NordVal International / NMKL
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NordVal International Certificate

Issued for:	HyServe Compact Dry EC Method for the Enumeration of *Escherichia coli* and coliforms
NordVal No:	036
First approval date:	1 December 2008
Renewal date:	1 December 2016
Valid until:	1 December 2018

HyServe Compact Dry EC

Manufactured by:
Nissui Pharmaceutical Co.Ltd,
3-23-9 Ueno,
Taito-ku, Tokyo, 110-8736
Japan

Supplied by:
HyServe GmbH & Co. KG,
Hechenrainerstr 24,
82449 Uffing,
Germany

The reference methods used are:

- ISO 16649-2:2001: "Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of beta-glucuronidase-positive Escherichia coli. Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide."

- ISO 4832:2006: "Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of coliforms. Colony-count technique."

The validation studies have been conducted by CCFRA Technology Limited, Chipping Campden, UK, according to the design of ISO 16140:2003. NordVal International has recalculated the data according to the ISO 16140-2:2016, and concludes Compact Dry EC provide equivalent results to the reference methods.

Date: 1/12 2016

Yours sincerely,
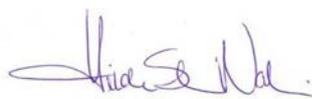
Hilde Skår Norli
Chair of NordVal International

Nina Skall Nielsen
NMKL Secretary General

PRINCIPLE OF THE METHOD

HyServe Compact Dry EC is a ready-to-use dry chromogenic plate for enumeration of *E.coli* and coliforms. The medium contains two kinds of chromogenic enzyme substrates: Magenta-Gal and X-Gluc. *E.coli* forms blue colonies. The total coliform group count is the sum of both the red and blue colonies.

An aliquot of 1 ml of an appropriate dilution is plated onto Compact Dry EC plate. The incubation conditions tested in the study were 37 ± 1°C for 24 ± 2h.

FIELD OF APPLICATION

The method has been tested on enumeration of *Escherichia coli* and coliforms in foods.

HISTORY

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

June 2016, a new edition of ISO 16140 for validation of alternative methods was published, which included new validation design and statistical evaluation of the results. NordVal International has a transition period of two years for additional studies required according to the new protocol. In this certificate the data obtained according to ISO 16140 has been recalculated using statistical models given in the new standard.

COMPARISON STUDIES

### COMPLIANCE BETWEEN COMPACT DRY EC METHOD AND THE REFENCE METHODS

The comparison studies were carried out by CCFRA Technology Limited in 2007 on cooked chicken, frozen fish, lettuce, milk powder and raw beef. Five levels of contamination were used for each food matrix. For all foods, except milk powder, naturally contaminated samples were tested. Five replicates were analysed at each level.

RELATIVE TRUENESS

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.

In this certificate the Bland-Altman Plot is conducted on results obtained in the validation carried out according to the ISO 16140:2003, i.e. 5 food categories, 5 levels and 5 replicates. The Bland-Altman Plot in Figure 1, illustrates the difference obtained in the enumeration of *E.coli* and total count by the alternative and the reference method, respectively.

Figure 1 Bland-Altman Plot of the enumeration of E.coli and total count in foods
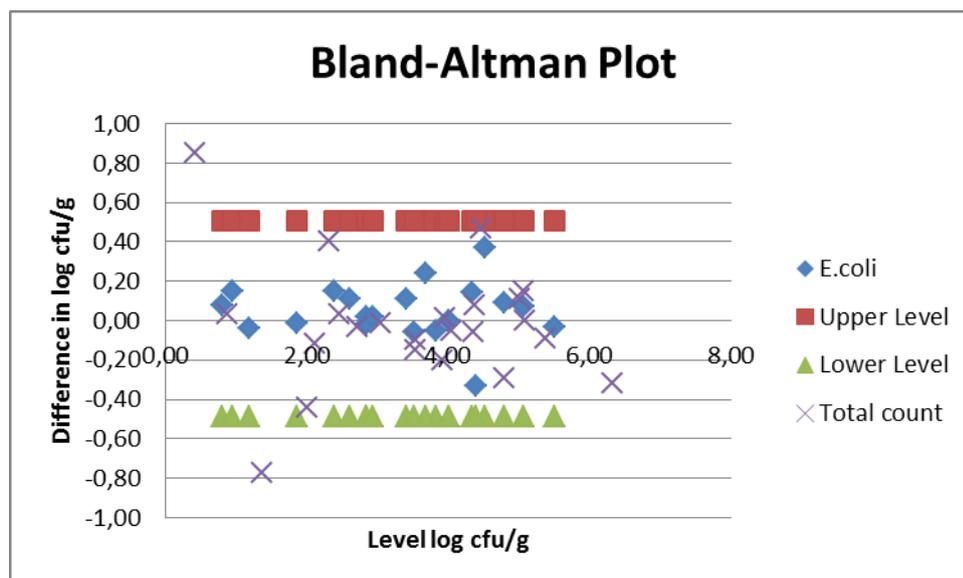
It is expected that no more than 1 in 20 data values will lie outside the 95% confidence levels (upper limit and lower limits). The results obtained are in accordance with the expectations.

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. As the results are based on data obtained according to ISO 16140:2003, the mean of the replicates are used rather than the median. The five food categories, including five levels each category and five replicates each level were analysed by using the Compact Dry EC, ISO 16649 and ISO 4832.

Cooked chicken

Four of the five levels were enumerated for *E.coli* and coliforms. The lowest level was not countable in neither of the methods. The results for the enumeration of E.coli and total count in cocked chicken are given in Table 1, and illustrated by an Accuracy Profile in Figure 2.

Table 1 Results in log cfu/g of the enumeration in chicken by the reference methods and TC Dry EC

	E.coli		Coliforms	
Methods	**ISO 16649**	**Dry EC**	**ISO 4832**	**Dry EC**
Concentration range (mean) log cfu/g:	0,76 - 4,27	0,84 - 4,41	0,87 - 4,33	0,90 - 4,41
Precision; standard deviation, log cfu/g:	0,16 - 0,27	0,071 - 0,16	0,13 - 0,38	0,070 - 0,12
Bias log cfu/g		0,08 - 0,15		-0,15 - 0,08
Lower Level - Upper Level		-0,14 / 0,37		-0,35 / -0,12
Acceptance Level		±0,50		±0,50

Figure 2 Accuracy Profiles for the enumeration of E.coli and total count in chicken

Results E.coli:
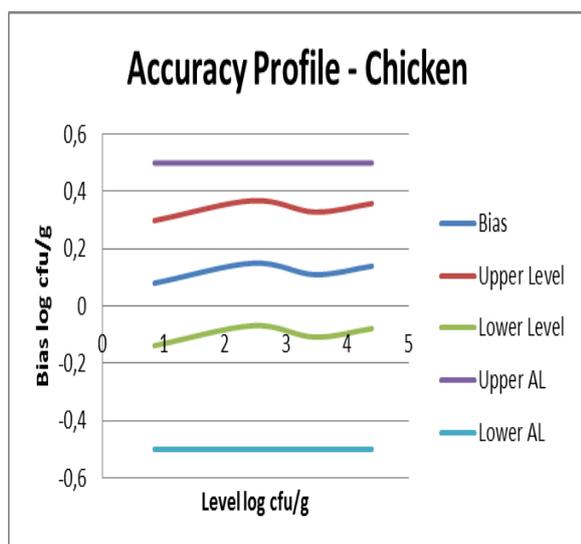
Results coliforms:
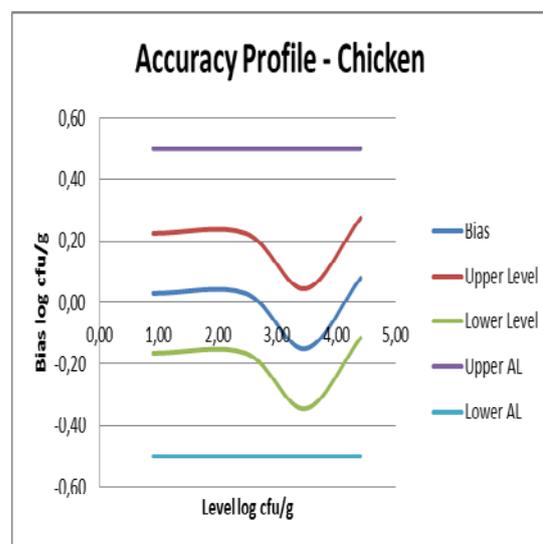
Whenever no biases exist, the results would be on y=0. In the figure above, the acceptability limits (AL = ± 0,5) are represented by the purple and the light blue lines. The levels where the results might be expected to vary between (upper and lower levels) are given as red and green lines. The bias (the difference obtained by the results obtained by the alternative method and the reference method) is given as the blue line.

As the upper level (red line) is below the upper AL (purple line), and the lower level (green line) is above the lower AL (light blue line) the alternative method is accepted as being equivalent to the reference method.

Frozen fish

Four of the five levels were enumerated for *E.coli*. The lowest level was not countable for *E.coli* in neither of the methods. All five levels were enumerated for coliforms.
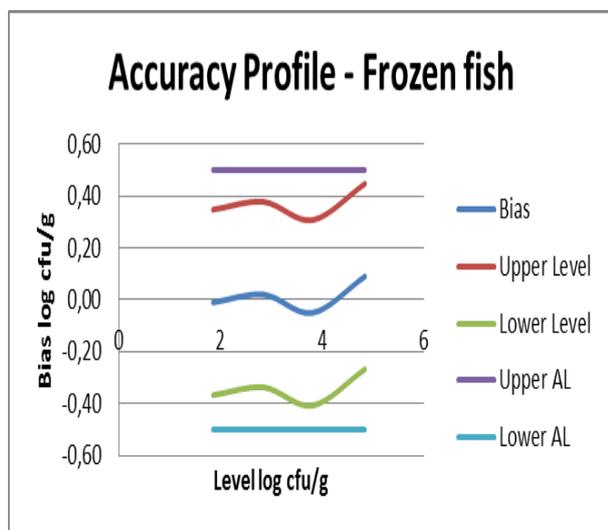
The results for the enumeration of E.coli and total count in frozen fish are given in Table 2, and illustrated by an Accuracy Profile in Figure 3.

*Table 2 Results in log cfu/g of the enumeration in fish by the reference methods and TC Dry EC*

	E.coli		Coliforms	
Methods	**ISO 16649**	**Dry EC**	**ISO 4832**	**Dry EC**
Concentration range (mean) cfu/g:	1,86 – 4,75	1,85 – 4,84	1,76 – 5,09	0,99 – 5,09
Precision; standard deviation, cfu/g:	0,11 - 0,16	0,12 - 0,22	0,081 - 0,23	0,11 - 0,23
Bias log cfu/g		-0,05 - 0,09		0,00 - 0,77
Lower Level - Upper Level		-0,41 / 0,45		-1,10 / -0,33
Acceptance Level		± 0,50		± 0,50

Figure 3 Accuracy Profiles for the enumeration of E.coli and total count in fish

Results E.coli:

Results coliforms:
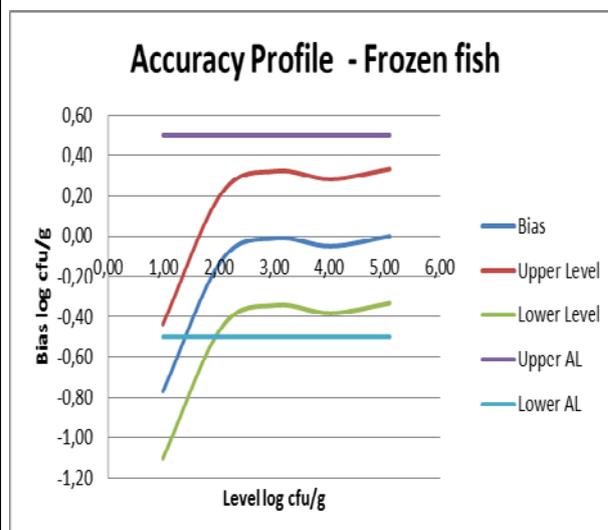
The lowest level was not countable for *E.coli* by neither of the methods. The results obtained for this level for total coliforms are also very uncertain, i.e. the bias (blue line) and the lower level (green line) are below the lower AL (light blue line). However, for all other levels the results fall within the upper and lower AL.

<u>Lettuce</u>

Four of the five levels were enumerated for *E.coli* and coliforms, respectively. The lowest level was not countable in neither of the methods. The results for the enumeration of E.coli and total count in lettuce are given in Table 3, and illustrated by an Accuracy Profile in Figure 4.


Table 3 Results in log cfu/g of the enumeration in lettuce by the reference methods and TC Dry EC

|	*E.coli*		coliforms	
Methods	ISO 16649	Dry EC	ISO 4832	Dry EC
concentration range (mean) cfu/g:	0,88 – 4,34	1,03 – 4,71	2,22 – 4,95	1,78 – 5,06
precision; standard deviation, cfu/g:	0,15 - 0,28	0,29 - 0,31	0,13 - 0,55	0,094 - 0,65
Bias log cfu/g		0,11 – 0,37		-0,44 - 0,47
Lower Level - Upper Level		-0,46 / 0,94		-1,16 / - 0,25
Acceptance Level		± 0,81		± 1,17

For the lowest countable level (level 2), the standard deviation is high (0,55 and 0,65 respectively). Thus the range of Upper and Lower Level as well as the Acceptance Levels are wide.

Figure 4 Accuracy Profiles for the enumeration of E.coli and total count in lettuce

Results E.coli:
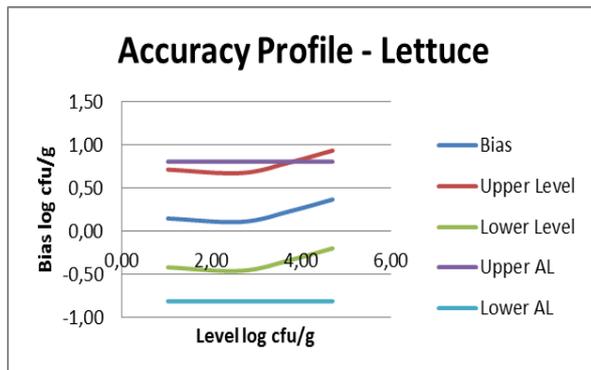
Results coliforms:
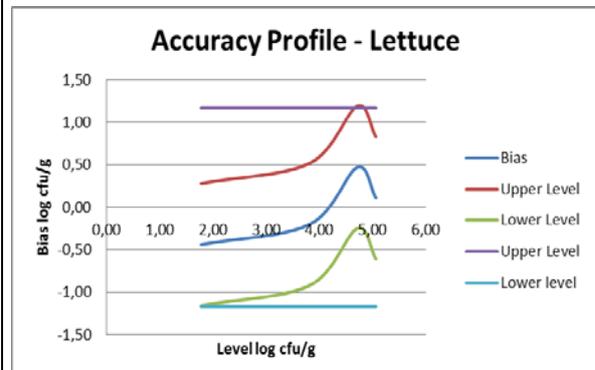
As the upper level (red line) is below the upper AL (purple line), and the lower level (green line) is above the lower AL (light blue line) the alternative method is accepted as being equivalent to the reference method.

Milk powder

Four of the five levels were enumerated for *E.coli* and coliforms, respectively. The lowest level was not countable in neither of the methods. The results for the enumeration of E.coli and total count in milk powder are given in Table 4, and illustrated by an Accuracy Profile in Figure 5.

Table 4 Results in log cfu/g of the enumeration in milk powder by the reference methods and TC Dry EC

|	*E.coli*		Coliforms	
Methods	**ISO 16649**	**Dry EC**	**ISO 4832**	**Dry EC**
concentration range (mean) cfu/g:	2,86 – 5,52	2,84 - 5,49	2,12 – 4,94	0,85 – 4,65
precision; standard deviation, cfu/g:	0,10 - 0,25	0,076 - 0,28	0,066 - 0,71	0,078 - 0,61
Bias log cfu/g		-0,33 - -0,02		-0,29 - 0,85
Lower Level - Upper Level		-0,74 – 0,39		-0,88 / 1,44
Acceptance Level		$\pm$ 0,77		$\pm$ 1,55

For the enumeration of total count, the standard deviations were high for both methods for the lowest countable level, resulting in higher AL than ± 0,50 log cfu/g.

Figure 5 *Accuracy Profiles for the enumeration of E.coli and total count in milk powder*

Results E.coli:
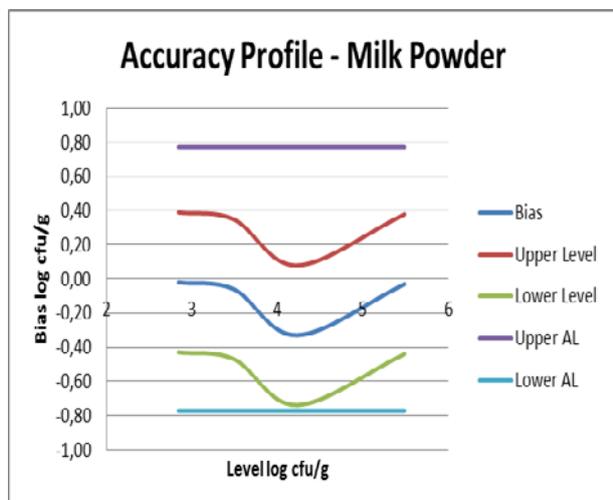
Results coliforms:
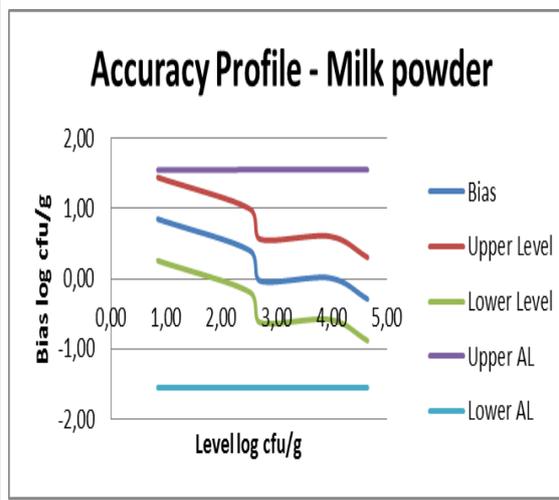
As the upper level (red line) is below the upper AL (purple line), and the lower level (green line) is above the lower AL (light blue line) the alternative method is accepted as being equivalent to the reference method.

Raw beef:

Four of the five levels were enumerated for *E.coli.* The lowest level was not countable in neither of the methods. All the five levels were enumerated for coliforms. The results for the enumeration of E.coli and total count in raw beef are given in Table 5, and illustrated by an Accuracy Profile in Figure 5.


Table 5 Results in log cfu/g of the enumeration in raw beef by the reference methods and TC Dry EC

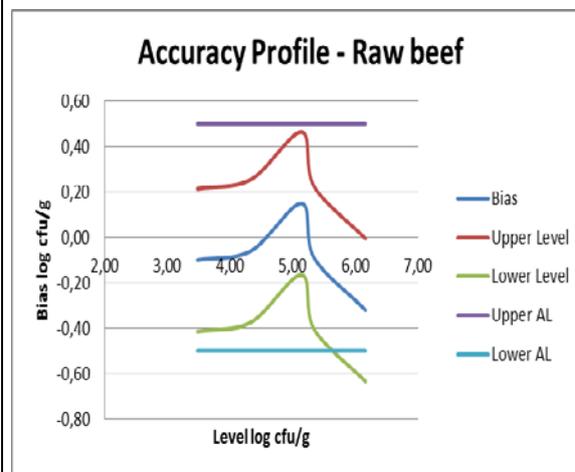|	*E.coli*		Coliforms	
Methods	**ISO 16649**	**Dry EC**	**ISO 4832**	**Dry EC**
concentration range (mean) cfu/g:	1,20 – 5,04	1,16 – 5,11	3,58 – 6,48	3,48 – 6,16
precision; standard deviation, cfu/g:	0,17 - 0,34	0,22 – 0,36	0,077 - 0,23	0,029 – 0,26
Bias log cfu/g		-0,04 – 0,07		-0,32 - 0,15
Lower Level - Upper Level		-0,60 / 0,63		-0,63 / 0,46
Acceptance Level		± 0,97		± 0,50

For the enumeration of E.coli, the combined standard deviations were high for the reference method, resulting in higher AL than ± 0,50 log cfu/g.

Figure 6 Accuracy Profiles for the enumeration of E.coli and total count in raw beef
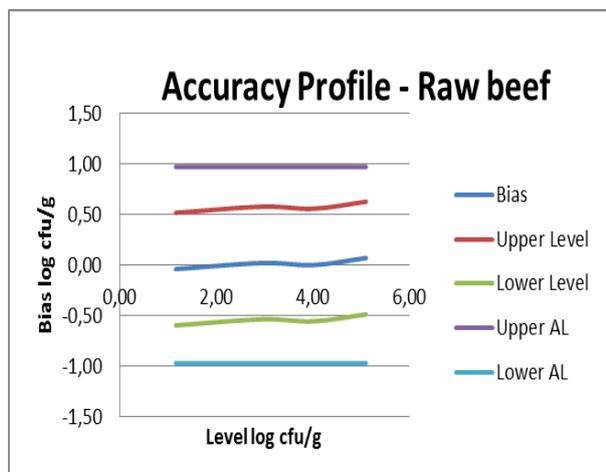
Results E.coli:	*Results coliforms:*

For the highest level for total coliforms, there was a negative bias resulting in that the lower level (green line) is below the lower AL (light blue line). For all the other results, the levels falls within the upper and lower AL.

SELECTIVITY (INCLUSIVITY/EXCLUSIVITY)

The selectivity study was performed according to ISO 16140:2003.

Inclusivity is the ability of an alternative method to detect the target analyte from a wide range of strains.

For E.coli: 31 strains (at 2-3 log cfu/ml) were studied. All 31 strains grew and produced typical colonies on the Compact Dry EC medium. By comparison, 5 strains failed to grow in the TBX medium (ISO16649-2:2001) and one strain yielded atypical colonies. This could be attributed to the composition of this medium or the sensitivity of these strains to the temperature of the molten medium, which was not investigated further.

For coliforms: 31 strains (at 2-3 log cfu/ml) were studied. All 32 strains grew and produced typical colonies in VRBGA (ISO 4832) and on Compact Dry EC medium.

Exclusivity is the lack of interference from a relevant range of non-target strains of the alternative method.

For E.coli: 21 strains (at 2-3 log cfu/ml) were studied. 18 of the 20 strains did not interfere. Two strains of *Shigella* did yield typical colonies by both methods which are not surprising, because strains of *Shigella* have E-glucuronidase activity which would give rise to typical conies with chromogenic media developed to show this activity.

For coliforms: 20 strains (at 2-3 log cfu/ml) were studied. 10 strains did not grow on the EC medium, 8 were atypical and 2 (both *Shigella sonnei*) appeared typical. By comparison, 7

stains failed to grow in VRBA (ISO 4832), 4 strains were atypical and 8 were typical in appearance.

CONCLUSION OF THE COMPARISON STUDIES

For E.coli: The results of the method comparison study showed that the Compact Dry EC provide equivalent results to the reference method ISO 16649-2:2001. The lowest validated level with satisfactory precision varies from 0,8 – 2,8 log cfu/g depending on the matrix.

For coliforms: The results of the method comparison study showed that the Compact Dry EC provide equivalent results to the reference method ISO 4832:2006. The lowest validated level with satisfactory precision varies from 0,9 – 3,6 log cfu/g depending on the matrix.

INTERLABORATORY STUDY OF *E.COLI*:

The interlaboratory study was conducted in November 2007.

Number of laboratories: 9 [13 labs participated. 2 were excluded as the analysis were not performed on the agreed date and further 2 labs failed to test their samples for *E.coli* by the reference method.]
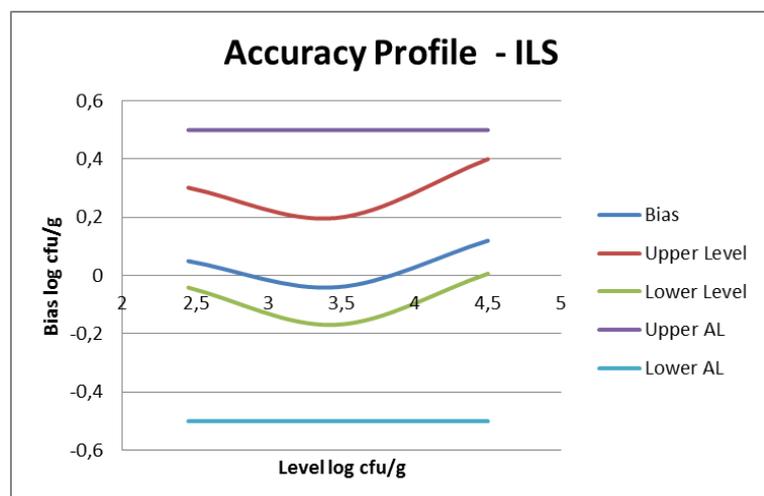
Samples: Pasteurised milk artificially contaminated with defined numbers of *E.coli.* The laboratories performed the analyses according to ISO 16649-2:2001 and Compact Dry EC method.

Table 6 Results (log cfu/g) – interlaboratory study of E.coli

	Referance method		Alternative method			Upper	Lower	Upper	Lower
Level	Median	S_R	Alt method	S_R	Bias	Level	Level	AL	AL
1	2,40	0,17	2,45	0,18	0,05	0,30	-0,04	0,50	-0,50
2	3,50	0,17	3,46	0,17	-0,04	0,20	-0,17	0,50	-0,50
3	4,38	0,34	4,50	0,20	0,12	0,40	0,01	0,50	-0,50

The results show that the bias is small and that the precision is satisfactory.

Figure 7 Accuracy Profile of the interlaboratory study for E.coli

According to the comparison and the interlaboratory study no substantial differences were found between the Compact Dry EC method and the reference method (ISO 16649-2:2001) for the enumeration of *Escherichia coli*.

INTERLABORATORY STUDY OF COLIFORMS
The interlaboratory study was conducted in November 2007.
Number of laboratories: 11
Samples: Pasteurised milk artificially contaminated with defined numbers of *E.coli.* The laboratories performed the analyses according to ISO 4832:2006 and Compact Dry EC method.

Table 7 Results (log cfu/g) – interlaboratory study of coliforms

	Referance method		Alternative method			Upper	Lower	Upper	Lower
Level	Median	S_R	Alt method	S_R	Bias	Level	Level	AL	AL
1	2,53	0,16	2,55	0,13	0,02	0,20	-0,09	0,50	-0,50
2	3,59	0,11	3,57	0,19	-0,02	0,25	-0,10	0,50	-0,50
3	4,48	0,075	4,59	0,15	0,11	0,32	0,00	0,50	-0,50

The results show that the bias is small and that the precision is satisfactory.

Figure 8 Accuracy Profile of the interlaboratory study for coliforms
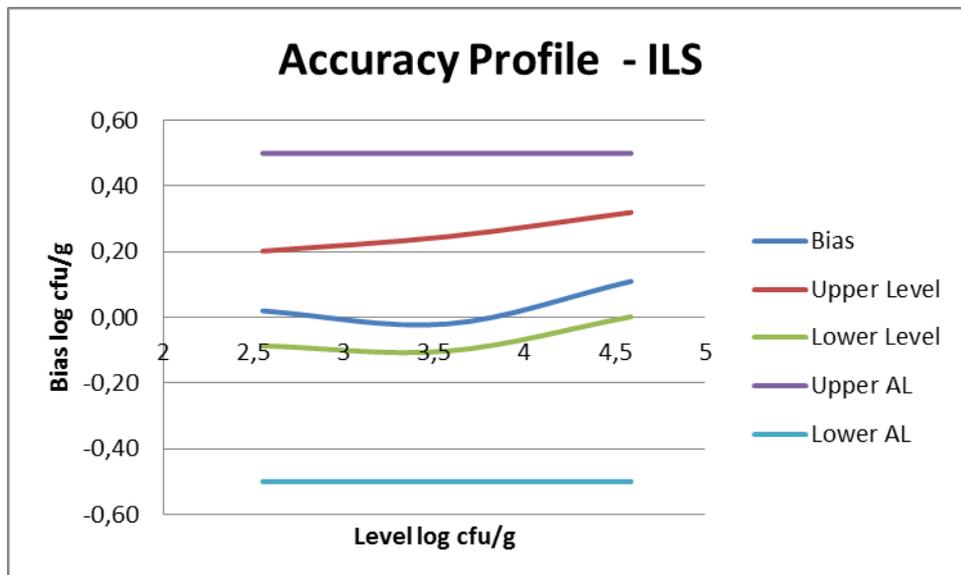
According to the comparison and the interlaboratory study no substantial differences were found between the HyServe Compact Dry EC method and the reference method (ISO 4832:2006) for the enumeration of coliforms.