Method Comparison Study Report for the ISO 16140-2:2016 validation of Brilliance™ *Staph 24* Agar for the enumeration of coagulase positive *Staphylococci* species in a broad range of foods

MicroVal study number: 2008LR11 renewal

Method/Kit name: Brilliance™ *Staph 24* Agar

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

Standardized report - Quantitative methods - Method Comparison Study and ILS 2008LR11 renewal Brilliance™ *Staph 24* 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
Wade Road,
Basingstoke,
Hampshire,
UK

Expert Laboratory: Campden BRI

Method/Kit name: Brilliance™ *Staph 24* 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 6888-1:1999 Incorporating Ammendment No 1 and corrigendum No 1. Microbiology of food and animal feeding stuffs- Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) —Part 1: Technique using Baird-Parker agar medium

ISO 6888-1:1999 DAM2:2017(E). Microbiology of food and animal feeding stuffs- Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) —Part 1: Technique using Baird-Parker agar medium. AMENDMENT 2 Inclusion of an alternative confirmation procedure

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

1. Dairy products
2. Fishery products,
3. Chocolate, bakery and confectionery products,
4. Meat and meat products
5. Multi-component foods

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
MVTC	MicroVal Technical Committee
EL	Expert Laboratory
n	number of samples
na	not applicable
neg	negative (target not detected)
NG	no growth
nt	not tested
RT	Relative Trueness
SD	standard deviation of differences
10^{-1} dilution	10-fold dilution of original food
10^{-2} dilution	100-fold dilution of original food
PSD	Peptone salt diluent
BSA	Brilliance™ Staph 24 Agar
BPA	Baird parker Agar
RPFA	Rabbit plasma fibrinogen Agar
BHIB	Brain Heart Infusion Broth

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Contents

1 Introduction

In this project a MicroVal validation study, based on ISO 16140-2:2016, of alternative method(s) for the enumeration of coagulase positive *Staphylococci* species in five different food categories was carried out by Campden BRI as the MicroVal Expert Laboratory.

This is a renewal of a method that was originally validated according to the superseded ISO16140:2003 standard for enumeration of coagulase-positive staphylococci in all foods. The original study was done by University of Ghent.

Five levels of contamination were used for the 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 can be partially used for the RT part of the renewal study as it covers two of the required food types within each category. Additional RT data was needed for a third type of food matrix within each 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 and therefore new data was collected for this part (Table 4).

There is insufficient Incl/Excl data available from the original study to cover the requirements of ISO16140-2:2016 so some additional strains were tested in this renewal study. There were 36 inclusivity cultures tested so a further 14 were needed and there were 28 exclusivity cultures so a minimum of 2 more were required

The alternative method used was:

- Brilliance™ *Staph 24* Agar for Enumeration of coagulase positive *Staphylococci* species following incubation at 37±1°C for 24±2h

The reference method used was:

- ISO 6888-1:1999 Microbiology of food and animal feeding stuffs- Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species) —Part 1: Technique using Baird-Parker agar
- ISO 6888-1:1999 DAM2:2017(E). Microbiology of food and animal feeding stuffs- Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) —Part 1: Technique using Baird-Parker agar medium. AMENDMENT 2 Inclusion of an alternative confirmation procedure

Categories included:

- Dairy products
- Fishery products,
- Chocolate, bakery and confectionery products,
- Meat and meat products
- Multi-component foods

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™ *Staph 24* Agar shows comparable performance to the reference methods (ISO 6888-1:1999, ISO 6888-1:1999 DAM2:2017(E) for the enumeration of coagulase positive *Staphylococci* species in a broad range of foods.

2 Method protocols

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

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

2.1 Reference method

See the flow diagram in Annex A.

Sample preparations used in the reference method were done according to ISO 6887-series parts 1, 2, 3, 4 and 5.

2.2 Alternative method

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

See the Brilliance™ *Staph 24* Agar kit insert in Annex B.

The alternative method principle is based on chromogenic media:

Brilliance™ *Staph 24* Agar: *is a spread plate method intended to enumerate coagulase-positive Staphylococci. The agar is a transparent medium which is a highly selective and diagnostic chromogenic medium. Target organisms grow as dark blue colonies on a clear background. A picture is provided in Figure 1.*

Figure 1: BSA

2.3 Study design

Samples of product containing the target organism were diluted 1 in 10 with an appropriate diluent according to ISO 6887 and homogenised in a stomacher.

Appropriate serial dilutions were made, and all relevant dilutions were analysed using the reference method and alternative method.

3 Method comparison study

3.1 Relative trueness study

The trueness study is a comparative study between the results obtained by the reference method and the results of the alternative method. This study was conducted using naturally or artificially contaminated samples. Different categories, types and items were tested for this.

A total of 5 categories were included in this validation study. A minimum of 15 items for each category were tested by both the reference method and the alternative method in the relative trueness study, with a minimum of 15 interpretable results per category.

Each category was made up of 3 types, with at least 5 items representative for each type.

3.1.1 Number of samples

The categories, the types and the number of samples analyzed are presented in Table 1.

Table 1 – Categories, types and number of samples analyzed

Category	Types		Number of interpretable samples			
			Old study		Renewal study	Total
			Artificially inoculated	Naturally contaminated		
Dairy products (combined category ; raw milk and heat processed)	a	Dry milk powders	10	0	4	14
	b	Raw milk and raw milk cheeses	10	6	0	16
	c	Dairy desserts e.g. chilled custard, trifle	0	0	5	5
		Total	**20**	**6**	**9**	**35**
RTE/RTRH Fishery products	a	Raw processed fish e.g. smoked mackerel	10	0	4	14
	b	RTE cooked fish i.e. prawns	10	0	4	14
	c	Frozen RTE / RTRH products	0	0	5	5
		Total	**20**	**0**	**13**	**33**
Chocolate, bakery products and confectionery	a	Confectionery: Cream patisserie, Chocolate mousse	10 10	0 0	4	24
	b	Dry powders e.g. cake mixes	0	0	5	5
	c	Low moisture products e.g. cakes, cookies, crackers	0	0	5	5
		Total	**20**	**0**	**14**	**34**
Meat (combined category; raw/RTC products and RTE/RTRH products)	a	Cured processed meat i.e. bacon	10	1	3	14
	b	Raw unprocessed meat i.e. mince	10	1	3	14
	c	RTE meats e.g. hams, pate	0	0	10	10
		Total	**20**	**2**	**16**	**38**
Multi component foods	a	RTE Deli salads with Mayonnaise	10	0	4	14
	b	RTRH chilled foods e.g. Pre-packed pancakes	10	0	4	14
	c	Composite processed meals	0	1	4	5
		Total	**20**	**1**	**12**	**33**
TOTAL DATA POINTS			**100**	**9**	**64**	**173**

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3.1.2 Test sample preparation

It is preferable to test naturally contaminated samples. In order to attempt to use naturally contaminated samples, all fifteen samples from each category were first tested for the presence of naturally occuring target organism making a total of seventy five samples which were tested. In the original study there were 13 naturally positive samples for the RT study.

For the renewal study a further 71 samples were anlaysed for the RT. All these samples were screened for the presence of naturally occuring coagulase-positive staphylococci. However none of the sample were positive and so artificial contamination was needed. In order to ensure as wide a range of conditions were tested as possible, 15 different strains were used from 15 different food items and were either chilled frozen, heated or lyophilised.

All samples which were negative for natural contamiantion had <10cfu/g coagulase-positive staphylococci on both the reference method and alternative method.

Artificial contaminations were obtained by:

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

In the renewal study, the same strain was not used to inoculate more than 5 samples.

3.1.3 Protocols applied during the validation study

A single protocol was applied for the study.

Reference method plates were incubated at 37±1°C for 24±2h plus a further 24±2h.

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

In all cases the minimum incubation times were used.

<u>Alternative method confirmation</u>

Five typical colonies from each plate used in the calculations were confirmed using coagulase tests. Either ISO 6888-1:1999: BHI and rabbit plasma or ISO 6888-1:1999 DAM2:2017(E) - RPF Agar

3.1.4 Test results

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

3.1.5 Calculation and interpretation of relative trueness study

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

Figures 2 to 7 shows the scatter plots for the individual categories and all categories.

Figure 2 - Scatter plot of the reference method versus alternative method results for Milk and dairy products

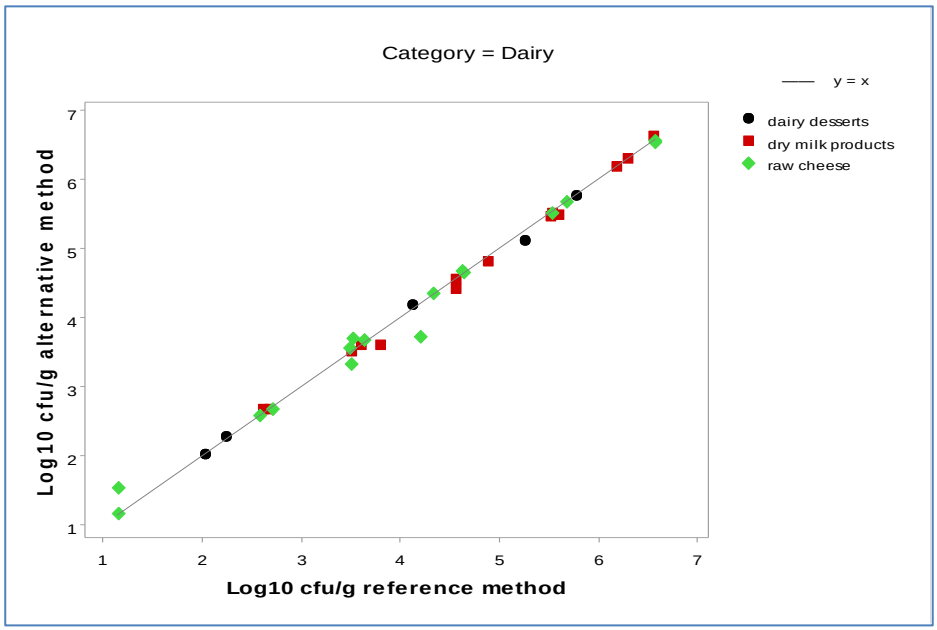


Figure 3- Scatter plot of the reference method versus alternative method results for Chocolate and bakery

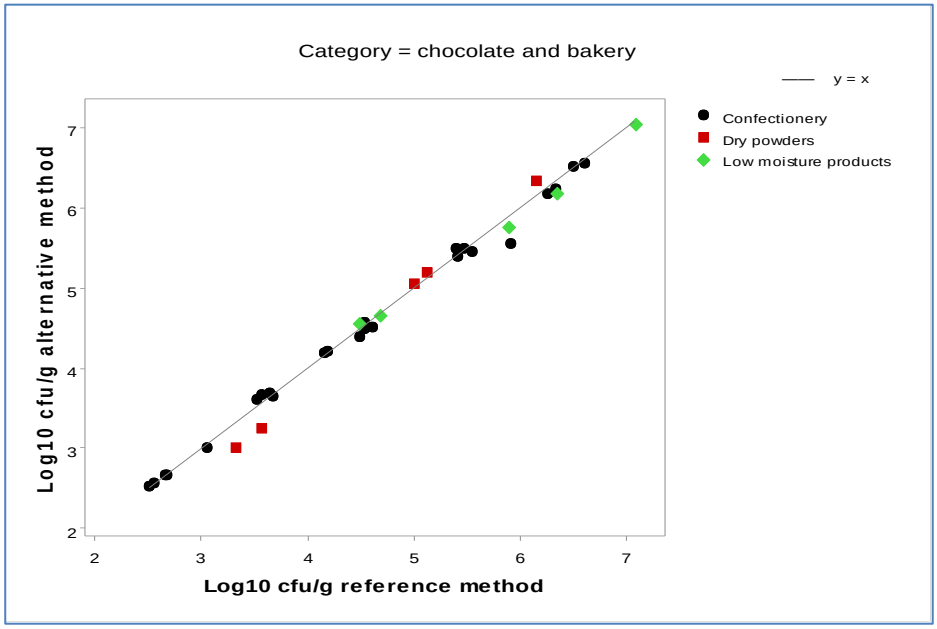


Figure 4- Scatter plot of the reference method versus alternative method results for Meat (combined category; raw/RTC products and RTE/RTRH products)

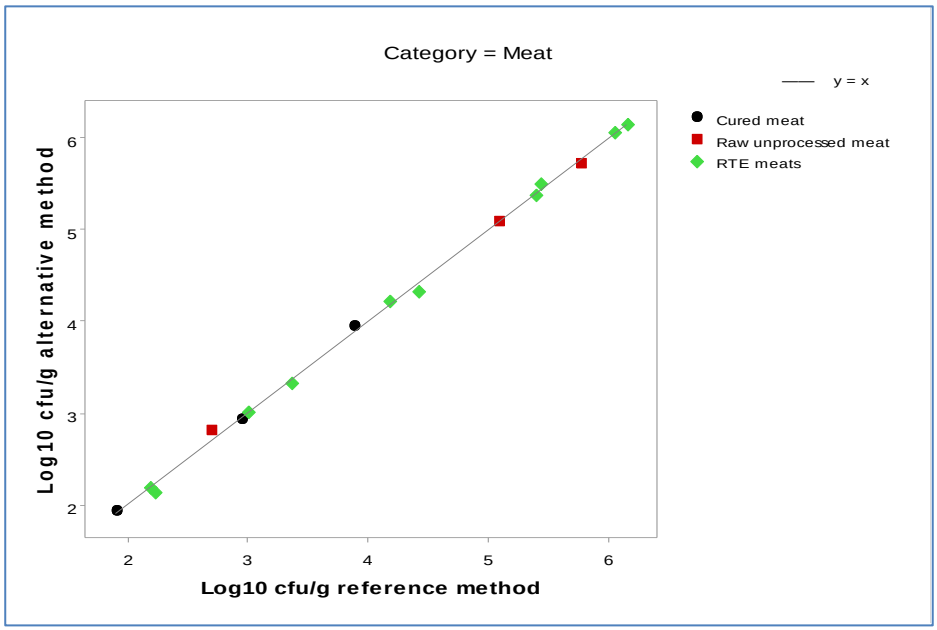


Figure 5- Scatter plot of the reference method versus alternative method results for Ready to eat fish

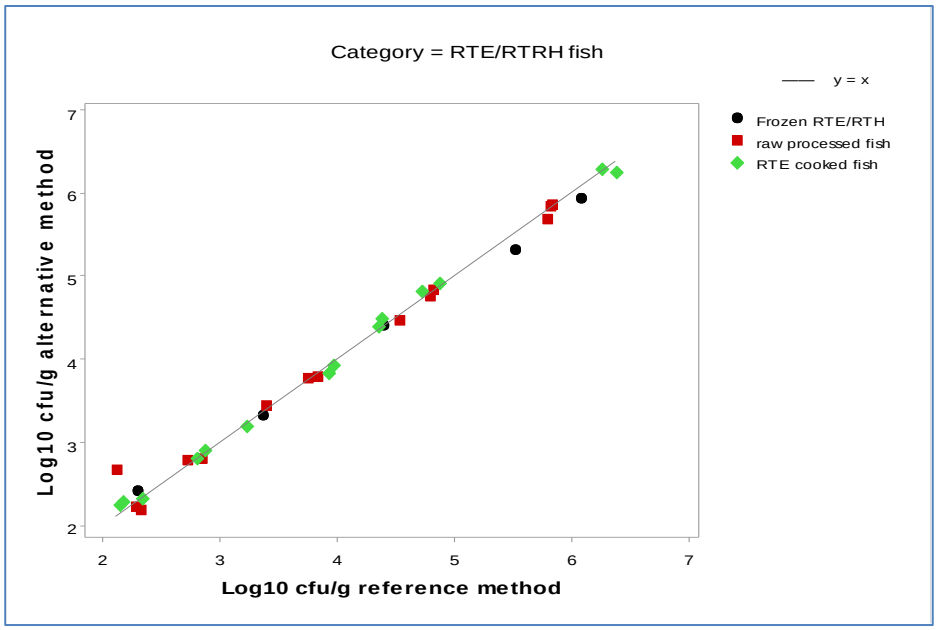


Figure 6- Scatter plot of the reference method versus alternative method results for Multi component foods

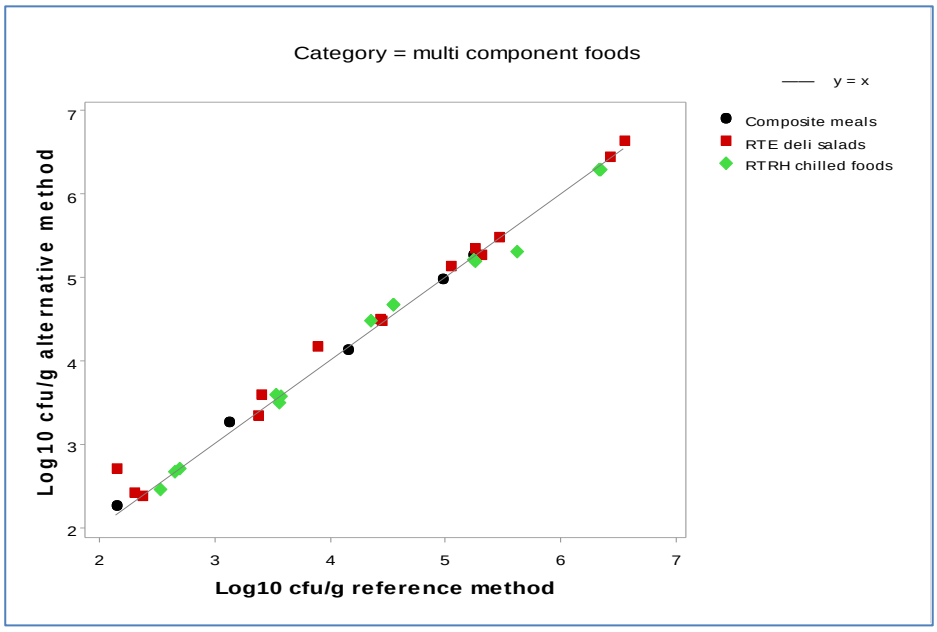
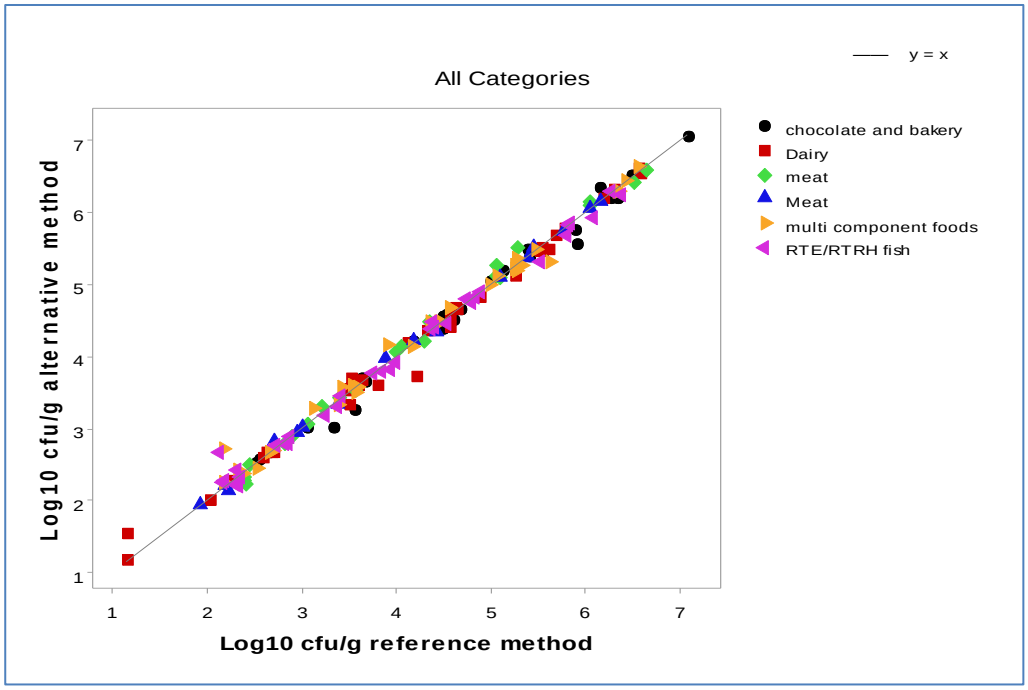


Figure 7 - Scatter plot of the reference method versus alternative method results for all categories



According to ISO 16140-2:2016 6.1.2.3 the results of the scatter plot are interpreted based on a visual observation on the amount of bias and extreme results.

There 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

Table 2- Summary of the calculated values per category –combined renewal and original data

Category.	n	$\overline{D}$	S_D	95% Lower limit	95% Upper limit
chocolate and bakery	34	-0.038	0.119	-0.283	0.208
Dairy	35	-0.025	0.128	-0.289	0.239
Meat	38	0.011	0.085	-0.164	0.187
multi component foods	35	0.039	0.134	-0.239	0.317
RTE/RTRH fish	33	-0.012	0.127	-0.274	0.250
All Categories	173	-0.005	0.121	-0.244	0.234

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

Figure 8 – Bland-Altman difference plot for all the samples

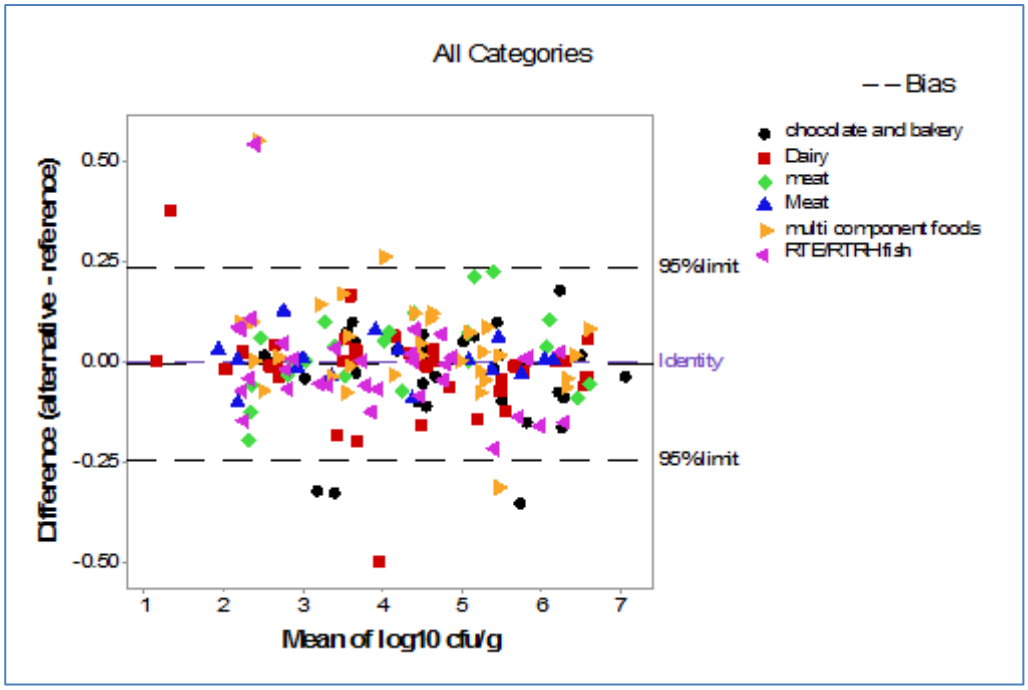


Table 3 - Data which are outside of the accepted limits

Category	Types	Code	Food item	strain	Spiking/ seeding	Log (Ref)	Log (Alt)	Mean	Difference
RTE/RTRH fish	raw processed fish	10	smoked mackerel	*S.aureus* 1208	chill 2-3 days	2.114	2.653	2.384	0.539
chocolate and bakery	Dry powders	30	sponge mix	*S.aureus* ATCC 29213	Lyophilised cells	3.556	3.230	3.393	-0.326
chocolate and bakery	Confection ery	23	cream cake	*S.aureus* 1215	chill 2-3 days	5.898	5.544	5.721	-0.354
chocolate and bakery	Dry powders	29	muffin mix	*S.aureus* ATCC 29213	Lyophilised cells	3.322	3.000	3.161	-0.322
multi component foods	RTE deli salads	48	chargrilled sweetcorn coleslaw	*S.aureus* 3098	chill 2-3 days	3.886	4.146	4.016	0.260
multi component foods	RTE deli salads	47	salad with sweetcorn	*S.aureus* 3098	chill 2-3 days	2.146	2.695	2.420	0.548
multi component foods	RTRH chilled foods	54	southern fried chicken wings	*S.aureus* 1994	chill 2-3 days	5.613	5.301	5.457	-0.312
Dairy	raw cheese	170	Raw milk	Natural strain	chill 2-3 days	1.146	1.519	1.332	0.372
Dairy	raw cheese	174	Raw milk cheese	Natural strain	chill 2-3 days	4.204	3.708	3.956	-0.497

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

In this study there were 9 data points from a total of 173 data points which were outside of the accepted limits. This meets the expectation. The data covered 4 different food categories, 5 different *Staphylococcus aureus* strains, and naturally present coagulase positive staphylococci. The differences between the results of the compared methods are in most of the case below the absolute value of 0,5 Log CFU/g, and only two are slightly above.

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. It possible to use either 2 separate batches of a single item for each food type. Or it is possible to use a single batch of 2 different items for each food type.

In this study five food categories were tested with a single batch of two different food types using 6 samples per type. Two samples were contaminated at a low level, 2 at intermediate level, 2 at a high level. For each sample, 5 replicates (5 different test portions) were tested. A total of 30 samples were analysed per food type. 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
Dairy products (combined category; raw milk and heat processed)	Dairy desserts	*S. aureus* CRA 1215 from cheese	Chilled custard	Level 1x5: 100-250 cfu /g
				Level 2x5: 1000- 5000 cfu/g
				Level 3x5 10,0000- 50,0000 cfu/g
			Whipped cream	Level 1x5: 100-250 cfu /g
				Level 2x5: 1000- 5000 cfu/g
				Level 3x5 10,0000- 50,0000 cfu/g
RTE/RTRH Fishery products	RTE fish products	*S.aureus* CRA 1208 from smoked fish	Smoked salmon	Level 1x5: 100-250 cfu /g
				Level 2x5: 1000- 5000 cfu/g
				Level 3x5 10,0000- 50,0000 cfu/g
			Tuna pate	Level 1x5: 100-250 cfu /g
				Level 2x5: 1000- 5000 cfu/g
				Level 3x5 10,0000- 50,0000 cfu/g
Chocolate, bakery products and confectionery	Pastries	*S.aureus* CRA 2078 Milk powder	Chilled patisserie	Level 1x5: 100-250 cfu /g
				Level 2x5: 1000- 5000 cfu/g
				Level 3x5 10,0000- 50,0000 cfu/g
			Chocolate filled pain au chocolate	Level 1x5: 100-250 cfu /g
				Level 2x5: 1000- 5000 cfu/g
				Level 3x5 10,0000- 50,0000 cfu/g
Meat (combined category; raw/RTC products and RTE/RTRH products)	RTE meats	*S.aureus* CRA 1219 beef	Sliced ham	Level 1x5: 100-250 cfu /g
				Level 2x5: 1000- 5000 cfu/g
				Level 3x5 10,0000- 50,0000 cfu/g
			Pastrami	Level 1x5: 100-250 cfu /g
				Level 2x5: 1000- 5000 cfu/g
				Level 3x5 10,0000- 50,0000 cfu/g
Multi component foods	Composite foods with raw /processed ingredients	*S.aureus* CRA 5932 from pasta	Pasta salad	Level 1x5: 100-250 cfu /g
				Level 2x5: 1000- 5000 cfu/g
				Level 3x5 10,0000- 50,0000 cfu/g
			Sandwich spread	Level 1x5: 100-250 cfu /g
				Level 2x5: 1000- 5000 cfu/g
				Level 3x5 10,0000- 50,0000 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= 150

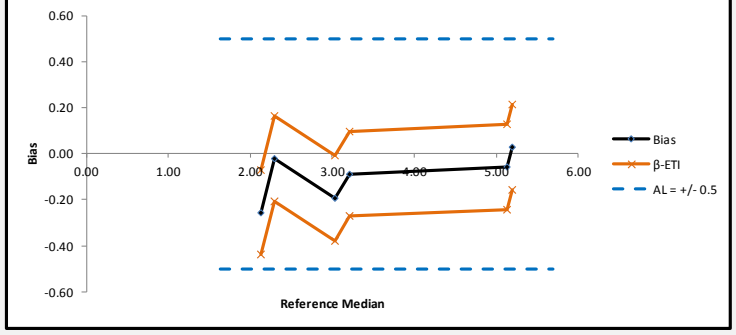
3.2.2 Calculations and interpretation of accuracy profile study

The statistical results and the accuracy profiles are provided in Figures 9 to 13.

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 9 Accuracy profile for Category : Milk and dairy products (types custard and cream)

(Food) Category	Dairy
(Food) Type	custard and whiping cream

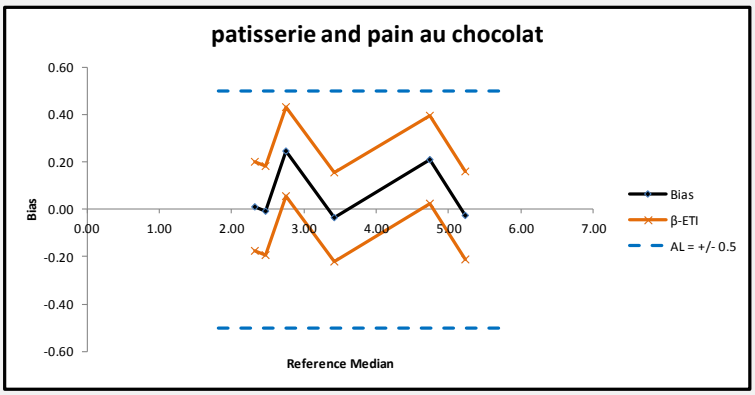


Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
1a-e	2.13	-0.255	-0.440	-0.070	YES	YES
4a-e	2.30	-0.022	-0.207	0.163	YES	YES
2a-e	3.03	-0.193	-0.377	-0.008	YES	YES
5a-e	3.21	-0.088	-0.273	0.097	YES	YES
3a-e	5.13	-0.058	-0.243	0.126	YES	YES
6a-e	5.19	0.027	-0.158	0.212	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.109	0.128	YES	+/- 0.500

Figure 9 Accuracy profile for Category : Chocolate and bakery(types patisserie)

(Food) Category	bakery
(Food) Type	patisserie and pain au chocolat

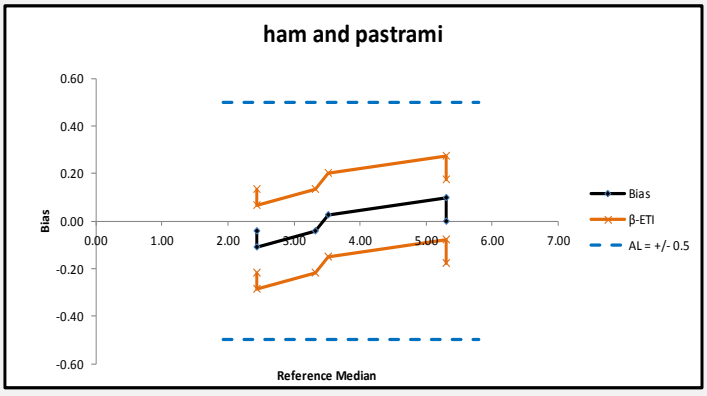


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
13 a-e	2.32	0.010	-0.177	0.198	YES	YES
16 a-e	2.47	-0.007	-0.195	0.180	YES	YES
17a-e	2.76	0.244	0.056	0.432	YES	YES
14a-e	3.41	-0.035	-0.222	0.153	YES	YES
18a-e	4.74	0.209	0.021	0.397	YES	YES
15a-e	5.23	-0.026	-0.214	0.161	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.105	0.130	YES	+/- 0.500

Figure 10 Accuracy profile for Category : Raw poultry and meats (types RTE meats)

(Food) Category	RTE meat
(Food) Type	ham and pastrami

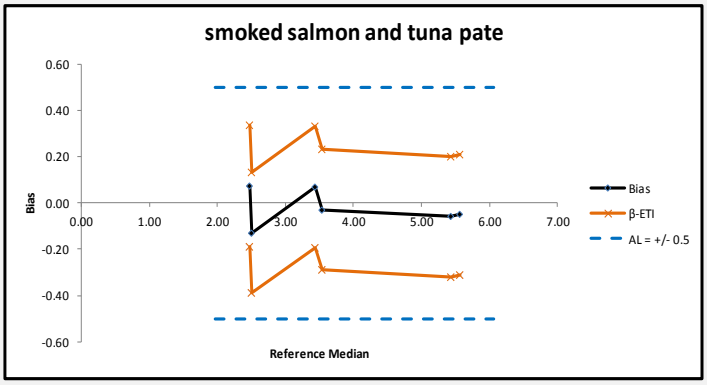


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
19 a-e	2.43	-0.042	-0.218	0.134	YES	YES
22 a-e	2.43	-0.109	-0.285	0.067	YES	YES
23 a-e	3.32	-0.043	-0.220	0.133	YES	YES
20 a-e	3.52	0.026	-0.151	0.202	YES	YES
21 a-e	5.30	0.097	-0.079	0.273	YES	YES
24 a-e	5.30	0.000	-0.176	0.176	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.115	0.122	YES	+/- 0.500

Figure 11 Accuracy profile for Category : Ready to eat fish (types RTE fish)

(Food) Category	RTE fishery products
(Food) Type	smoked salmon and tuna pate

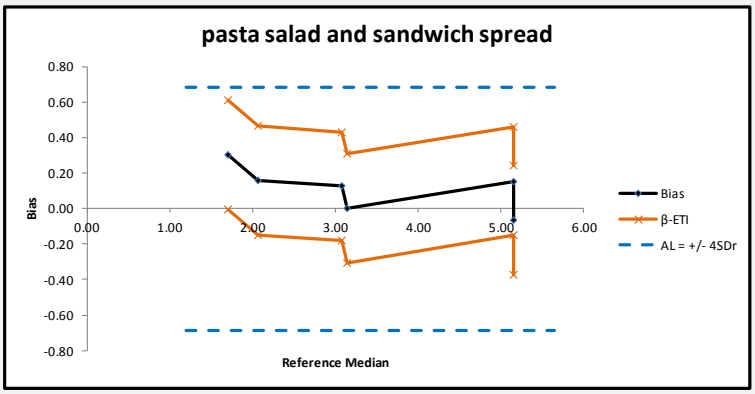


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
10a-e	2.47	0.074	-0.187	0.336	YES	YES
7a-e	2.51	-0.129	-0.390	0.132	YES	YES
11a-e	3.44	0.069	-0.192	0.331	YES	YES
8a-e	3.54	-0.029	-0.290	0.232	YES	YES
9a-e	5.44	-0.059	-0.320	0.202	YES	YES
12a-e	5.57	-0.050	-0.311	0.212	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.096	0.181	YES	+/- 0.500

Figure 12 Accuracy profile for Category : Multi component foods (types products with mayonaise)

(Food) Category	Multi component foods
(Food) Type	pasta salad and sandwich



Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
25 a-e	1.70	0.301	-0.006	0.609	NO	YES
28 a-e	2.06	0.157	-0.151	0.464	YES	YES
26 a-e	3.08	0.125	-0.183	0.432	YES	YES
29 a-e	3.15	0.000	-0.307	0.307	YES	YES
27 a-e	5.15	0.155	-0.153	0.462	YES	YES
30 a-e	5.15	-0.067	-0.374	0.241	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.171	0.213	NO	+/- 0.684

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

In this study all four categories met the AL of 0.5log. These were; Dairy products; Fishery products; Chocolate, bakery and confectionery products; Meat and meat products.

For Multi-component foods, the standard deviation of the reference method was >0.125 and one out of 12 limits was exceeded. This was the upper CL for the low level pasta salad.

The additional calculations were carried out and the reference method met the newly calculated AL of ± 0.684.

The accuracy of the Alternative method is satisfied as the all categories met the 0.5log AL or in the case of Multi-component foods, 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.

In this study, the target organism is coagulase positive Staphylococci which consists of three species:- *Staphylococcus aureus*, - *Staphylococcus hyicus*, - *Staphylococcus intermedius*.

3.3.1 Protocols

- Inclusivity
  A minimum of fifty strains of coagulase positive staphylococci needed to be tested to meet the requirements of ISO16140-2:2016.
  Thirty four strains of coagulase positive staphylococci were evaluated in the original study.
  In addition, a further seventeen cultures were tested here making a total of fifty one strains tested.
  In this renewal study each strain was grown in Nutrient Broth in at 30±1°C for 18-24h and appropriate dilutions were made for testing. Each strain was tested once with the alternative method, the reference method and a non-selective agar.

- Exclusivity
  A minimum of thirty strains *of* non target organisms needed to be tested to meet the requirements of ISO16140-2:2016. Twenty eight strains of non-coagulase positive staphylococci were evaluated in the original study.

  In addition, a further five cultures were tested here making a total of thirty three strains tested. All strains were grown in appropriate selective broths and incubation conditions and appropriate dilutions were made for testing. Each strain was tested once with the alternative method, the reference method and a non-selective agar.

3.3.2 Results

- Inclusivity

Of the 51 inclusivity strains tested, 48 were detected by the reference method and the alternative method. One strain (*Staph. intermedius* code 407) was detected by the alternative method but not the reference method. And two strains (*Staph. hyicus* code 285 , *Staph.hyicus* code 406) were not detected by either method.

- Exclusivity

Of the 35 exclusivity strains tested, 2 were detected by the reference method and the alternative method. (*Staph.caprae* code 21 from Goats Milk; *Staph epidermidis* code 402 from Goats Milk).

A further three strains were detected by the reference method only (*Staph. carnosus*, CRA 284 from Fermented sausage; *Staph saprophyticus*, CRA 3191 from dry sausage; *Staph.warneri* CRA 262 from Salami) but these colonies were not confirmed and therefore would not be detected as coagulase positive Staphylococci

3.3.3 Conclusion
The alternative method Brilliance™ *Staph 24* Agar for enumeration of coagulase positive *Staphylococci* species in foods was shown to be specific and selective and give comparable performance to the reference method.

3.4 Limit of quantification (LOQ)

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

3.5 Conclusion (MCS)

Overall, the conclusions for the Method Comparison are:

- The alternative method Brilliance™ *Staph 24* Agar for enumeration of coagulase positive *Staphylococci* species shows satisfactory results for relative trueness;
- The alternative method Brilliance™ *Staph 24* Agar for enumeration of coagulase positive *Staphylococci* species shows satisfactory results for accuracy profile;
- The alternative method Brilliance™ *Staph 24* Agar for enumeration of coagulase positive *Staphylococci* species is selective and specific.

4 Interlaboratory study

The inter-laboratory study is a study performed by multiple laboratories testing identical samples at the same time, the results of which are used to estimate alternative-method performance parameters.

4.1 Study organisation

4.1.1 Collaborators
Samples were sent to 11 laboratories in four different countries.

4.1.2 Matrix and strain used

Pasteurised milk samples were inoculated with *Staph. aureus* LFMFP-UGent N°532 isolated from raw milk cheese. Samples were individually inoculated with the relevant dilution of the *Staph. aureus* strain.

4.1.3 Sample preparation

Samples were prepared and inoculated and despatched as described below:

Each laboratory received eight samples of 25 g, i.e. two samples per inoculation level L1, L2, and L3 and 2 samples of the blank L0. All samples were blind-coded.

The target contamination levels were:

Level 0 < 10 cfu/g (Blank)
Level 1: 500 cfu/ml
Level 2: 5000 cfu/ml
Level 3: 50,000cfu/ml

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

Before dispatch, each set of samples were packed into isothermal boxes, containing cooling blocks, and express-shipped to the different laboratories. It was aimed to have the samples delivered within 24-48h.

Also, an additional vial containing a temperature monitoring sensor was packed with each set of samples. These were sent back to the expert lab to provide a profile of the temperature during transportation and receipt at the collaborators.

Upon receipt, each collaborating laboratory tested each sample according to the reference method and alternative method following the instruction sheets which were sent prior to the start of the study. 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

Collaborative study laboratories and the expert laboratory carried out the analyses on Wednesday October 7th, 2009 with the alternative and reference methods. The analyses by the reference method and the alternative method were performed on the same day.

4.2 Experimental parameter controls

4.2.1 Detection of coagulase positive staphylococci in the matrix before inoculation
In order to ensure the absence of coagulase positive staphylococci in the food matrix, the reference method was performed on five portions (25 g) before the inoculation. All the results were negative.

4.2.2 Strain stability during transport
Duplicate 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 coagulase positive staphylococci (Log_{10} cfu/g) in stability samples stored at 4°C

Level and time	Reference: BPA			Alternative: BSA		
	0h	24h	48h	0h	24h	48h
low a	2.51	2.68	2.56	2.56	2.68	2.64
low b	2.54	2.66	2.61	2.56	2.72	2.66
medium a	3.62	3.48	3.53	3.73	3.47	3.61
medium b	3.58	3.53	3.51	3.53	3.51	3.69
high a	4.48	4.50	4.51	4.48	4.50	4.53
high b	4.45	4.43	4.54	4.53	4.42	4.36

The data showed that the levels of coagulase positive staphylococci were not affected by the storage conditions and were stable during chill storage with no increase after 48h at 4°C.

4.2.3 Logistic conditions
The temperatures measured at receipt by the collaborators, the temperatures registered by the thermo-probe, and the receipt dates are given in Table 6.

Table 6 - Sample temperatures at receipt

Organising laboratory	Temperature measured at receipt (°C)	Receipt date and time	Analysis date
1	5.0°C	Day 0 11.10	7th October 2009
2	3.5°C	Day 0 10.30	7th October 2009
3	7.3°C	Day 0 12.00	7th October 2009
4	3.8°C	Day 1 12.15	7th October 2009
5	4.9°C	Day 0 09.45	7th October 2009
6	6.6°C	Day 1 11.00	7th October 2009
7	9.5°C	Day 0 13.00	7th October 2009
8	Forgotten to measure	Day 0 11.15	7th October 2009
9	5.0°C	Day 1 11.15	7th October 2009
10	1.6°C	Day 0 11.00	7th October 2009
11	3.4°C	Day 1 11.00	7th October 2009
EL	5.0°C	Day 0 08.00	7th October 2009

No problems were encountered during the transport or at receipt for the 11 collaborators.

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

Temperatures during shipment and at receipt were all correct. The temperature reading at receipt from the water sample was <8°C except for laboratory 7 which had a measured temperature of 9.5ºC

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

Level	Reference method	Alternative method
Blank	<10	<10
Low	2.67	2.68
Low	2.66	2.72
Medium	3.48	3.48
Medium	3.53	3.52
High	4.51	4.51
High	4.43	4.41

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 13 and the statistical analysis of the data shown in Table 9.

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

		Reference method (Log cfu/g)		Alternative method (Log cfu/g)	
Collaborator/level		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
1	low	2.60	2.57	2.53	2.62
2	low	2.80	2.81	2.84	2.83
3	low	2.85	2.85	2.85	2.83
4	low	2.72	2.74	2.76	2.72
5	low	2.52	2.58	2.60	2.52
6	low	2.92	2.82	2.76	2.86
7	low	2.65	2.41	2.65	2.48
8	low	2.40	2.43	2.51	2.49
9	low	2.45	2.48	2.45	2.48
10	low	2.54	2.51	2.59	2.57
11	low	2.60	2.72	2.70	2.59
1	medium	3.60	3.81	3.72	3.86
2	medium	3.91	3.72	3.63	3.73
3	medium	3.80	3.85	3.85	3.90

4	medium	3.80	3.89	3.83	3.81
5	medium	3.52	3.51	3.54	3.40
6	medium	3.79	3.84	3.81	3.81
7	medium	3.59	3.69	3.64	3.53
8	medium	3.45	3.41	3.45	3.51
9	medium	3.32	3.49	3.48	3.45
10	medium	3.62	3.45	3.51	3.49
11	medium	3.49	3.45	3.56	3.52
1	high	4.56	4.86	4.68	4.88
2	high	4.95	4.65	4.91	4.72
3	high	4.88	4.70	4.81	4.75
4	high	4.90	4.85	4.91	4.88
5	high	4.56	4.54	4.58	4.45
6	high	4.82	4.95	4.85	4.93
7	high	4.66	4.69	4.69	4.54
8	high	4.56	4.46	4.58	4.40
9	high	4.36	4.48	4.51	4.41
10	high	4.71	4.72	4.72	4.69
11	high	4.53	4.59	4.48	4.46

Figure 14. Accuracy profile of Brilliance™ *Staph 24* Agar from the ILS

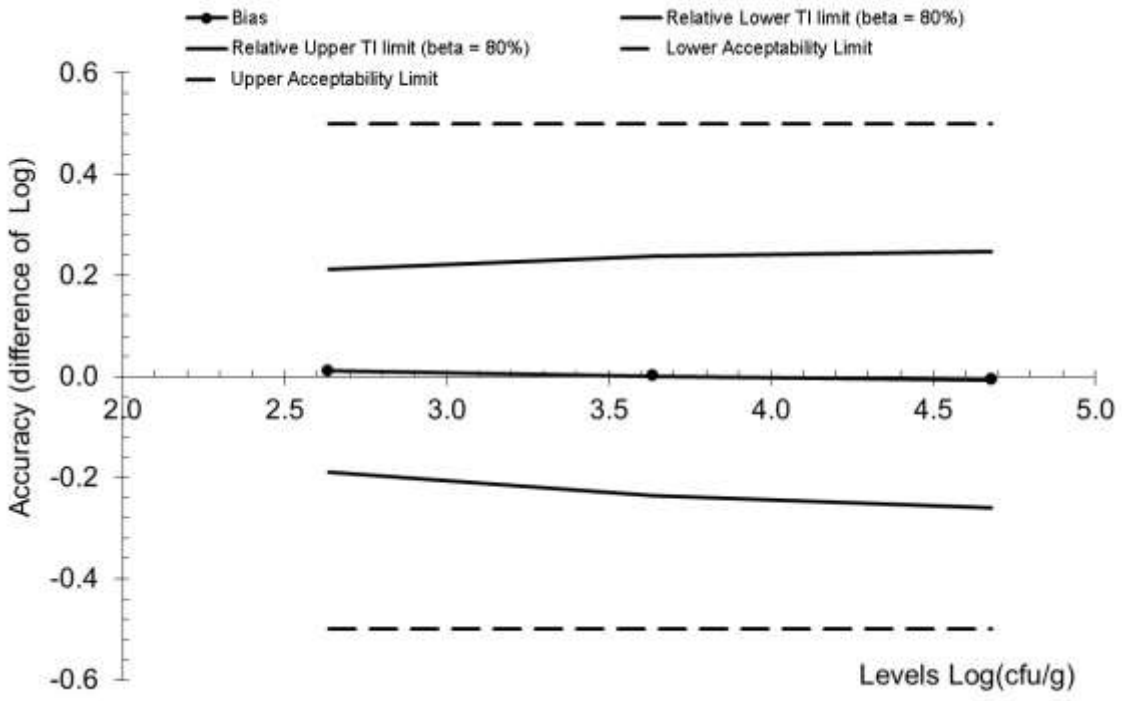


Table 9. Statistical analysis of the ILS data according to the ISO spreadsheet

Accuracy profile	0.5						
Study Name	Staph Brilliance						
Date	calculated 09/01/2018 from existing data						
Coordinator	Campden BRI						
Tolerance probability (beta)	80%	80%	80%				
Acceptability limit in log (lambda)	0.50	0.50	0.50				
	Alternative method				**Reference method**		
Levels	**Low**	**Medium**	**High**		**Low**	**Medium**	**High**
Target value	**2.635**	**3.636**	**4.680**				
Number of participants (K)	11	11	11		11	11	11
Average for alternative method	2.646	3.637	4.674		2.635	3.636	4.680
Repeatability standard deviation (sr)	0.057	0.057	0.086		0.063	0.086	0.109
Between-labs standard deviation (sL)	0.130	0.156	0.158		0.152	0.161	0.132
Reproducibility standard deviation (sR)	0.142	0.166	0.180		0.164	0.182	0.172
Corrected number of dof	11.746	11.237	12.570		11.604	12.489	14.845
Coverage factor	1.414	1.419	1.406				
Interpolated Student t	1.358	1.362	1.353				
Tolerance interval standard deviation	0.1477	0.1734	0.1874				
Lower TI limit	2.445	3.401	4.420				
Upper TI limit	2.847	3.873	4.927				
Bias	**0.011**	**0.001**	**-0.007**				
Relative Lower TI limit (beta = 80%)	**-0.190**	**-0.235**	**-0.260**	FALSE			
Relative Upper TI limit (beta = 80%)	**0.212**	**0.237**	**0.247**	FALSE			
Lower Acceptability Limit	**-0.50**	**-0.50**	**-0.50**				
Upper Acceptability Limit	**0.50**	**0.50**	**0.50**				
New acceptability limits may be based on reference method pooled variance							
Pooled repro standard dev of reference	0.173						

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.

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

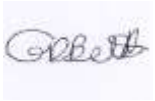
5 Overall conclusions of the validation study

- The alternative method Brilliance™ Staph 24 Agar for enumeration of coagulase positive Staphylococci species shows satisfactory results for relative trueness;
- The alternative Brilliance™ Staph 24 Agar for enumeration of coagulase positive Staphylococci species shows satisfactory results for accuracy profile;
- The alternative Brilliance™ Staph 24 Agar for enumeration of coagulase positive Staphylococci species is selective and specific.
- The alternative Brilliance™ Staph 24 Agar for enumeration of coagulase positive Staphylococci species shows satisfactory performance in the ILS

The alternative Brilliance™ Staph 24 Agar shows comparable performance to the reference method ISO 6888-1:1999 DAM2:2017 for enumeration of coagulase positive Staphylococci species

Date, 28/03/2019

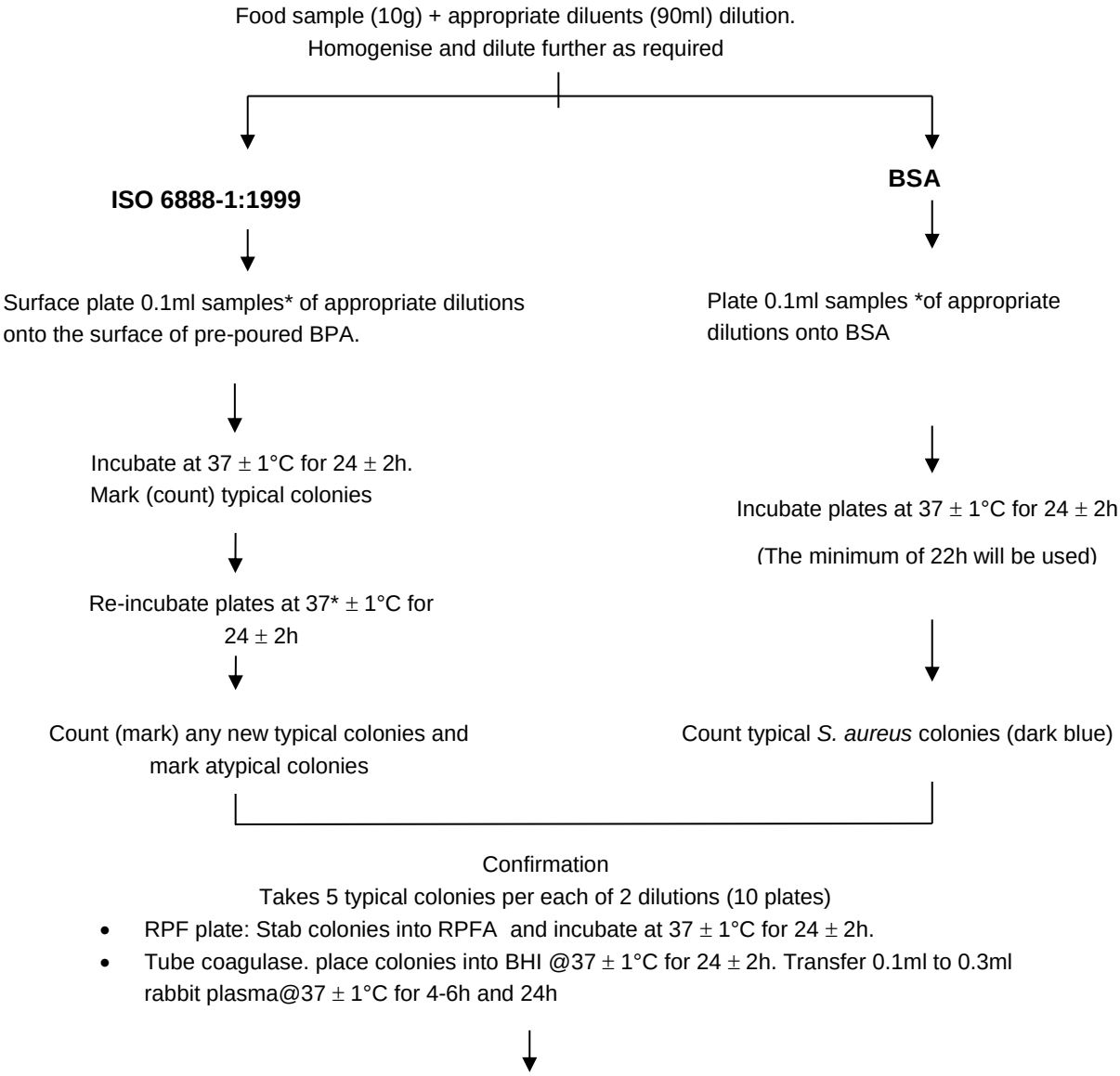
Signature



Annexes

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

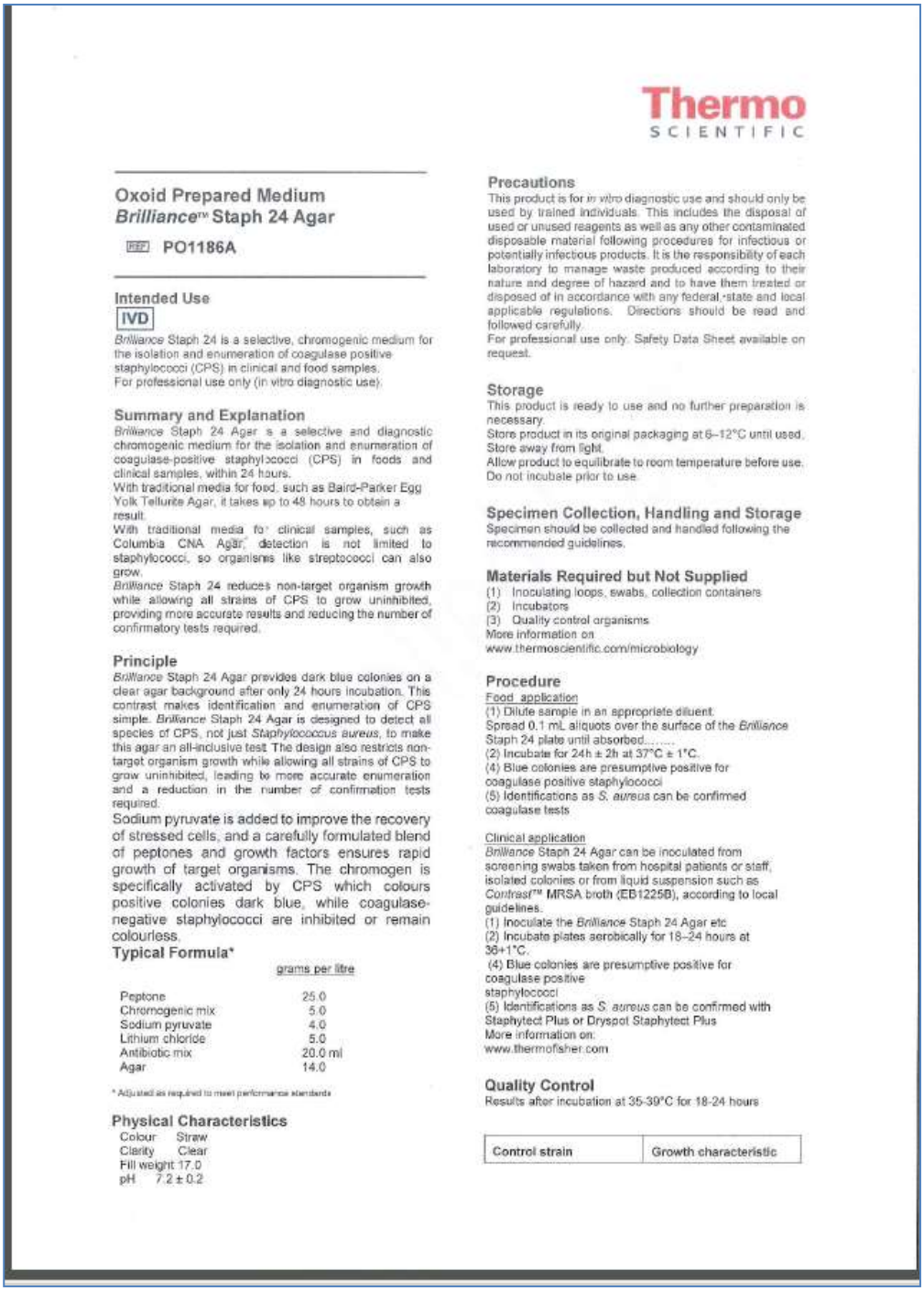
ANNEX A: Flow diagram of the alternative method and reference methods



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

Note RPF agar and Tube coagulase both used for inclusivity exclusivity cultures. RPF only used for RT and AP

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



Thermo SCIENTIFIC

Oxoid Prepared Medium
Brilliance™ Staph 24 Agar

REF PO1186A

Intended Use

IVD

Brilliance Staph 24 is a selective, chromogenic medium for the isolation and enumeration of coagulase positive staphylococci (CPS) in clinical and food samples.
For professional use only (in vitro diagnostic use).

Summary and Explanation

Brilliance Staph 24 Agar is a selective and diagnostic chromogenic medium for the isolation and enumeration of coagulase-positive staphylococci (CPS) in foods and clinical samples, within 24 hours.
With traditional media for food, such as Baird-Parker Egg Yolk Tellurite Agar, it takes up to 48 hours to obtain a result.
With traditional media for clinical samples, such as Columbia CNA Agar, detection is not limited to staphylococci, so organisms like streptococci can also grow.
Brilliance Staph 24 reduces non-target organism growth while allowing all strains of CPS to grow uninhibited, providing more accurate results and reducing the number of confirmatory tests required.

Principle

Brilliance Staph 24 Agar provides dark blue colonies on a clear agar background after only 24 hours incubation. This contrast makes identification and enumeration of CPS simple. *Brilliance* Staph 24 Agar is designed to detect all species of CPS, not just *Staphylococcus aureus*, to make this agar an all-inclusive test. The design also restricts non-target organism growth while allowing all strains of CPS to grow uninhibited, leading to more accurate enumeration and a reduction in the number of confirmation tests required.
Sodium pyruvate is added to improve the recovery of stressed cells, and a carefully formulated blend of peptones and growth factors ensures rapid growth of target organisms. The chromogen is specifically activated by CPS which colours positive colonies dark blue, while coagulase-negative staphylococci are inhibited or remain colourless.

Typical Formula*

	grams per litre
Peptone	25.0
Chromogenic mix	5.0
Sodium pyruvate	4.0
Lithium chloride	5.0
Antibiotic mix	20.0 ml
Agar	14.0

* Adjusted as required to meet performance standards

Physical Characteristics

Colour Straw
Clarity Clear
Fill weight 17.0
pH 7.2 ± 0.2

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 6–12°C until used. Store away from light.
Allow product to equilibrate to room temperature before use. Do not incubate prior to use.

Specimen Collection, Handling and Storage

Specimen should be collected and handled following the recommended guidelines.

Materials Required but Not Supplied

(1) Inoculating loops, swabs, collection containers
(2) Incubators
(3) Quality control organisms
More information on
www.thermoscientific.com/microbiology

Procedure

Food application
(1) Dilute sample in an appropriate diluent.
Spread 0.1 mL aliquots over the surface of the *Brilliance* Staph 24 plate until absorbed.....
(2) Incubate for 24h ± 2h at 37°C ± 1°C.
(4) Blue colonies are presumptive positive for coagulase positive staphylococci
(5) Identifications as *S. aureus* can be confirmed coagulase tests

Clinical application
Brilliance Staph 24 Agar can be inoculated from screening swabs taken from hospital patients or staff, isolated colonies or from liquid suspension such as *Contrast*™ MRSA broth (EB1225B), according to local guidelines.
(1) Inoculate the *Brilliance* Staph 24 Agar etc
(2) Incubate plates aerobically for 18–24 hours at 36+1°C.
(4) Blue colonies are presumptive positive for coagulase positive staphylococci
(5) Identifications as *S. aureus* can be confirmed with Staphytect Plus or Dryspot Staphytect Plus
More information on:
www.thermofisher.com

Quality Control

Results after incubation at 35-39°C for 18-24 hours

Control strain	Growth characteristic

Staphylococcus aureus ATCC® 25923	Blue colonies
Staphylococcus aureus ATCC® 6538	Blue colonies
Staphylococcus saprophyticus ATCC® 15305	White colonies
Escherichia coli ATCC® 25922	No growth
Bacillus cereus ATCC® 10876	No growth
Enterococcus faecalis ATCC® 29212	No growth
Staphylococcus epidermidis ATCC® 12228	Inhibited as slight background film

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[1]

Linearity study:

A total of 120 samples (consisting of meat products, fish products, bakery products, composite food and dairy products) were artificially contaminated. Each food type was contaminated within the ranges of: 1×10^2 to 5×10^2 CFU/g, 5×10^2 to 5×10^3 CFU/g, 5×10^3 to 5×10^4 CFU/g, 5×10^4 to 5×10^5 CFU/g and 5×10^5 to 5×10^6 CFU/g. Samples were analysed by both the ISO 6888-1:1999/Amd 1:2003 method and *Brilliance* Staph 24 Agar method.

Food category	Food product/strain pair	Regression line
Meat	Bacon	y=0.986 + 1.050 x
	Minced meat	y=0.013 + 1.005 x
Fish products	Cooked peeled shrimps	y=0.082 + 0.977 x
	Smoked mackerel	y=0.120 + 0.978 x
Bakery products	Chocolate mousse	y = 0.077 + 0.983 x
	Pastry cream	y= 0.045 + 0.956 x
Composite food	Pre-packed pancakes	y=0.104 + 0.879 x
	Raw mayonnaise based salad	y=0.061 + 0.936 x
Dairy	Dried milk powder	y=0.030 + 0.987 x
	Unpasteurised milk based cheese	y= 0.045 + 0.968 x

Relative accuracy study

One hundred and twenty artificially contaminated samples and 18 naturally contaminated samples were tested. The contamination (concentration) ranges were as follows:

Food category	Contamination range (in log CFU/g)
Meat products	2.39-6.57
Fish products	2.16-6.32
Bakery products	2.60-6.26
Composite food	2.30-6.54
Dairy products	2.64-6.58

The equation of the regression line between the *Brilliance* Staph 24 Agar and the ISO reference method for all categories combined is:
y=0.0074+0.9981 x
R2=0.9985
y=log (N *Brilliance* Staph 24 Agar)
x=log (N ISO reference method)

The linearity and relative accuracy study proved *Brilliance* Staph 24 Agar to have a good degree of equivalence to Baird Parker Egg Yolk Tellurite Agar medium for the enumeration of coagulase positive staphylococci.

Selectivity (inclusivity/exclusivity)

A total of 36 coagulase positive staphylococci (31 *S. aureus*, 2 *S. intermedius*, 2 *S. caprae*, 1 *S. epidermidis*) were tested, all gave positive results on *Brilliance* Staph 24 Agar. Twenty eight strains of coagulase negative staphylococci and non-staphylococci gave a negative result with *Brilliance* Staph 24 Agar. In comparison, 13 of the exclusivity isolates produced typical colonies on Baird Parker Egg Yolk Tellurite Agar medium which required confirmation. These were later identified by the tube-coagulase test as coagulase negative.

Practicability

The *Brilliance* Staph 24 Agar medium is an agar based culture medium shown to be of practical use in the lab as the blue typical colonies enabled convenient reading of the plates and enumeration of presumptive coagulase positive staphylococci.

Interlaboratory study

Eleven different laboratories tested samples of pasteurised milk that were artificially contaminated with a single strain to provide samples with low (5×10^2 CFU/ml), medium (5×10^3 CFU/ml) and high (5×10^5 CFU/ml) contamination levels. Uninoculated samples were used to provide the fourth contamination level (0 CFU/ml). Each laboratory received duplicate blind-coded samples for each contamination level which were tested by both the ISO 6888-1:1999/Amd 1:2003 method and *Brilliance* Staph 24 Agar method.

Contamination level	No. samples taken into account	ISO reference method		Brilliance Staph 24 Agar		
		r (sd)	R (sd)	r (sd)	R (sd)	Bias
low	22	0.031	0.189	0.042	0.146	0.010
Medium	22	0.094	0.279	0.052	0.197	-0.005
high	22	0.105	0.202	0.105	0.281	-0.010

r (sd) = repeatability
R (sd) = reproducibility

The results from the method comparison study and the interlaboratory study revealed that there were no substantial differences between *Brilliance* Staph 24 Agar and Baird Parker Egg Yolk Tellurite Agar medium according to the reference method ISO 6888-1:1999/Amd 1:2003 for enumeration of coagulase positive Staphylococci.

Clinical study

Five hundred fifty-eight wound swabs from a UK hospital were tested on *Brilliance* Staph 24 Agar and a competitor chromogenic agar for detection of *S. aureus* (plate 'A'). Performance of the two media is summarized below:

Performance	*Brilliance*	*Plate 'A'*

Thermo SCIENTIFIC
A Thermo Fisher Scientific Brand