



NordVal International Certificate

Issued for:	RAPID' <i>E.coli</i> 2 Agar
NordVal No:	020
First approval date:	24 June 2005
Renewal date:	1 December 2017
Valid until:	1 December 2019

RAPID'*E.coli* 2 Agar

Manufactured and supplied by:
Bio-Rad Laboratories,
3 Blvd Raymond Poincaré,
92430 Marnes-la-Coquette,
France

fulfils the requirements of the NordVal validation protocol. The reference methods were ISO 4832:2006 for the enumeration of coliforms in foods at 37 °C, and ISO 16649-2:2001 for the enumeration of *E.coli* in foods at 37 °C and 44 °C.

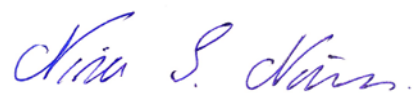
NordVal International has studied the enclosures to the application and evaluated the results obtained in the validations conducted by ISHA in accordance with ISO 16140-2:2016. NordVal has concluded that it has been satisfactorily demonstrated that the requirements of the NordVal validation protocol are fulfilled for broad range of foods. The results document no statistical difference in the performances between the RAPID'*E.coli* 2 Agar and the reference methods.

Date: 1 December 2017

Yours sincerely



Hilde Skår Norli
Chair of NordVal International



Nina Skall Nielsen
NMKL Secretary General



PRINCIPLE OF THE METHOD:

RAPID'*E.coli* 2 is based on simultaneous detection of two enzyme activities; β -D-Glucuronidase (GLUC) and β -D-Galactosidase (GAL).

RAPID'*E.coli* 2 totally inhibits growth of Gram-positive bacteria and of the principal Gram-negative bacteria other than *Enterobacteriaceae*. *Escherichia coli* specificity is obtained by means of simultaneous detection of β -D-Glucuronidase (GLU) (specific to *E. Coli*) and β -D-Galactosidase (GAL) enzymatic activities.

The medium contains 2 chromogenic substrates:

- √ one substrate specific to GLU, causing pink coloration of colonies positive for this enzyme,
- √ one substrate specific to GAL, causing blue coloration of colonies positive for this enzyme.

Coliforms other than *E. coli* (GAL+/GLUC-) form blue to green colonies, *E. coli* (GLU+/GAL+) form violet to pink colonies.

FIELD OF APPLICATION:

The method is applicable for the determination of *E.coli* and total coliforms in a broad range of foods.

HISTORY:

June 1995: The RAPID'*E.coli* 2 was approved using the reference method ISO 4832 (1991): Horizontal method for the enumeration of coliforms – colony-count technique.

2006: ISO 4832 was revised, however it was considered that the validation of alternative methods based on the previous edition were not affected by this revision.

2003: The certificate was renewed with additional studies from 2001 using the reference method ISO 16649-2 (2001): 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.

2004: Interlaboratory Study was carried out on coliforms using RAPID'*E.coli* 2 and ISO 4832.

2017: Additional studies were performed to assure compliance with the new NMKL protocol and the standard ISO 16140-2:2016.

RESULTS OF THE COMPARISON STUDY

Selectivity; inclusivity and exclusivity

For coliforms:

Sixty nine (69) positive strains and thirty (30) negative strains were tested in duplicate by the alternative method. All coliforms strain were cultivated on media and gave characteristic colonies, except for few *Hafnia alvei* strain which give white colonies. These colonies are ONPG negative, that's explained the non-characteristic aspect of the colonies.

Two strains ONPG negative were tested with the reference method (with VRBL incubated at 37°C). One strain gave non characteristics colonies on VRBL and the other didn't grow.

All the negative strains, when they cultivated on the agar media of the alternative method, showed a non-typical aspect, except for a strain of *Shigella sonnei* (β -glucuronidase positive) and two strains of *Salmonella arizonae* (lactose positive).

These three strains were tested with the reference method (with VRBL incubated at 37°C). They gave a typical aspect on the VRBL media. In conclusion, the inclusivity and the exclusivity of the alternative method are satisfactory.

For *E. coli*:

Fifty (50) positive strains and fifty four (54) negative strains were tested in duplicate by the alternative method. All the β -glucuronidase positive *Escherichia coli* strains cultivated on the agar media of the alternative method and gave typical colonies. All the negative strains, when they cultivated on the agar media of the alternative method, showed a non-typical aspect, except for a strain of *Shigella sonnei* (β -glucuronidase positive) and two strains of *Salmonella arizonae* (lactose positive). These three strains were tested with the reference method (pouring in TBX agar media). They also gave a typical aspect on the TBX agar media (blue colonies). These results were observed at both temperatures.

In conclusion, the inclusivity and the exclusivity of the alternative method are satisfactory.

Relative trueness

The relative trueness is the degree of correspondence between the response obtained by the alternative method and the response obtained by reference methods on identical samples. According to ISO 16140-2, five categories should be tested including three different types and at least 15 samples each category. RAPID'*E.coli* 2 is compared against both reference methods, fulfilling the requirement to the number of samples. The results are illustrated in so called Bland-Altman plot for the difference in the response (bias), including 95% confidence interval of the bias. Bland-Altman difference plot for all categories are shown in Figure 1-3, for the different reference methods and temperatures.

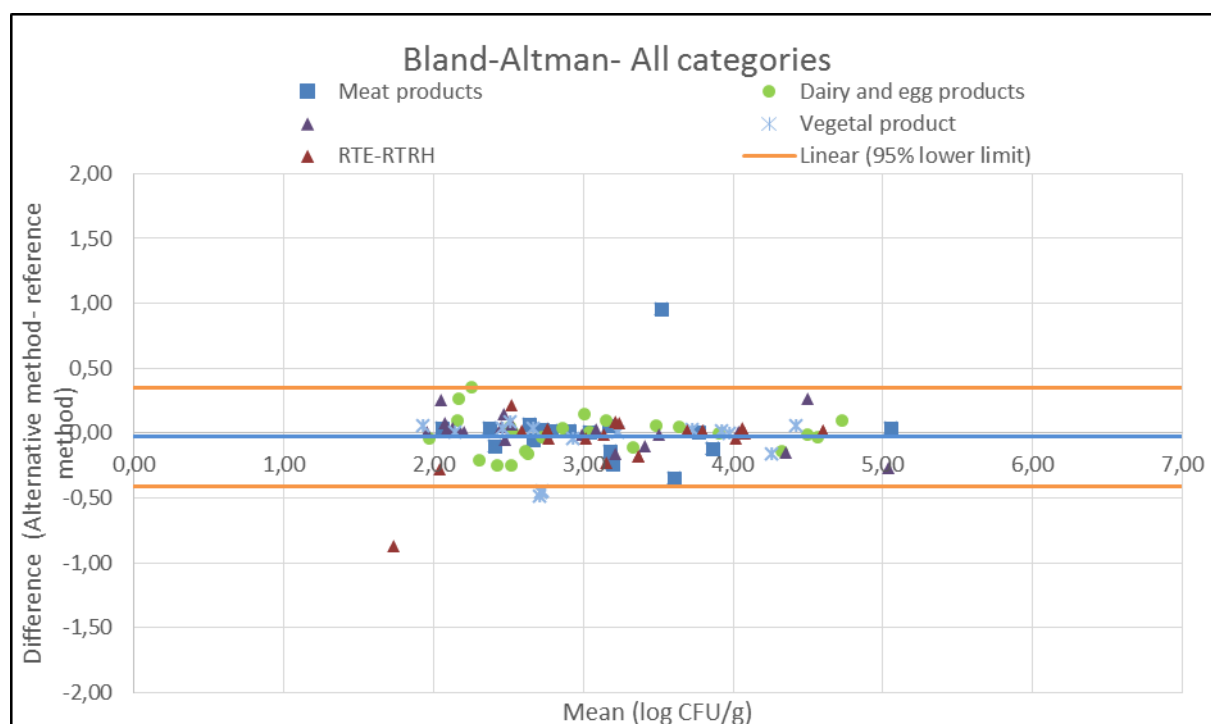


Figure 1. Comparison to reference method ISO 4832

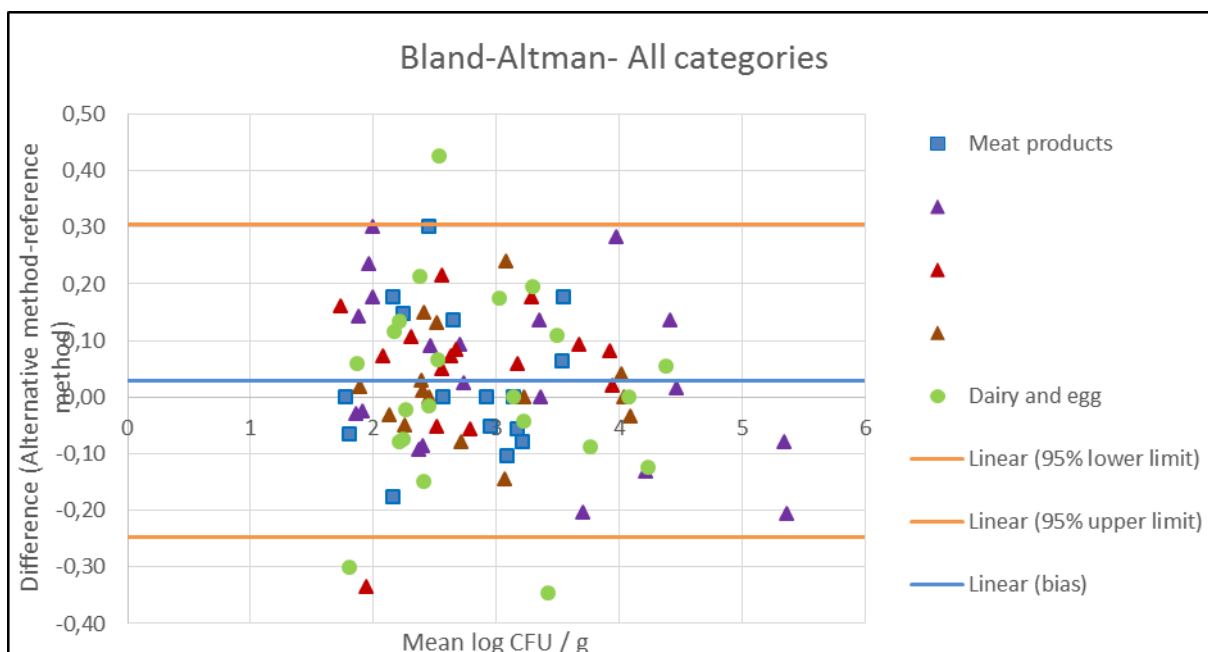


Figure 2. Comparison to reference method ISO 16649-2 at 37 °C

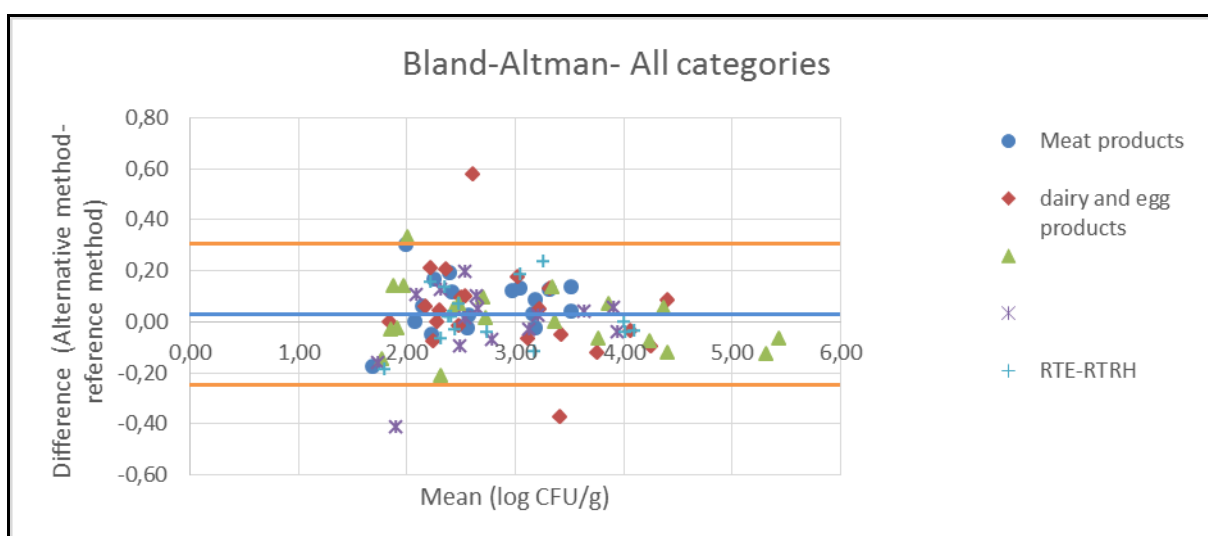


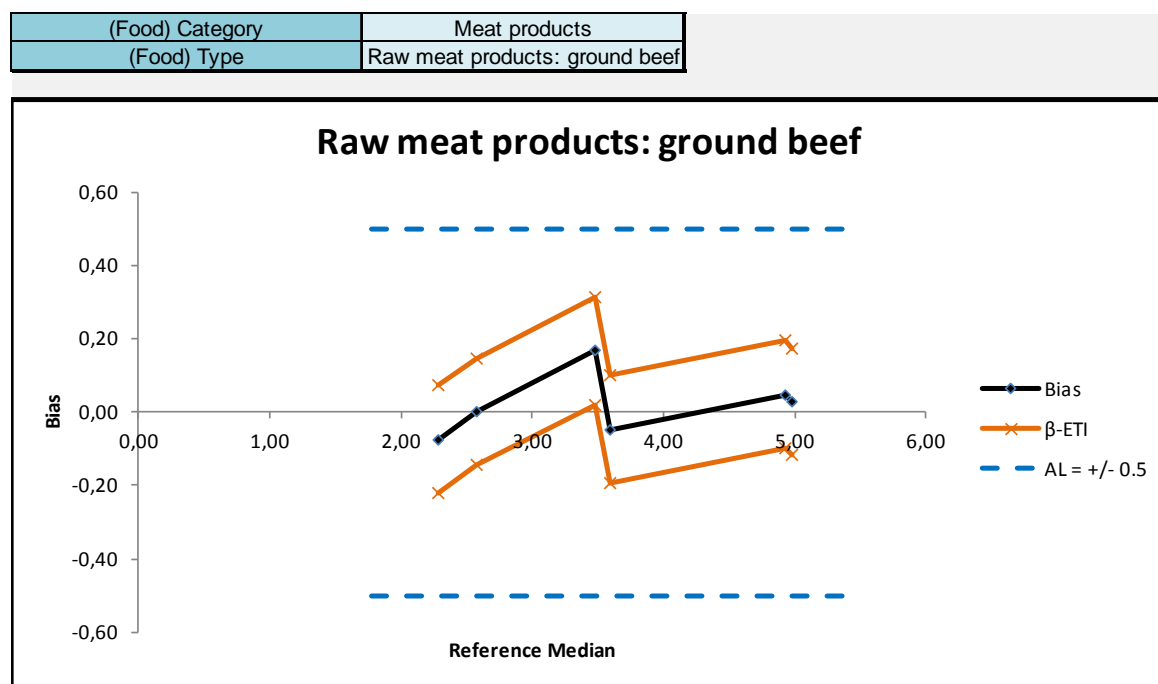
Figure 3. Comparison to reference method ISO 16649-2 at 44 °C

It will be expected that no more than 1 in 20 data values will lie outside the 95% confidence levels (upper limit and lower limits). In conclusion, the relative trueness of the alternative method is satisfactory compared to reference method ISO 4832, and to ISO 16649-2 regardless of temperature (37 or 44 °C).

Accuracy profiles

For determination of accuracy profiles, the five different categories, meat products, dairy products, seafood products, vegetable products and composite foods were inoculated at low, medium and high level i.e. 2.5, 3.5 and 5.0 log CFU/g with a coliform strain for comparison with reference method ISO 4832 or with an *E. coli* strain for comparison with reference method ISO 16649-2.

Figure 4 is an example of the accuracy profiles for the reference methods against the alternative method.



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
MML1T1	2,28	-0,075	-0,220	0,071	YES	YES
MML2T1	2,58	0,000	-0,146	0,146	YES	YES
MML1T2	3,48	0,166	0,021	0,312	YES	YES
MML2T2	3,59	-0,047	-0,193	0,099	YES	YES
MML1T3	4,93	0,048	-0,098	0,194	YES	YES
MML2T3	4,97	0,027	-0,119	0,173	YES	YES

	Reference method	Alternative method	SD repeatability of reference method ≤ 0.125	Final AL
SD Repeatability	0,210	0,101	NO	+/- 0,500

Figure 4: accuracy profiles ($\beta = 80\%$, AL = 0.5) (reference method, ISO 4832, versus alternative method).

The accuracy profiles of all the categories, with β at 80% and AL (Acceptable Limit) at 0.5, the upper limits of the β -expectation tolerance interval (β -ETI) are inferior to the positive AL of 0.5 and the lower limits of the β -ETI are superior to the negative AL of -0.5. For all samples, the results fall within the AL ± 0.5 . In conclusion, the accuracy profiles are satisfactory in the range of contamination for coliforms and *E. coli* enumeration.

RESULTS OF THE COLLABORATIVE STUDY

For coliform bacteria

An interlaboratory study was performed in November 2004. The samples were sent to 16 collaborators. The collaborators received 8 samples of pasteurized milk (2 vials per contamination level; 4 contamination levels) to perform the analyses with the reference method ISO 4832 and with the alternative method RAPID'*E.Coli* 2. The two strains used for the contamination of the samples were 1) an *Escherichia coli* β -glucuronidase positive isolated from a pastry and 2) an *Enterobacter cloacae* isolated from milk powder.

Table 1: Results of the interlaboratory test for coliforms at 37°C. Reference method was ISO 4832.

Levels	Alternative method			Reference method		
	Low	Medium	High	Low	Medium	High
Target value	1,872	2,934	3,961			
Number of participants	10	10	10	10	10	10
Average for alternative method	1,950	2,977	4,017	1,872	2,934	3,961
Repeatability standard deviation (sr)	0,082	0,037	0,049	0,050	0,046	0,069
Between-labs standard deviation (sL)	0,000	0,065	0,062	0,083	0,082	0,092
Reproducibility standard deviation (sR)	0,082	0,075	0,079	0,097	0,094	0,115
Bias	0,078	0,042	0,056	11,697	11,409	12,803
Relative Lower TI limit (beta = 80%)	-0,034	-0,064	-0,054			
Relative Upper TI limit (beta = 80%)	0,190	0,149	0,166			
Lower Acceptability Limit	-0,50	-0,50	-0,50			
Upper Acceptability Limit	0,50	0,50	0,50			

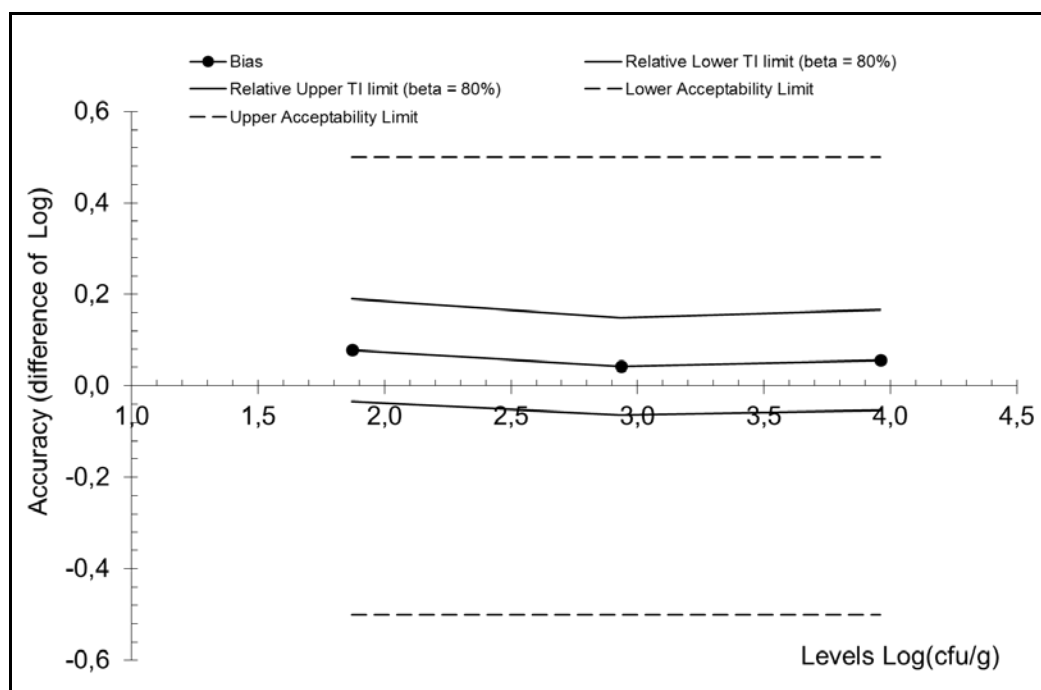


Figure 5. Graphical representation of the accuracy profile of the interlaboratory study for coliform test at 37°C. Reference method was ISO 4832.

For *E. Coli*

An interlaboratory study was performed in November 2001. The samples were sent to 16 collaborators. The collaborators received 8 samples of pasteurized milk (2 vials per contamination level ; 4 contamination levels) to perform the analyses with the reference method ISO 16649-2 and with the alternative method RAPID'*E.Coli* 2. The strain used for the contamination of the samples was an *Escherichia coli* β -glucuronidase positive isolated from pastry. Comparison was performed at 37 and 44°C.

Table 2: Results of the interlaboratory study for RAPID'*E. coli* 2 at 37°C. Reference method was ISO 16649-2

Levels	Alternative method			Reference method		
	Low	Medium	High	Low	Medium	High
Target value	1,605	2,659	3,721			
Number of participants	10	10	10	10	10	10
Average for alternative method	1,724	2,715	3,751	1,605	2,659	3,721
Repeatability standard deviation (sr)	0,094	0,042	0,061	0,113	0,050	0,093
Between-labs standard deviation (sL)	0,000	0,070	0,000	0,113	0,117	0,082
Reproducibility standard deviation (sR)	0,094	0,082	0,061	0,160	0,127	0,124
Bias	0,119	0,057	0,030	14,503	10,473	15,296
Relative Lower TI limit (beta = 80%)	-0,009	-0,059	-0,053			
Relative Upper TI limit (beta = 80%)	0,247	0,173	0,112			
Lower Acceptability Limit	-0,50	-0,50	-0,50			
Upper Acceptability Limit	0,50	0,50	0,50			

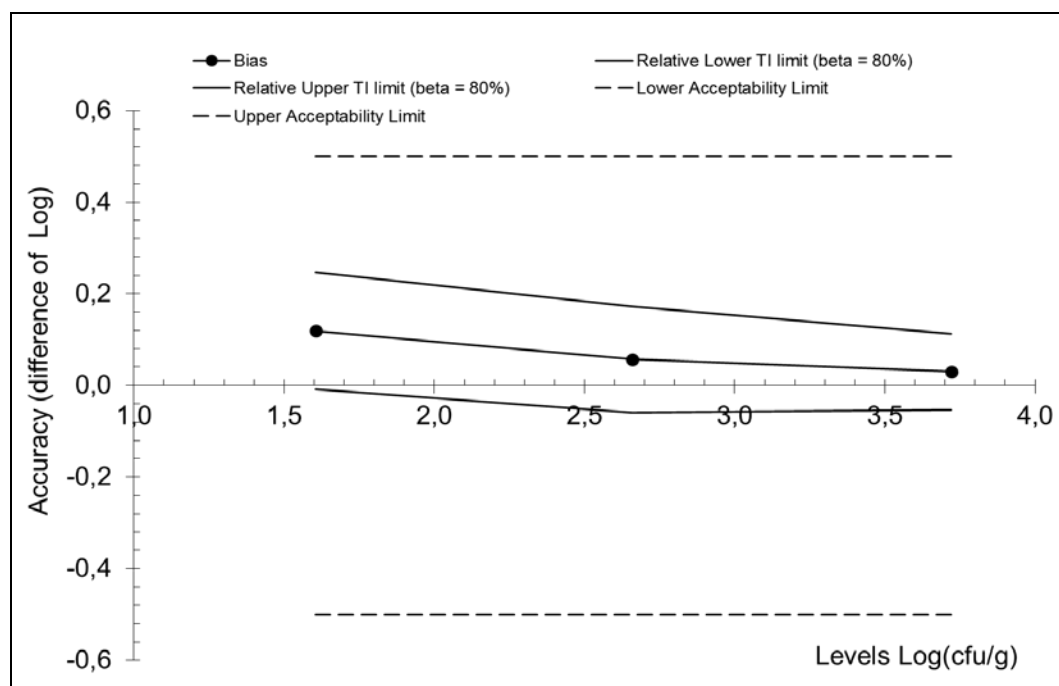


Figure 6. Graphical representation of the accuracy profile of the interlaboratory study for *E. coli* test at 37°C. Reference method was ISO 16649-2

Table 3: Results of the interlaboratory for *E. coli* test at 44°C. Reference method was ISO 16649-2

Levels	Alternative method			Reference method		
	Low	Medium	High	Low	Medium	High
Target value	1,485	2,571	3,676			
Number of participants	13	14	14	13	14	14
Average for alternative method	1,597	2,662	3,711	1,485	2,571	3,676
Repeatability standard deviation (sr)	0,067	0,060	0,058	0,173	0,119	0,114
Between-labs standard deviation (sL)	0,106	0,055	0,085	0,000	0,179	0,097
Reproducibility standard deviation (sR)	0,125	0,081	0,103	0,173	0,215	0,150
Bias	0,112	0,091	0,035	24,960	17,615	22,388
Relative Lower TI limit (beta = 80%)	-0,061	-0,019	-0,106			
Relative Upper TI limit (beta = 80%)	0,285	0,201	0,176			
Lower Acceptability Limit	-0,50	-0,50	-0,50			
Upper Acceptability Limit	0,50	0,50	0,50			

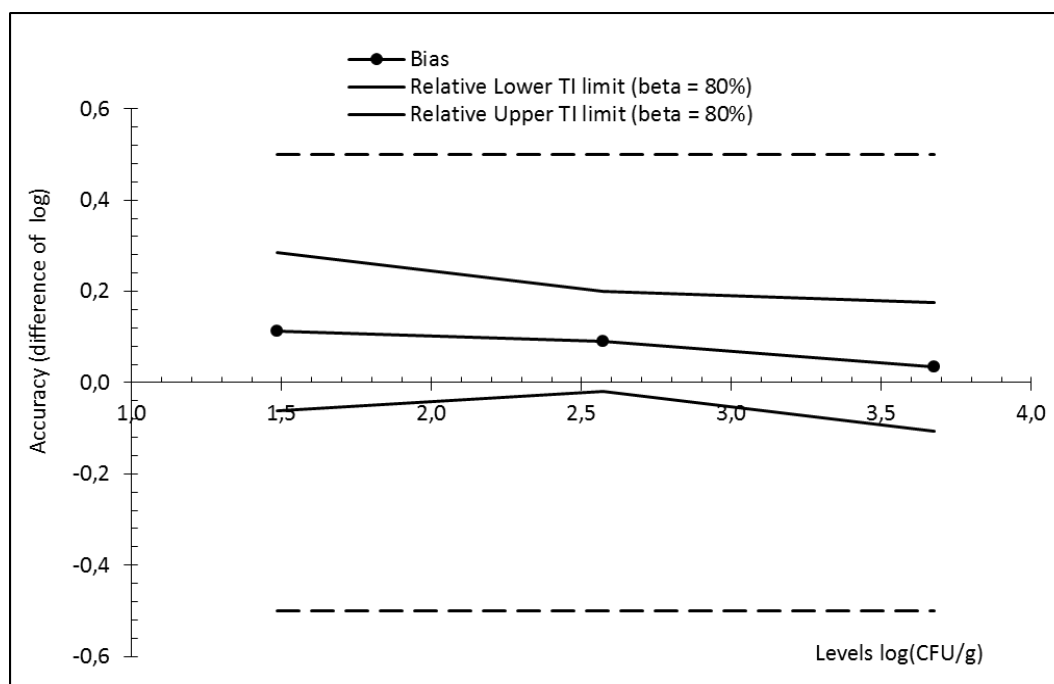


Figure 7. Graphical representation of the accuracy profile of the interlaboratory study for *E. coli* test at 44°C. Reference method was ISO 16649-2

CONCLUSION:

According to the comparison and the collaborative study no statistical differences were found between RAPID'*E. coli* 2 Agar and the reference methods, ISO 4832:2006 and ISO 16649-2: 2001.