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NordVal International Certificate

Issued for:	Compact Dry ETB Method for the Enumeration of *Enterobacteriaceae*
NordVal No.:	034
First approval date:	1 December 2008
Renewal date:	1 December 2020
Valid until:	1 December 2022

Manufactured by:
Nissui Pharmaceutical Co.Ltd,
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Compact Dry ETB

The principle of this method is growth of dedicated bacteria on specific chromogen media.

The performance of this method has been compared to the reference method ISO 21528-2:2004: “Microbiology of foods and animal feeding stuffs. Horizontal method for the detection and enumeration of *Enterobacteriaceae* - part 2: Colony Count Method”.

The validation studies have been conducted by Campden BRI, UK, according to ISO 16140-2:2016 and NordVal International Protocol 1.

NordVal International concludes that Compact Dry ETB provides equivalent results to ISO 21528-2:2004 for a broad range of foods. The production of Compact Dry ETB is certified according to ISO 9001 and ISO 13485.

Date: 1/12 2020

Yours sincerely

Hilde Skår Norli
Chair of NordVal International

Eystein Oveland
NMKL Secretary-General

PRINCIPLE OF THE METHOD

Compact Dry ETB is a ready-to-use selective plate containing glucose for the enumeration of *Enterobacteriaceae*. Pre-treat the samples according to ISO 6687 or NMKL 91. An aliquot of 1 ml of an appropriate dilution is plated onto Compact Dry ETB plate. The plate is incubated at 37 ± 1°C and colonies (red/purple) were counted after 24 ± 2h.

FIELD OF APPLICATION

The method has been tested on enumeration of *Enterobacteriaceae* in a broad range of foods.

HISTORY

In 2007, the method was validated according to the ISO 16140:2003. Every two years until 2018 the method has been renewed without any additional studies.

In 2018 a renewal study was performed to comply with the requirements for relative trueness and accuracy profile in the new standard ISO 16140-2:2016. As the design of the Inter-laboratory study (ILS) is the same for the 2003 and 2016 versions of ISO16140, the data from the ILS data of 2007 are re-evaluated using the new statistical approach outlined in ISO16140-2:2016.

COMPARISON STUDY

Relative trueness study

The trueness study is a comparative study between results obtained by the reference method and the results of the alternative method. Different categories, types and items were tested as shown in Table 1 below.

Table 1 – Categories and types tested

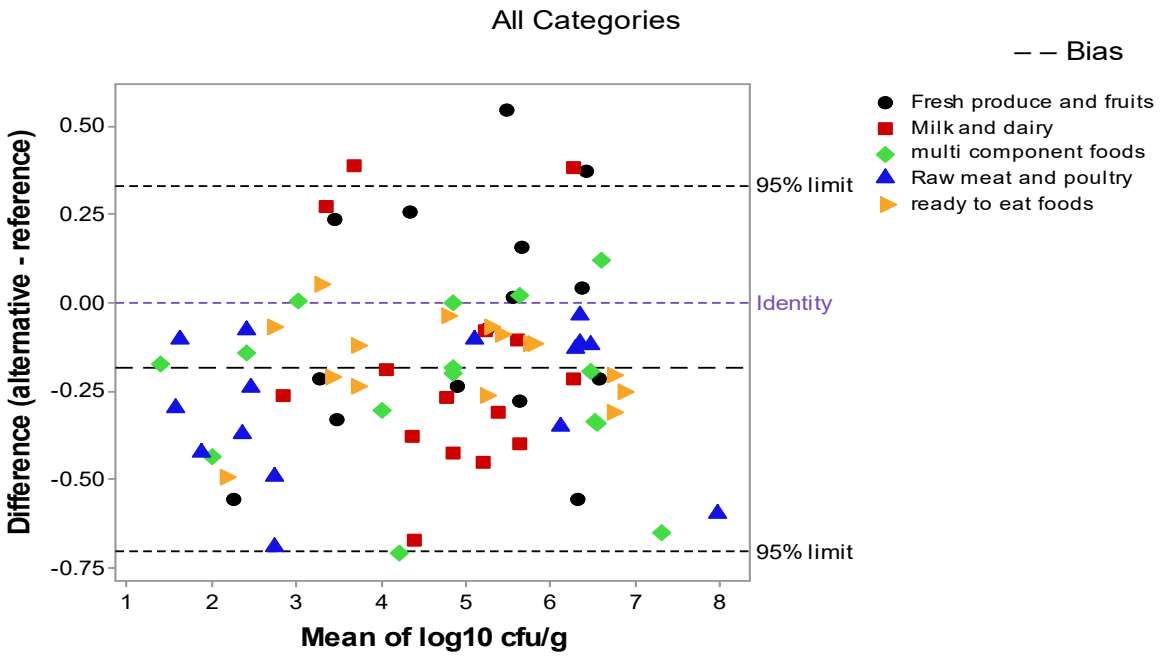
Category	Types	No. of samples
Heat processed milk and dairy products	Dry milk product e.g. milk powder, powder for milk based desserts, dried infant formula	5
	Dairy products e.g. ice-cream, yogurts, cream, hard cheese, soft cheese, raw milk cheese	5
	Pasteurised milk products e.g. skimmed, semi-skimmed, full fat and flavoured milks	5
Fresh produce and fruits	Cut ready to eat fruit e.g. fruit mixes, fruit juices	5
	Cut ready to eat vegetables e.g. Bagged pre-cut salads and shredded carrot, cabbage, vegetable juices	5
	Leafy greens/Sprouts e.g. soy, mung, alfalfa,	5
Raw poultry and meats (Combined category raw/ RTC meats and poultry)	Fresh poultry cuts e.g. turkey breast, turkey fillet	5
	Fresh mince e.g. lamb, beef, pork	5
	Processed ready to cook e.g. frozen patties, marinated kebabs, seasoned chicken breasts	5
Ready to eat foods (Combined category RTE/RTRH meats and poultry)	Ready to eat poultry e.g. turkey fillet, chicken sausage, pate	5
	Cooked fish products e.g. prawns, terrine, pate, smoked fish	5
	Cooked meat e.g. ham, salami, pate, corned beef	5
Multi component foods or meal components	Ready to re-heat refrigerated food e.g. cooked chilled foods, rice and pasta, products	5
	Ready to re-heat food frozen e.g. fries, pizza	5
	Composite foods with substantial raw ingredients e.g. .pasta salads, sandwiches, deli-salads	5

75 samples were analysed, whereof 60 samples were artificially contaminated and 15 samples were naturally contaminated.

The relative trueness is illustrated by the use of a Bland-Altman plot, i.e. the difference (bias) between paired samples analysed with the reference method and the alternative method respectively, plotted against the mean values obtained by the reference method. In the plot, Upper and Lower limits are included as the bias ± 2 times the standard deviation of the bias.

The Bland-Altman Plot in Figure 1, illustrates the difference obtained in the enumeration of *Enterobacteriaceae* in foods by the alternative and the reference method, respectively.

Figure 1 Bland-Altman Plot of the food categories tested



For 'All Categories' there are five in 75 values (7%) which lie outside the CLs. This is a little more than the expectation of less than one in 20 (5%). Of the five points outside of the CLs, the data covered 3 different food categories, and 4 different inoculated strains. Although there was a general slight negative bias to the data, only one data point was outside the lower CL and 4 were outside the upper CL.

NordVal International considers the relative trueness for satisfactory.

ACCURACY PROFILE

The accuracy profile study is a comparative study between the results obtained by the reference method and the results of the alternative method. Each item used were artificially contaminated obtaining three target levels; low (10^2 cfu/g), medium (10^4 cfu/g) and high (10^6 cfu/g). Five test portions of each level of each item were analysed, resulting in 150 samples. The tested categories, types, items and inoculated strains are provided in the Table 2.

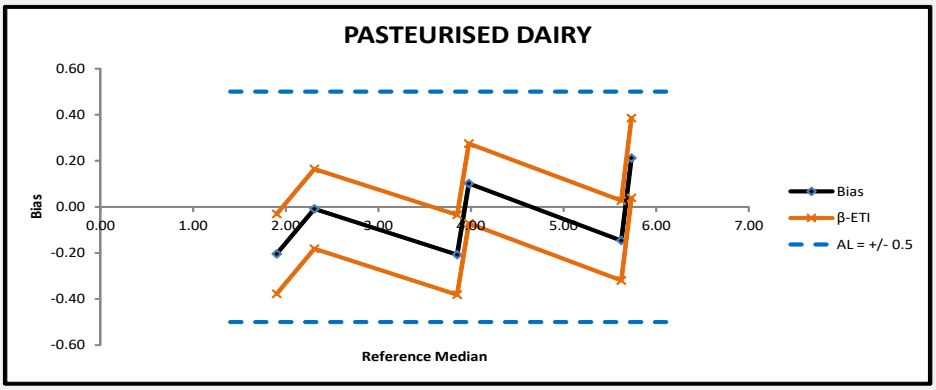
Table 2 - Categories, types and food items

Category	Types	Strain	Item
Dairy products	Pasteurised dairy products	*E. coli* CRA 1476 from dried milk	Pasteurised cream
		Enterobacter agglomerans CRA 5613 from milk powder	Cream cheese
Fruits and vegetables	Fresh produce	*E.hermanii* CRA 7477 from sesame seeds	Ready to cook Vegetable preparation
		Citrobacter amalonaticus CRA 7458 from beansprouts	Vegetable juice
Raw poultry and meats (Combined category raw/ RTC meats and poultry)	Fresh meat	*Salmonella Brandenberg* CRA 1070 from beef	Pork mince
		Proteus mirabilis CRA 1588 from poultry	Raw bacon
Ready to eat foods (Combined category RTE/RTRH meats and poultry)	Cooked fish products e.g. prawns	*E.coli* CRA 2003 from fish	Fresh prawns
		Klebsiella oxytoca ATCC 15926	Fish pate
Multi component foods	Composite foods with raw ingredients	*Hafnia alvei* CRA 400 from sandwich	Sandwiches
		E. adecarboxylata CRA 5501 from skimmed milk powder	Cooked chilled rice

The statistical results and the accuracy profiles are provided in the Figures 2 to 6.

Figure 2 Dairy Products

(Food) Category	DAIRY
(Food) Type	PASTEURISED DAIRY

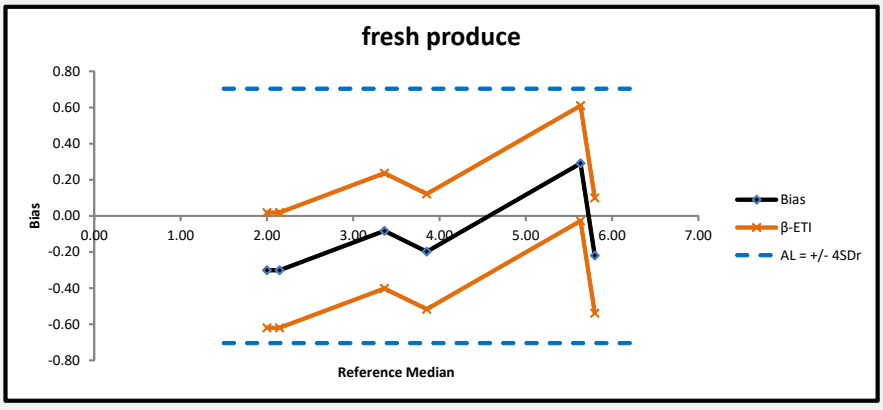


Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
142 21 248 188 53	1.90	-0.204	-0.377	-0.031	YES	YES
107 B45B 75B 342B 322B	2.31	-0.009	-0.182	0.165	YES	YES
31 10 97 13 273	3.85	-0.208	-0.381	-0.035	YES	YES
314 346 25 63 328	3.98	0.101	-0.072	0.275	YES	YES
196 229 57 102 259	5.62	-0.146	-0.319	0.027	YES	YES
58 141 172 83 109	5.73	0.212	0.039	0.385	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.129	0.120	NO	+/- 0.500

Figure 3 Fruit and vegetable products

(Food) Category	fruit and veg
(Food) Type	fresh produce

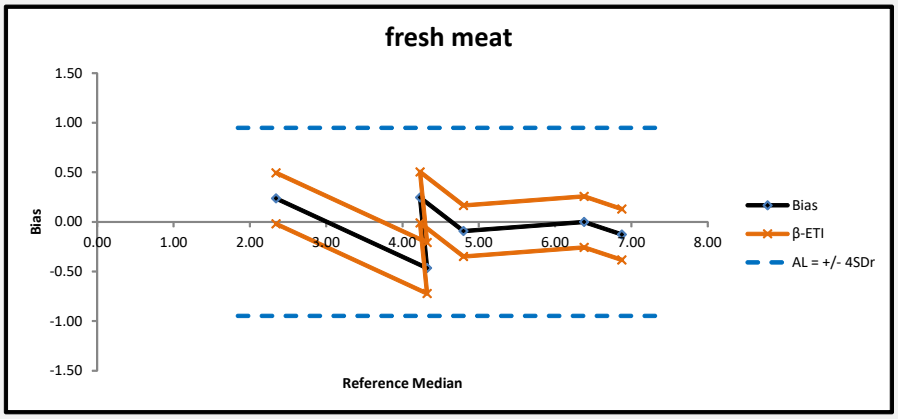


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
8 17 327 246 37	2.00	-0.301	-0.620	0.018	NO	YES
264 252 139 300 294	2.15	-0.301	-0.620	0.018	NO	YES
312 301 64 267 340	3.36	-0.083	-0.402	0.236	YES	YES
236 92 223 9 158	3.85	-0.198	-0.517	0.121	NO	YES
316 86 323 117 203	5.63	0.291	-0.028	0.610	NO	YES
74 234 272 34 144	5.80	-0.220	-0.539	0.099	NO	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.176	0.221	NO	+/- 0.704

Figure 4 Meat and poultry

(Food) Category	raw poultry and meat
(Food) Type	fresh meat

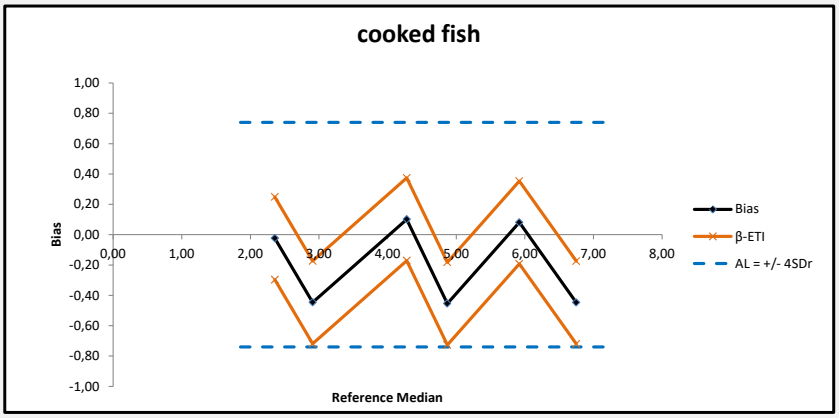


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
250 80 337 56 238	2.34	0.237	-0.020	0.494	YES	YES
194 72 177 12 270	4.32	-0.465	-0.722	-0.208	NO	YES
200 339 120 274 106	4.23	0.247	-0.010	0.504	NO	YES
243 124 297 268 347	4.80	-0.092	-0.349	0.165	YES	YES
183 197 2 40 5	6.38	0.000	-0.257	0.257	YES	YES
232 217 313 89 147	6.88	-0.127	-0.384	0.130	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.237	0.178	NO	+/- 0.948

Figure 5 Ready to eat foods

(Food) Category	RTE foods
(Food) Type	cooked fish

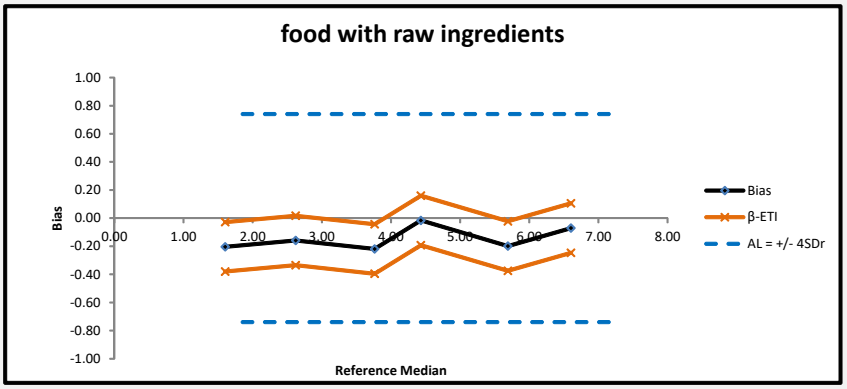


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
51 247 218 87 287	2,36	-0,024	-0,296	0,249	YES	YES
111 155 255 186 202	2,91	-0,446	-0,719	-0,173	NO	YES
289 68 23 309 226	4,28	0,101	-0,171	0,374	YES	YES
256 192 295 16 298	4,87	-0,454	-0,727	-0,181	NO	YES
195 261 42 61 320	5,92	0,081	-0,192	0,354	YES	YES
319 189 78 82 182	6,75	-0,447	-0,720	-0,174	NO	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0,185	0,189	NO	+/- 0,740

Figure 6 Multi component foods

(Food) Category	Mulit component
(Food) Type	food with raw ingredients



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
329 30 146 275 242	1.60	-0.204	-0.380	-0.028	YES	YES
307 290 157 288 32	2.62	-0.159	-0.335	0.017	YES	YES
242 184 211 334 331	3.76	-0.219	-0.395	-0.043	YES	YES
282 60 159 308 235	4.43	-0.016	-0.193	0.160	YES	YES
131 59 161 103 209	5.69	-0.199	-0.375	-0.023	YES	YES
239 179 29 251 128	6.60	-0.071	-0.247	0.106	YES	YES

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

The observed profiles are within the 0.5 log AL or the recalculated AL limit calculated according to ISO16140-2:2015 section 6.1.3.3.

All the accuracy profiles fulfil the performance criteria after the permitted recalculation, and the alternative method is accepted as being equivalent to the reference method.

THE SELECTIVITY OF THE METHOD (INCLUSIVITY/EXCLUSIVITY)

The selectivity study was performed according to ISO 16140-2:2016.

Inclusivity is the ability of an alternative method to detect the target analyte from a wide range of strains. In the original study 32 strains were studied. One of the 32 strains failed to grow on Compact Dry ETB. In the renewal study from 2016, 18 of the 23 strains tested were detected by both methods. Those not detected by either method were *Erwinia amylovora* 8037 and *Erwinia herbicola* 7057. Three strains were detected by the reference method but not by the alternative method- these were: *Serratia liquefaciens* 10670, *Rahnella aqualatis* NCIMB 13365 and *Yersinia intermedia* 380.

Exclusivity is the lack of interference from a relevant range of non-target strains of the alternative method. In the original study, 21 of the 23 strains did not grow on either methods. The two strains that did grow in VRBGA included a strain of *Aeromonas hydrophila* (strain 4111) which appeared typical in this medium, a strain of *Vibrio parahaemolyticus* (strain 15737) which grew but was atypical in appearance on the ETB medium and which produced typical colonies in VRBGA, although growth was poor. One strain of *Pasteurella bettyae* yielded typical colonies by both methods whereas tests with other *Pasteurella* strains, including an additional *P. bettyae* strain showed inhibition of these bacteria by both media. *Pasteurella* spp belong to the family Pasteurellaceae and not the Enterobacteriaceae, and both members of these families are capable of fermenting glucose, and although their optimum growth temperature is 37°C most are fastidious in their growth requirements. However, unlike members of the Enterobacteriaceae *Pasteurella* spp. are oxidase-positive.

In the 2018 study, of the 10 exclusivity strains tested, one strain was detected by both the alternate method and by the reference method, *A.sobria* CRA 8390.

CONCLUSION OF THE COMPARISON STUDY

The results of the method comparison study showed that the Compact Dry ETB provide equivalent results to the reference method ISO 21528-2:2004. The lowest validated level is 2.0 cfu/g.

INTERALBORATORY STUDY

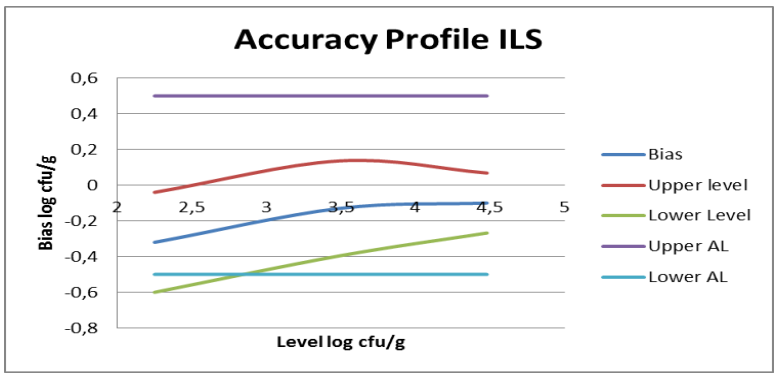
The interlaboratory study was conducted in November 2007. Ten laboratories analysed samples of pasteurised milk artificially contaminated with defined numbers of *Esherichia coli* and *Enterobacter aerogenes* according to ISO 21528-2:2004 and Compact Dry ETB respectively.

The obtained results (log cfu/g) is given in Table 3, and illustrated by an Accuracy Profile in Figure 7.

Table 3 The interlaboratory study results in log cfu/g

	Reference method		Alternative method			Upper	Lower	
Level	Median	S_R	Median	S_R	Bias	Level	Level	± AL
1	2.57	0.12	2.25	0.20	-0.32	-0.04	-0.60	0.50
2	3.62	0.13	3.49	0.19	-0.13	0.14	-0.40	0.50
3	4.58	0.069	4.48	0.12	-0.1	0.07	-0.27	0.50

Figure 7 Accuracy Profile of the interlaboratory study



The lowest level has a negative bias, and thus the lower level is below –AL.

CONCLUSION

According to the comparison and the interlaboratory study no substantial differences were found between the Compact Dry ETB method and the reference method ISO 21528-2:2004 for the enumeration of *Enterobacteriaceae.*