exclusivity)

A total of 37 target thermotolerant campylobacter isolates (19 *C. jejuni*, subspp. *jejuni*, 1 *C. jejuni* subspp. *doylei*, 10 *C. coli*, 3 *C. lari*, 3 *C. upsaliensis*, 1 *C. hyointestinalis*,) were tested. All 19 *C. jejuni*, subspp. *jejuni* and all 10 *C. coli* isolates gave typical growth. The *C. jejuni* subspp. *doylei* failed to grow (as expected due to the incubation temperature) on either mCCDA (with the ISO reference method) or *Brilliance* CampyCount Agar. None of the *C. upsaliensis* or *C. hyointestinalis* isolates grew on either mCCDA or *Brilliance* CampyCount Agar. 2 of the 3 *C. lari* isolates grew on *Brilliance* CampyCount Agar.

A total of 21 non-target organisms were analysed. 19 of the 21 exclusivity isolates gave negative results (no growth) on *Brilliance* CampyCount Agar. An isolate of *Acinetobacter baumanii* and an ESBL-producing isolate of *Escherichia coli* grew on both *Brilliance* CampyCount Agar (as red colonies) and mCCDA (as white atypical colonies). Confirmation tests showed that these two isolates were not *Campylobacter* spp.

In conclusion the *Brilliance* CampyCount Agar plate showed comparable results to the use of mCCDA in the reference method detailed in ISO 10272-2:2006.

Limitations

Species of *Acinetobacter* may grow as large dark red colonies on the plate. These are easily distinguished as they appear far larger (>3mm diameter) than *Campylobacter* and are surrounded by a clear mucoid halo.

Packaging

PO1185A	Ten 90mm plates, film wrapped.

Bibliography

1. ISO 6887 Parts 1-5; Microbiology of food and animal feeding stuffs-Preparation of test samples, initial suspension and decimal dilutions for microbiological examination.
2. MicroVal ISO 16140 validation study report-please contact our Technical Support Team by email: microbiology.techsupport.uk@thermofisher.com or telephone: +44 (0)1256 694238.

Symbol Legend

Symbol	Meaning
	Catalogue number
	In Vitro Diagnostic Medical Device
	Manufacturer
	Temperature limitation (storage temp.)

Thermo SCIENTIFIC
A Thermo Fisher Scientific Brand