Method Comparison Study and ILS Report for the validation of Compact Dry, for the enumeration of *Pseudomonas aeruginosa* in a broad range of water types intended for human consumption according to ISO 17994, and parts of ISO16140-2:2016

MicroVal study number: 2017LR66

Method/Kit name: Compact Dry PA

Report version:MCS/ILS summary report

MicroVal Expert Laboratory:Campden BRI

Foreword

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

Company: Nissui Pharmaceutical Co Ltd

Expert Laboratory: Campden BRI

Method/Kit name: Compact Dry PA

Validation standard: ISO 17994-2014 and ISO 16149-2:2016

Reference method: ISO 16266:2008

Scope of validation: Broad range of water for human consumption

Certification orgnization: Lloyd's Register

List of abbreviations

- AL Acceptability Limit
- AP Accuracy Profile
- Art. Cont. Artificial contamination
- CFU Colony Forming Units
- CL confidence limit (usually 95%)
- EL Expert Laboratory
- $\overline{D}$ 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

And, in *Pseudomonas aeroginosa* studies:

- MRD Maximum Recovery Diluent
- NA Nutrient Agar
- NB Nutrient Broth
- PCA Plate count Agar
- SDW Sterile Distilled Water

Contents

1 Introduction

In this project a MicroVal validation study, based on the requirements of ISO 17994 and parts of ISO 16140-2:2016, of alternative method(s) for the enumeration of *Pseudomonas aeruginosa* in 5 different (water) categories was carried out by Campden BRI as the MicroVal Expert Laboratory. The alternative method used was: Compact Dry PA – *Pseudomonas aeruginosa*

- Water samples (100ml or 250ml) were filtered through a membrane filtration system onto and placed onto pre-moistened Compact Dry plates
- Incubation was done at 36±1°C for 48h ±3h

The reference method used was: ISO 16266-2008 Detection and Enumeration of Pseudomonas aeruginosa- by Membrane Filtration[1]

Scope of the validation study is: A broad range of water intended for human consumption. Categories included:

- Potable tap water
- Bottled still water
- Drinking fountain water
- Bottled water containing gas
- Bottled mineral water

Criteria evaluated during the study have been:

Section of ISO 16140-2:2016	Proposed approach
Relative difference study	According to ISO 17994-2014 sections 5 and 6
Accuracy Profile study	According to ISO 16140-2:2016 sections 6.1.3
Inclusivity/Exclusivity	According to ISO 16140-2:2016 section 6.1.5
Inter-laboratory study (ILS)	According to ISO 17994-2014 sections 5 and 6 with a minimum of 8 collaborators and 16140:2 section 6.2

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

The alternative method (Compact Dry PA) shows comparable performance to the reference method for the enumeration of *Pseudomonas aeruginosa* in broad range of water types intended for human consumption.

2 Method protocols

The Method Comparison Study was carried out using 100 and 250 ml portions of sample material as described below;

- Potable tap water 100ml
- Bottled still water 250ml
- Drinking fountain water 100ml
- Bottled water containing gas 250ml
- Bottled mineral water 250ml

Sample volumes of 100ml or 250ml (bottled waters) were chosen as required in *Council Directive 98/83/EC of 3rd November 1998 on the quality of water intended for human consumption.*

According to ISO 16140-2 the reference method and alternative methods were performed with, as far as possible , exactly the same sample. Each sample (500 or 200ml) was split into two equal portions, one of which was filtered and analysed using the reference method and the other filtered and analysed using the alternative. Each 250ml or 100ml subsample was passed through a sterile microfunnel filter unit containing a 0.45 µm pore size gridded cellulose ester membrane filter. The filter was placed onto the surface of the relevant method plate.

2.1 Reference method

See the flow diagram in Annex A.

Sample preparations used in the reference method were done according to ISO 16266-2008 Detection and Enumeration of *Pseudomonas aeruginosa* by Membrane filtration

2.2 Alternative method

See the flow diagram of the alternative method in Annex B. The plates were incubated for 45h which is the shortest time quoted for the Alternative method.

See the Compact Dry PA kit insert in Annex C.

The alternative method principle is based on chromogenic media

This is a quantitative sheet method using a ready to use, selective and chromogenic plate for detection and enumeration of *Pseudomonas aeruginosa*. The cap of the Compact Dry plate is removed, the media is reconstitiuted by adding 1ml of SDW. The sample is filtered and the filter placed onto the reconstituted media, the cap refitted, the plate inverted and then incubated. The target microorganisms, if present, grow as red colonies with a yellow/green halo or as blue-green colonies.

2.3 Study design

Samples of product containing the target organism were divided into 2 equal subsamples. Each subsample was filtered and the resultant filters were analysed using the reference method and alternative method.

3 Method comparison study

3.1 Relative trueness study

This is not a relative trueness study according to ISO16140-2:2016 but is the relative difference study according to ISO17994:2014. The study is a comparative study between the results obtained by the reference method and the results of the alternative method. The relative difference study as described in ISO 17994 (sections 5 and 6) assesses the performance of an alternative method based on a comparison study of a single data set.The format for this study included data from the accuracy profile study and interlaboratory study, to provide 211 samples for analysis.

This study was conducted using artificially contaminated samples. Different categories, types and items were tested for this. A total of five 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.

3.1.1 Number of samples
The categories, the types and the number of samples analyzed are presented in Table 1. Only two types are available for each category as the data were gathered from the Accuracy profile part of the study and were not obtained in an ISO16140-6 Relative Trueness design. This format was agreed during the protocol development

Table 1 – Categories, types and number of samples analyzed

Water Type	Item		Number of samples analyzed in accuracy profile	Number of sample analyzed in ILS	Number of sample excluded from analysis (bla samples)	Total Number of samples analyzed
Gaseous	a	Ashbeck	20	n/a	5	15
	b	Value	20	n/a	5	15
		Total	40	n/a	10	30
Mineral	a	Ashbeck	20	n/a	5	15
	b	Evian	20	n/a	5	15
		Total	40	n/a	10	30
Potable	a	Wash up	20	n/a	5	15
	b	Laboratory	20	n/a	5	15
		Total	40	n/a	10	30
Still	a	Ice Valley	20	n/a	5	15
	b	Nestlé	20	n/a	5	15

Water Type	Item		Number of samples analyzed in accuracy profile	Number of sample analyzed in ILS	Number of sample excluded from analysis (bla samples)	Total Number of samples analyzed
		Total	40	n/a	10	30
Fountain	a	Chemistry corridor	20	n/a	5	15
	b	Goods in roo	20	n/a	5	15
	c	Chemistry corridor (Inte study)	n/a	79	18	61
		Total	40	79	28	81
Total			**200**	**79**	**68**	**211**

279 samples were analyzed, leading to 211 exploitable results.

3.1.2 Test sample preparation
Strains of *Pseudomonas aeruginosa* were inoculated into 50% nutrient broth (nutrient broth:water, 50:50) , incubated, overnight at 37ºC. The cultures were then diluted in MRD to a level of approximately 1 x10^3 cfu/ml and then diluted in 120ml water to produce a stock culture solution, for each inoculum level, high, medium and low. From the stock solutions, 20 mls were inoculated into 190ml or 490ml (dependent on sample size) for each sample, dependent on sample size. From this sample 100ml or 250ml was taken to go through each method.

The same strain was not used to inoculate more than 6 samples.

None of the samples tested were naturally contaminated. Blank samples were analysed for the presence of the target organisms (ANNEX M) but all were negative.

3.1.3 Protocols applied during the validation study

Incubation time

An incubation of 36±1ºC for 45 – 51 hours was used for the alternative method. In this validation study the minimum time of 45 hours was used.

Confirmations if required for the alternative method

No confrimation steps were required in this study

3.1.4 Test results
All raw data per category are given in Annex D and the results for blank samples, not used in the calculations are given in Annex M.

3.1.5 Calculation and interpretation of relative difference study
The data were analysed using the methods given in 17994:2014- section 6

The results are as follows:

Number of samples 211

Mean relative difference = -2.78%

Standard uncertainty (standard deviation) = 43.22

Standard uncertainly (formerly standard error) = 2.96

Half- width of confidence interval = 5.92

Lower limit = -8.71

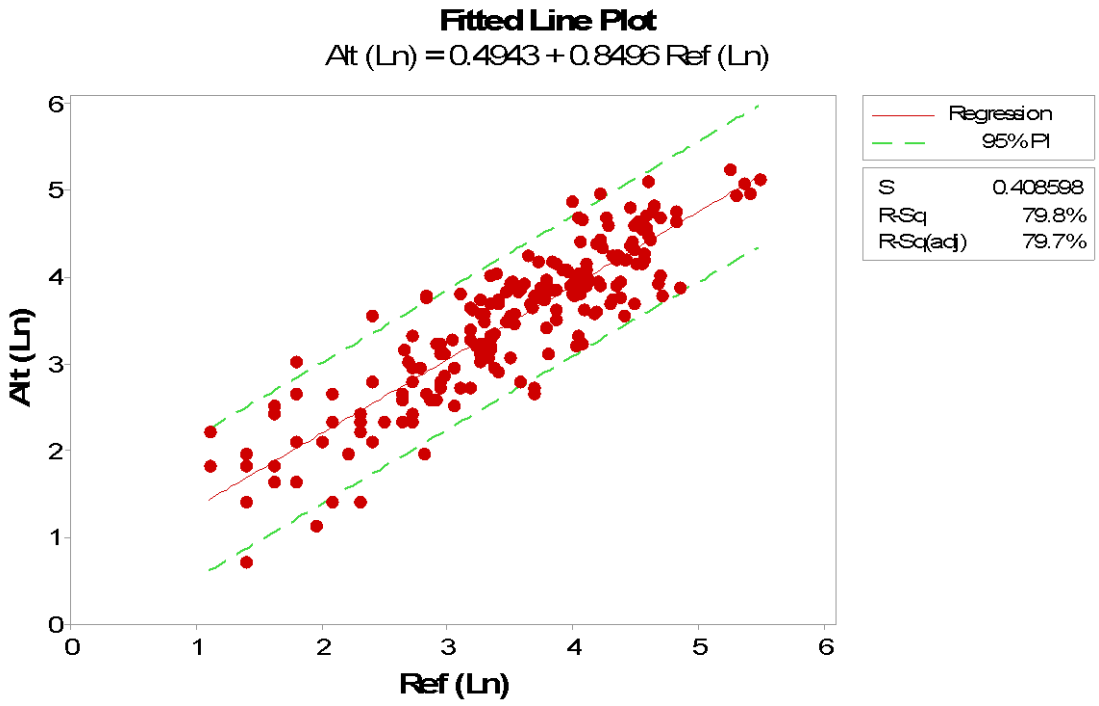
Upper limit = 3.14

The calculations are provided in Annex E.

The obtained data were analyzed using the scatter plot.

Figure 1 shows the scatter plot for all the categories.

.**Figure 1 - Scatter plot of the reference method versus alternative method results for all categories**



The above graph shows a fitted regression line and 95% Prediction interval. In the absence of outliers it would be expected that 5% of the data points will fall outside of the prediction interval, approximately 10-11 of the 211 data points. The observed number of points outside the lines of 12 is consistent with the expectation. The data points above the line (positive bias) are shown in Table 2 and those below the line (negative bias) are shown in Table 4.

The results of the evaluation, taking the stipulated limit as 2L = 10% when analysed according to section 7.2.2 of 17994:2014, is: **Methods not different**

Table 2 – Samples with a positve bias

Sample Code	Category	Strain	Reference log cfu/100ml	Alternate log cfu/100ml	Difference (Alt – Ref)
136	Nestle still water	NCTC 12924	1.79	2.99	1.2
44	Ashbeck gaseous water	NCTC 10701	2.39	3.53	1.14
205	Fountain water	NCIMB 13295	2.83	3.76	0.93
170	Fountain water	NCIMB 13295	3.98	4.84	0.86
197	Fountain water	NCIMB 13295	4.22	4.94	0.72

Table 3 – Samples with a negative bias

Sample Code	Category	Strain	Reference Logcfu/100ml	Alternate Logcfu/100ml	Difference (Alt – Ref)
97	Potable tap water	NCTC 13619	3.68	2.64	-1.04
98	Potable tap water	NCTC 13619	3.68	2.71	-0.97
156	Fountain water	NCIMB 13295	2.82	1.95	-0.87
62	Ashbeck gaseous water	NCTC 10701	2.30	1.39	-0.91
47	Sparkling water (Value)	NCIMB 10434	2.08	1.39	-0.69
61	Ashbeck gaseous water	NCTC 10701	1.95	1.10	-0.85
48	Sparkling water (Value)	NCIMB 10434	1.39	0.69	-0.70

3.1.6 Conclusion (RT study)

The relative trueness of the Alternative method is satisfied as the expectation of not more than 5% of the data points will fall outside of the prediction interval is met, and the results of the evaluation according to 17994:2014 is that the Methods are not different.

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

Five food types were tested in this study, with 2 items analysed per type.

Two samples of each item were contaminated at 4 different levels; low level, intermediate level, high level and control samples were also included. For each sample, 5 replicates (5 different test portions) were tested. A total of 40 samples were analysed per water type. The following food type/strain pairs were studied (See Table 4):

Each sample was inoculated from a bulk inoculum as described in section 3.1.2

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

Category	Sample size	Inoculated Strain	Item	Inoculation levels
Potable tap water	100 ml	NCTC 13619	Wash up (41,43,45)	Level 1x5: <1cfu/100ml
				Level 2x5: 1-10 cfu/100ml
				Level 3x5: 30-40 cfu/100ml
				Level 4x5 70-80 cfu/100ml
		NCIMB 8672	Laboratory (42,44,46)	Level 1x5: <1cfu/100ml
				Level 2x5: 1-10 cfu/100ml
				Level 3x5: 30-40 cfu/100ml
				Level 4x5 70-80 cfu/100ml
	250 ml	NCTC 13619		Level 1x5: <1cfu/250ml
				Level 2x5: 1-10 cfu/250ml
				Level 3x5: 30-40 cfu/250ml

Category	Sample size	Inoculated Strain	Item	Inoculation levels
Bottled still water			Ice Valley (12,14,16)	Level 4x5 70-80 cfu/250ml
		NCTC 12924	Nestlé(11,13,15)	Level 1x5: <1cfu/250ml
				Level 2x5: 1-10 cfu/250ml
				Level 3x5: 30-40 cfu/250ml
				Level 4x5 70-80 cfu/250ml
Drinking Fountain water	100 ml	NCIMB 13295	Chem corridor (20,22,24)	Level 1x5: <1cfu/250ml
				Level 2x5: 1-10 cfu/250ml
				Level 3x5: 30-40 cfu/250ml
				Level 4x5 70-80 cfu/250ml
		NCIMB 9038	Goods in (19,21,23)	Level 1x5: <1cfu/250ml
				Level 2x5: 1-10 cfu/250ml
				Level 3x5: 30-40 cfu/250ml
				Level 4x5 70-80 cfu/250ml
Bottled water containing gas	250 ml	NCTC 10701	Ashbeck (27,29,40)	Level 1x5: <1cfu/250ml
				Level 2x5: 1-10 cfu/250ml
				Level 3x5: 30-40 cfu/250ml
				Level 4x5 70-80 cfu/250ml
		NCIMB 10434	Value (28,30,32)	Level 1x5: <1cfu/250ml
				Level 2x5: 1-10 cfu/250ml
				Level 3x5: 30-40 cfu/250ml
				Level 4x5 70-80 cfu/250ml
Bottled mineral water	250 ml	NCIMB 10780	Ashbeck (11,13,15)	Level 1x5: <1cfu/250ml
				Level 2x5: 1-10 cfu/250ml
				Level 3x5: 30-40 cfu/250ml
				Level 4x5 70-80 cfu/250ml
		NCIMB 8295	Evian (35,37,39)	Level 1x5: <1cfu/250ml
				Level 2x5: 1-10 cfu/250ml
				Level 3x5: 30-40 cfu/250ml
				Level 4x5 70-80 cfu/250ml

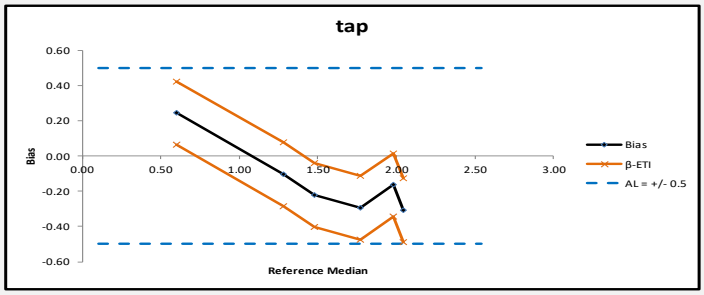
3.2.2 Calculations and interpretation of accuracy profile study

The raw data are provided in Annex G and the summary tables (in log CFU/g) in Annex E. The statistical results and the accuracy profiles are provided Figure 2.

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 2 – Accuracy profile

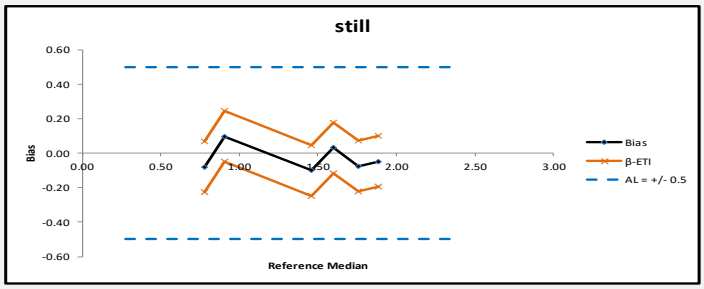
(Food) Category	potable
(Food) Type	tap



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
41	0.60	0.243	0.063	0.423	YES	YES
42	1.28	-0.103	-0.283	0.078	YES	YES
43	1.48	-0.222	-0.402	-0.041	YES	YES
44	1.77	-0.294	-0.474	-0.113	YES	YES
46	1.98	-0.165	-0.345	0.016	YES	YES
45	2.04	-0.309	-0.489	-0.129	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.104	0.125	YES	+/- 0.500

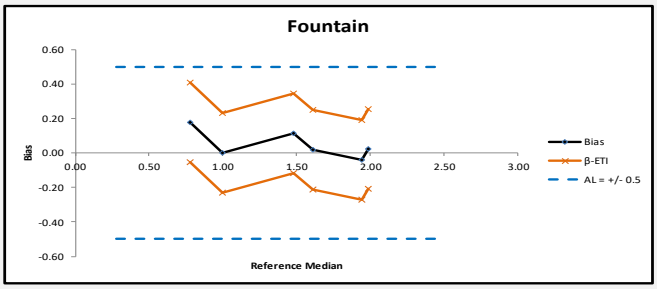
(Food) Category	still
(Food) Type	still



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
12	0.78	-0.079	-0.226	0.068	YES	YES
11	0.90	0.097	-0.050	0.244	YES	YES
14	1.46	-0.101	-0.248	0.047	YES	YES
13	1.60	0.031	-0.116	0.179	YES	YES
16	1.76	-0.075	-0.222	0.073	YES	YES
15	1.89	-0.048	-0.195	0.100	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.107	0.102	YES	+/- 0.500

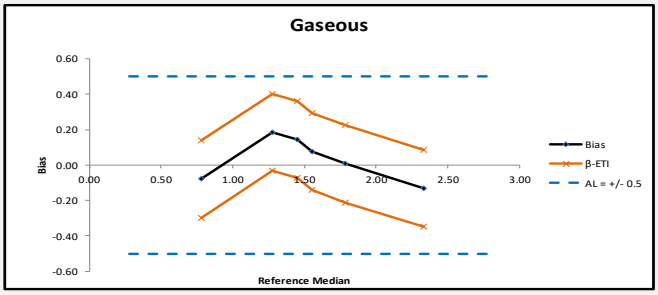
(Food) Category	Fountain
(Food) Type	Fountain



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
20	0.78	0.176	-0.055	0.407	YES	YES
19	1.00	0.000	-0.231	0.231	YES	YES
21	1.48	0.114	-0.117	0.345	YES	YES
22	1.61	0.021	-0.210	0.252	YES	YES
23	1.94	-0.041	-0.272	0.190	YES	YES
24	1.99	0.022	-0.209	0.253	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.179	0.160	NO	+/- 0.500

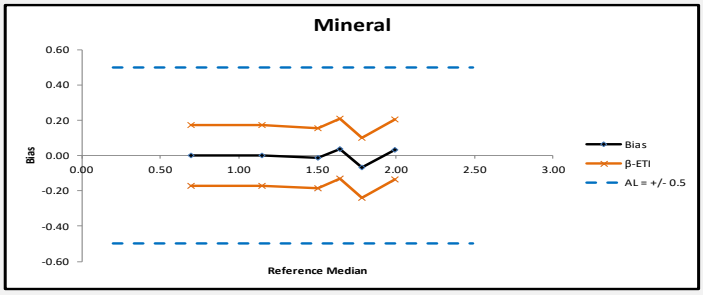
(Food) Category	Gaseous
(Food) Type	Gaseous



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
28	0.78	-0.079	-0.297	0.139	YES	YES
40	1.28	0.184	-0.034	0.402	YES	YES
30	1.45	0.144	-0.074	0.362	YES	YES
27	1.56	0.077	-0.141	0.295	YES	YES
32	1.79	0.007	-0.211	0.225	YES	YES
29	2.33	-0.131	-0.349	0.087	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.115	0.151	YES	+/- 0.500

(Food) Category	Mineral
(Food) Type	Mineral



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
11	0.70	0.000	-0.170	0.170	YES	YES
35	1.15	0.000	-0.170	0.170	YES	YES
13	1.51	-0.014	-0.184	0.157	YES	YES
37	1.64	0.038	-0.133	0.208	YES	YES
15	1.79	-0.069	-0.240	0.101	YES	YES
39	1.99	0.034	-0.136	0.204	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.118	0.118	YES	+/- 0.500

Comments

In this study the following categories met the AL of 0.5log : potable tap water, still water, fountain water, gaseous water and mineral water.

The accuracy of the Alternative method is satisfied as the all categories met the 0.5log AL.

3.3 Inclusivity / exclusivity

Inclusivity is the ability of the alternative method to detect the target analyte from a wide range of strains. Exclusivity is the lack of interference from a relevant range of non-target strains of the alternative method.

3.3.1 Protocols

- Inclusivity

50 cultures were grown in NB medium at 37°C. Each strain was tested once with the alternative method, the reference method and a non-selective agar.

- Exclusivity

30 cultures were grown in NB medium at either 30 or 37°C. 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

A total of 50 strains were tested for inclusivity. 48 of these strains showed a positive result. 2 strains showed a negative result: *Pseudomonas aeruginosa* NCIMB 10752 and 10753, the negative result was observed using both the reference and candidate method. This may be due to these strains requiring a lower growth temperature than that used in both methods, for the non-selective media a growth temperature of 25ºC was used for these strains, compared to the 35 and 36ºC for the reference and candidate methods respectively. It is also noted that these strains showed weak growth for identification and were unable to be identified using MALDI. In order to have sufficient inclusivity strains showing a positive result, 2 additional strains were tested ; CRA 4634 isolated from sesame seeds and CRA 4636 isolated from chicken. These both gave a positive reaction with both methods.

- Exclusivity

A total of 30 strains were tested for exclusivity. 26 of these strains showed a negative result. 4 strains showed a positive result: 1 strain in both the reference and candidate method *Pseudomonas putida* (CRA 8296) . Two strains, *Burkholderia cepacia* (NCTC 10661), and *Pseudomonas gingeri* (CRA 8081), gave a positive results in the candidate method only and 1 strain, *Pseudomonas stutzeri* (CRA 8252), gave a positive result in the reference method only.

The identity of all 4 discordant cultures was checked using MALDI ToF. The identity of the *Pseudomonas putida* (CRA 8296) and *Burkholderia cepacia* (NCTC 10661) strains was confirmed. *Pseudomonas gingeri* (CRA 8081) was identified as *Pseudomonas marginalis and Pseudomonas stutzeri* (CRA 8252) as *Pseudomonas citronellolis* using MALDI ToF.

3.3.3 Conclusion
The alternative, compact dry PA, enumeration method is selective and specific.

3.4 Limit of quantification (LOQ)

Providing the limit for quantification is only required for instrumental measurement.

The limit of Quantification (LOQ) analysis is not required for this study

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 7 laboratories; 2 collaborators were involved in the study for 2 of the Laboratories (See Annex K). Collaborator number 9 was sent the ILS samples but failed to do any analysis or return any results due to a lab closure.

4.1.2 Matrix and strain used
Freeze dried vials of *Pseudomonas aeruginosa* NCIMB 13295 were prepared to a required level.

It was originally planned to send samples inoculated with viable cultures but preliminary stability trials showed that it was not possible to achieve a stable and homognenous set of samples.
Therefore it was decided to use freeze dried vials to ensure the collaborators received a set of homogenous samples. Stability trials were done on the freeze dried culture after storage and rehydration.

Sample preparation
Samples of fountain water were aliquoted and sent to the participating laboratories on Thursday 12th July 2018 to be inoculated with the rehydrated vials as detailed below

Each collaborator was provided with a set of samples containing, 3 vials for preparation of samples for analysis labelled C, D and E and instructions on how to use the vials to inoculate the samples: Vial C was used to inoculate samples W2 and W6, Vial D was used to inoculate W1, W3, and W4, and Vial E was used to inoculate samples W5, W7 and W8. The target levels and codes are shown below:

Table 5: Contamination levels

Contamination level	**Sample code**
Uninoculated	2
Uninoculated	6
Low (1 -10 cfu/100ml)	1
Low (1 -10 cfu/100ml)	8
Medium (30 - 40 cfu/100ml)	4
Medium (30-40 cfu/100ml)	5
High (70-100 cfu/100ml)	3
High (70 - 100 cfu/100ml)	7

4.1.3 Labelling and shipping
Blind coded samples were placed in isothermal boxes, which contained cooling blocks, and express-shipped to the different laboratories.

A temperature control flask containing a sensor was added to the package in order to register the temperature profile during the transport, the package delivery and storage until analyses.

Samples were shipped in 24 h to 120 h to the involved laboratories. Although the samples were shipped chilled, a chilled temperature was not critical due to the nature of the samples and the fact that the inoculum was in a freeze dried format.

4.1.4 Analysis of Samples
Collaborative study laboratories and the expert laboratory carried out the analyses on 17th July 2018 with the alternative and reference methods. The analyses by the reference method and the alternative method were performed on the same day.

4.2 Experimental parameters controls

4.2.1 Detection of Pseudomonas aeruginosa in the matrix before inoculation
From historical experience it was known that this matirix was very unlikely to contain the target organism so this was not carried out.

4.2.2 Strain stability during transport
As the target organism was sent in freeze dried form to the participating laboratories, nine vials were rehydrated and tested using the reference and alternative methods before the samples were despatched to ensure consistent results were achieved between the vials. The results can be seen in Table 4,

Table 6 Freeze dried vial analysis

Vial	Reference cfu/100ml	Alternative cfu/100ml	Reference log cfu/100ml	Alternative log cfu/100ml	Difference Alt -ref
Low	14	4	1.15	0.60	-0.54
Low	4	1	0.60	0.00	-0.60
Low	11	16	1.04	1.20	0.16
Medium	17	43	1.23	1.63	0.40
Medium	42	47	1.62	1.67	0.05
High	77	69	1.89	1.84	-0.05
High	58	81	1.76	1.91	0.15
High	66	78	1.82	1.89	0.07
High	100	160	2.00	2.20	0.20
				Mean difference	**-0.02**

The data showed good performance between the two methods with, on average, a slight positive bias for the

alternate method.

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 10.

Table 7 - Sample temperatures at receipt

Collaborator	Average Temperature measured by the probe (°C)	Temperature measured at receipt (°C)	Receipt date and time	Analysis date
1	13.6	6.2	16/7/18 13:00	17/7/18
2	6.5	10.9	13/7/18 14:50	17/7/18
3	5.6	9.6	13/7/18 10:00	17/7/18
4	4.3	10.2	13/7/18 10:00	17/7/18
5	12.2	4.0	16/7/18 13:00	17/7/18
6	15.1	12.0	17/7/18 13:10	17/7/18
7	12.6	19.2	16/7/18 10:30	16/7/18
8	9.4	10.0	13/7/18 10:00	17/7/18

The average temperature measured by probe during transportation ranged between 4.3 and 15.1°C, the average temperature at receipt ranged from 4.0 to 19.2°C.

The temperature curves are given in Annex L.

4.3 Calculation and summary of data

The raw data are given in Annex I.

4.3.1 MicroVal Expert laboratory results
The results obtained by the expert laboratory are given in Table 6.

Table 8 – Results obtained by the expert lab.

Level	Reference method (cfu/100ml)	Alternative method (cfu/100ml)	Reference log cfu/ml	Alternative log cfu/ml	Difference Alt -ref
Blank	0	0	n/a	n/a	n/a
Blank	0	0	n/a	n/a	n/a
Low	71	28	1.85	1.45	-0.40
Low	52	19	1.72	1.28	-0.44
Medium	141	55	2.15	1.74	-0.41
Medium	136	64	2.13	1.81	-0.33
High	241	92	2.38	1.96	-0.42
High	211	119	2.32	2.08	-0.25
Mean difference					**-0.37**

The results from the expert lab data showed that there was an unexpected negative bias for the alternate method. This showed different performance between the two methods from that expected from the accuracy profile data and that shown in the stability trials (Table 4).

There was a negative bias of -0.37 for the alternate method in the ILS whereas there had been a +0.02 positive bias in the stability trials. A root cause analysis showed that the storage conditions of the freeze dried vials was ok and the methods had been carried out correctly. The only difference identified was that pre-poured plates purchased directly from the manufacture were used in the ILS whereas plates poured and dried by the expert lab were used for all other samples. The same manufacturer and product code were used and similar performance was expected.

Further investigations after the ILS was completed showed that the lot of pre-prepared CN agar plates used had been subject to a “customer notification” received after the trial due to incidence of bacterial contamination of the plates. No obvious contamination was observed on the plates used and as the water samples were filtered and the filters placed on the agar plates then there was unlikely to be any impact of the contaminating bacteria. However, the presence of non target bacteria suggests the plates were less selective than usual which could account for the higher counts seen.

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 from the collaborator labs showed a similar positive bias for the reference agar observed for the expert lab samples which was likely to be due to the lot of agar used as described above.

In order to assess the effect of the negative bias on the ILS results, each collaborator result was adjusted by -0.39 to adjust the difference between agars back to that expected from the stability trials. The mean difference of the stability trial was +0.02, compared to -0.37 of the ILS carried out by the same laboratory hence a cumulative adjustment of -0.39 was done to all reference agar data.

Table 8 gives a summary of the original data and adjusted data for the reference method.

The results obtained by the collaborators are shown in Table 8 and in Annex K.

Table 10: Summary of the results of the interlaboratory study per analyte level

Collaborator	**Level**	**Reference method (Log cfu/100ml) Original data**		**Reference method (Log cfu/100ml) Adjusted data***		**Alternative method (Log cfu/100ml)**	
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
1	low	1.65	1.63	1.26	1.24	1.11	1.11
2	low	1.56	1.71	1.17	1.32	1.30	1.41
3	low	1.26	1.81	0.87	1.42	0.90	1.40
4	low	1.66	1.83	1.27	1.44	1.20	1.32
5	low	1.57	1.68	1.18	1.29	1.28	1.23
6	low	1.61	1.72	1.22	1.33	0.85	1.08
7	low	1.78	1.57	1.39	1.18	1.57	1.43
8	low	1.54	1.68	1.15	1.29	1.36	1.34
10	low	1.85	1.72	1.46	1.33	1.45	1.28
1	medium	2.02	1.91	1.63	1.52	1.64	1.70
2	medium	1.86	1.95	1.47	1.56	1.75	1.70
3	medium	1.65	2.10	1.26	1.71	1.40	1.76
4	medium	1.85	2.29	1.46	1.90	1.45	1.71
5	medium	1.84	1.97	1.45	1.58	1.59	1.83
6	medium	1.98	2.07	1.59	1.68	1.58	1.52
7	medium	2.11	2.07	1.72	1.68	1.76	1.66
8	medium	1.92	1.91	1.53	1.52	1.71	1.53
10	medium	2.15	2.13	1.76	1.74	1.64	1.70
1	high	2.15	2.01	1.76	1.62	2.03	1.81
2	high	2.12	2.22	1.73	1.83	2.10	2.15
3	high	2.12	2.30	1.73	1.91	1.68	1.53
4	high	2.26	2.34	1.87	1.95	1.61	1.59
5	high	2.06	2.24	1.67	1.85	1.81	2.03
6	high	2.21	2.14	1.82	1.75	1.56	1.43

Collaborator	Level	Reference method (Log cfu/100ml) Original data		Reference method (Log cfu/100ml) Adjusted data*		Alternative method (Log cfu/100ml)	
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
7	high	2.30	2.28	1.91	1.89	1.81	1.69
8	high	2.16	2.25	1.77	1.86	2.02	1.99
10	high	2.38	2.32	1.99	1.93	1.96	2.08
1	blank	<10		<10	<10	<10	
2	blank	<10		<10	<10	<10	
3	blank	<10		<10	<10	<10	
4	blank	<10		<10	<10	<10	
5	blank	<10		<10	<10	<10	
6	blank	<10		<10	<10	<10	
7	blank	<10		<10	<10	<10	
8	blank	<10		<10	<10	<10	
10	blank	<10		<10	<10	<10	

Key - * data adjusted due to problem with over recovery of reference method media.

The accuracy profile analysis was carried out with the original data and the adjusted data.

The data is shown in Figure 3 and Table 9 for the original data and Figure 4 and Table 10 for the adjusted data.

Figure 3. Accuracy profile of Compact Dry PA from the ILS

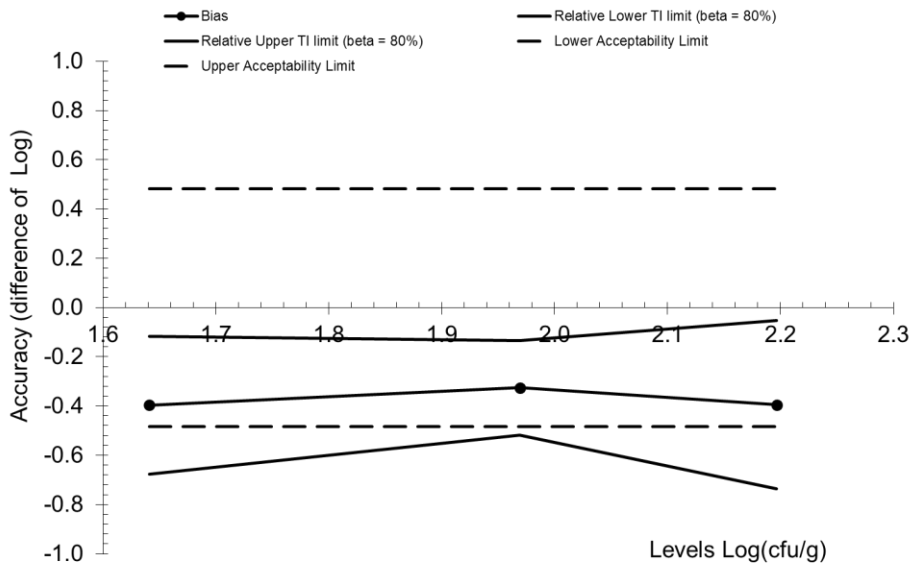


Table 9. Statistical analysis of the ILS data according to the ISO spreadsheet

Accuracy profile							
Study Name	Hyserve Compact Dry PA						
Date	19/02/2019						
Coordinator	Campden BRI						
Tolerance probability (beta)	80%	80%	80%				
Acceptability limit in log (lambda)	0.48	0.48	0.48				
				TRUE	Application of clause 6.2.3 Step 8: If any of the values for the β-ETI fall outside the acceptability limits, calculate the pooled average reproducibility standard deviation of the reference method. Step 9: Calculate new acceptability limits as a function of this standard deviation.		
	Alternative method				**Reference method**		
Levels	**Low**	**Medium**	**High**		**Low**	**Medium**	**High**
Target value	**1.641**	**1.969**	**2.197**				
Number of participants (K)	8	8	8		8	8	8
Average for alternative method	1.244	1.643	1.802		1.641	1.969	2.197
Repeatability standard deviation (sr)	0.147	0.139	0.098		0.166	0.166	0.085
Between-labs standard deviation (sL)	0.131	0.000	0.212		0.000	0.000	0.041
Reproducibility standard deviation (sR)	0.197	0.139	0.233		0.166	0.166	0.095
Corrected number of dof	11.927	14.933	8.345		14.933	14.933	14.107
Coverage factor	1.416	1.382	1.469				
Interpolated Student t	1.357	1.341	1.392				
Tolerance interval standard deviation	0.2057	0.1432	0.2460				
Lower TI limit	0.965	1.451	1.459				
Upper TI limit	1.523	1.835	2.144				
Bias	**-0.397**	**-0.326**	**-0.395**				
Relative Lower TI limit (beta = 80%)	**-0.676**	**-0.518**	**-0.737**	TRUE	Select ALL blue lines to draw the accuracy profile as illustrated in the worksheet "Graph Profile"		
Relative Upper TI limit (beta = 80%)	**-0.118**	**-0.135**	**-0.052**	TRUE			
Lower Acceptability Limit	**-0.48**	**-0.48**	**-0.48**				
Upper Acceptability Limit	**0.48**	**0.48**	**0.48**				
New acceptability limits may be based on reference method pooled variance							
Pooled repro standard dev of reference	0.146						

Figure 3. Accuracy profile of Compact Dry PA from the ILS (adjusted data)

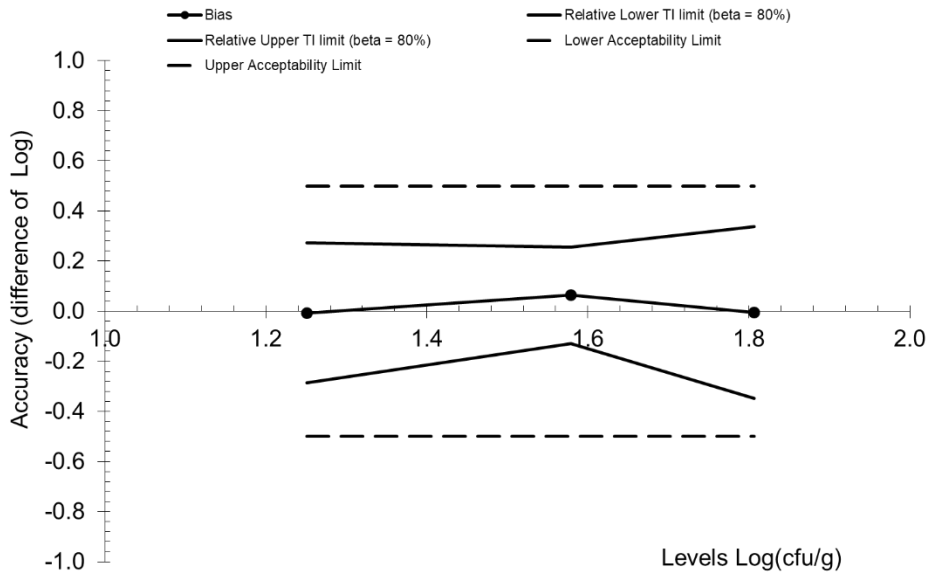


Table 10. Statistical analysis of the ILS data according to the ISO spreadsheet (adjusted data)

Accuracy profile	0.5		
Study Name	Hyserve Compact Dry PA		
Date	2/19/2019		
Coordinator	Campden BRI		
Tolerance probability (beta)	80%	80%	80%
Acceptability limit in log (lambda)	0.50	0.50	0.50

FALSE

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

	Alternative method			Reference method		
Levels	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
Target value	1.251	1.579	1.807			
Number of participants (K)	8	8	8	8	8	8
Average for alternative method	1.244	1.643	1.802	1.251	1.579	1.807
Repeatability standard deviation (sr)	0.147	0.139	0.098	0.166	0.166	0.085
Between-labs standard deviation (sL)	0.131	0.000	0.212	0.000	0.000	0.041
Reproducibility standard deviation (sR)	0.197	0.139	0.233	0.166	0.166	0.095
Corrected number of dof	11.927	14.933	8.345	14.933	14.933	14.107
Coverage factor	1.416	1.382	1.469			
Interpolated Student t	1.357	1.341	1.392			
Tolerance interval standard deviation	0.2057	0.1432	0.2460			
Lower TI limit	0.965	1.451	1.459			
Upper TI limit	1.523	1.835	2.144			
Bias	**-0.007**	**0.064**	**-0.005**			
Relative Lower TI limit (beta = 80%)	**-0.286**	**-0.128**	**-0.347**			
Relative Upper TI limit (beta = 80%)	**0.272**	**0.255**	**0.338**			
Lower Acceptability Limit	**-0.50**	**-0.50**	**-0.50**			
Upper Acceptability Limit	**0.50**	**0.50**	**0.50**			

FALSE
FALSE

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

New acceptability limits may be based on reference method pooled variance	
Pooled repro standard dev of reference	0.146

It was recommended at the 41st MVTC meeting to do an additional experiment to confirm that the Compact Dry PA and the reference Agar made by the expert lab gave the same agreement found in the MCS. It was not possible to test the pre-poured plates by the same manufacturer as they did not provide these any more.

The media tested were

1) Compact Dry PA
2) Reference agar Plates made in house by expert lab.

The results from these trials are shown in Table 11.

Table 11: Results from extra freeze dried vial analysis

Level	Vial	Reference cfu/100ml	CD PA cfu/100ml	Reference log cfu/100ml	CD PA log cfu/100ml	Diff Alt -ref
Low	W1	1	7	0.00	0.85	0.85
Low	W8	5	5	0.70	0.70	0.00
Medium	W4	4	1	0.60	0.00	-0.60
Medium	W5	10	9	1.00	0.95	-0.05
High	W3	18	17	1.26	1.23	-0.02
High	W7	37	20	1.57	1.30	-0.27
Total cfu		**75**	**59**			
Log cfu				1.88	1.77	
Log diff Alt -ref				**-0.11**		

These results showed that the two methods performed exactly the same as in the stability trials done in preparation for the ILS .

This confirms the suspicion that there was a lack of selectivity in the pre-poured plates purchased for the ILS compared to those made by the expert lab and used in the ILS. Therefore , making the adjustment to account for the lack of selectivity in the pre-poured plates used in the ILS, it is concluded that the ILS showed comparable performance between the reference method and alternative method

5 Overall conclusions of the validation study

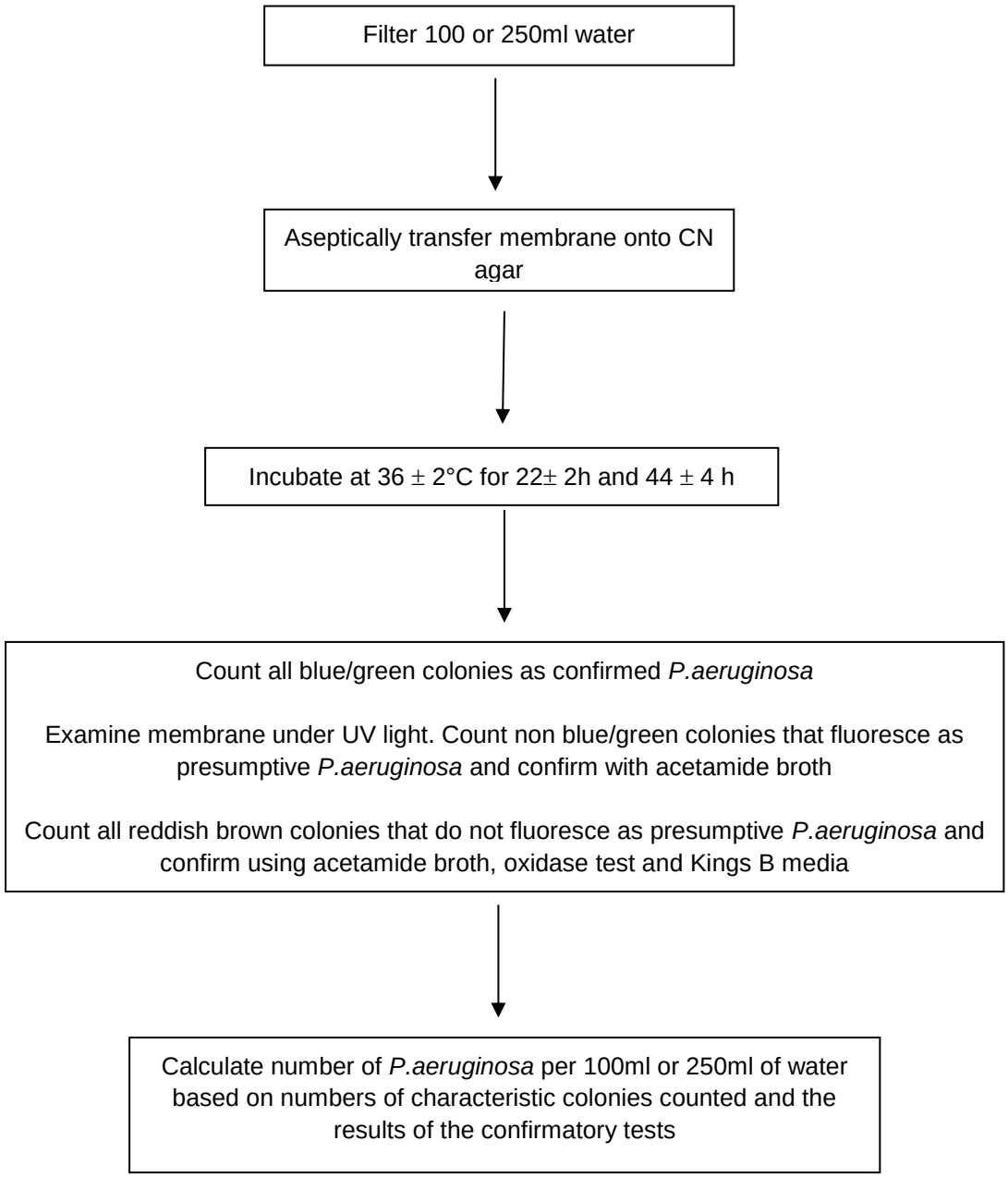
- The alternative method compact dry PA for enumeration of *Pseudomonas aeruginosa* shows satisfactory results for relative trueness;
- The alternative compact dry PA for enumeration of *Pseudomonas aeruginosa* shows satisfactory results for accuracy profile;
- The alternative compact dry PA for enumeration of *Pseudomonas aeruginosa* is selective and specific.
- The alternative compact dry PA for enumeration of *Pseudomonas aeruginosa* shows satisfactory performance in the ILS
- The alternative compact dry PA for enumeration of *Pseudomonas aeruginosa* shows comparable performance to the reference method ISO 16266-2008 Detection and Enumeration of *Pseudomonas aeruginosa* by Membrane filtration

Date, 14/08/19

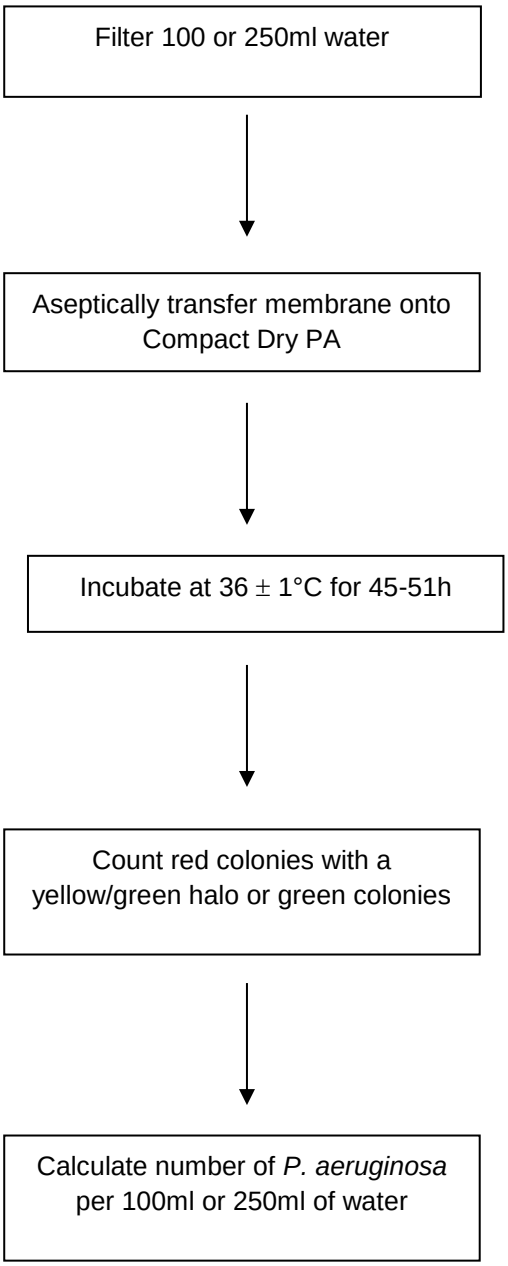
Signature Suzanne Jordan

ANNEX A: Flow diagram of the reference method

ISO 16266-2 008 Detection and Enumeration of *Pseudomonas aeruginosa* by Membrane filtration



ANNEX B: Flow diagram of the alternative method – Compact Dry PA



ANNEX C: Kit insert(s)