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NF VALIDATION
Validation of alternative analytical methods
Application in food microbiology

Summary report

EN ISO 16140 validation study of the D-Count® TVC method for enumeration of mesophilic aerobic flora

(BIO 12/36 – 10/14)

Quantitative method

This report includes 53 pages, with 9 appendixes.
Only copies including the totality of this report are authorized.

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Before comment

Quality assurance documents related to this study can be consulted upon request from bioMérieux.

The technical protocol and the result interpretation were realized according to the EN ISO 16140 and the AFNOR technical rules.

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✓ **Expert Laboratory**:	ADRIA Développement ZA Creac'h Gwen 29196 QUIMPER Cedex
✓ **Studied method:**	**D-Count® TVC method for enumeration of mesophilic aerobic flora**
✓ **Validation standard**:	EN ISO 16140 (October 2003) : Food microbiology – Protocol for the validation of alternative methods
✓ **Reference method**♦ :	ISO 4833-1 - Microbiology of the food chain -- Horizontal method for the enumeration of microorganisms -- Part 1: Colony count at 30 degrees C by the pour plate technique
✓ **Scope:**	**UHT milks, pasteurized dairy based desserts, ice creams prior to frozen process and meat products**
✓ **Certification organism**:	AFNOR Certification

♦ Analyses performed according to the COFRAC accreditation

1 INTRODUCTION

The D-*Count*® TVC method for enumeration of mesophilic aerobic flora was validated according to the EN ISO 16140 protocol and the AFNOR Technical Rules in October 2014.

During the validation study, the following criteria were evaluated:

- *Method Comparison Study*:
- Linearity study,
- Relative accuracy,
- Detection limit and quantification limit,
- Relative sensitivity,
- Praticability,

- *Inter-laboratory study*.

2 METHOD PROTOCOLS

2.1 Alternative method

The protocol of the alternative method is described in **Appendix 1**.

The D-*Count*® TVC method allows a direct detection of viable bacteria in samples.
Samples are treated with reagents that label viable micro-organisms when potentially present. Only viable micro-organisms are able to enzymatically cleave the non-fluorescent substrate inside the cell to liberate and to accumulate the resulting fluorochrome. Then, the sample is injected into the quartz flow cell of the D-*Count*® TVC, forming a narrow and laminar flow stream that ensures micro-organisms pass the laser excitation beam one by one.

2.2 Reference method ♦

The reference method used for this validation was the ISO 4833-1 “Microbiology of the food chain - Horizontal method for the enumeration of microorganisms - Part 1: Colony count at 30 degrees C by the pour plate technique” (See **Appendix 2**).

♦ Analysis performed according to the COFRAC accreditation

3 VALIDATION STUDY RESULTS

3.1 Method Comparison Study

3.1.1 Linearity study

Linearity is the ability of the method when used with a given matrix to give results that are in proportion to the amount of analyte present in the sample, that is an increase in analyte corresponds to a linear or proportional increase in results.

3.1.1.1 Food matrices and protocols

Three matrix/strain pairs were tested. Five contamination levels were done and five replicates were analyzed per level. The tested matrices, the inoculated strains, the contamination levels and the tested incubation times are listed in Table 1.

Table 1

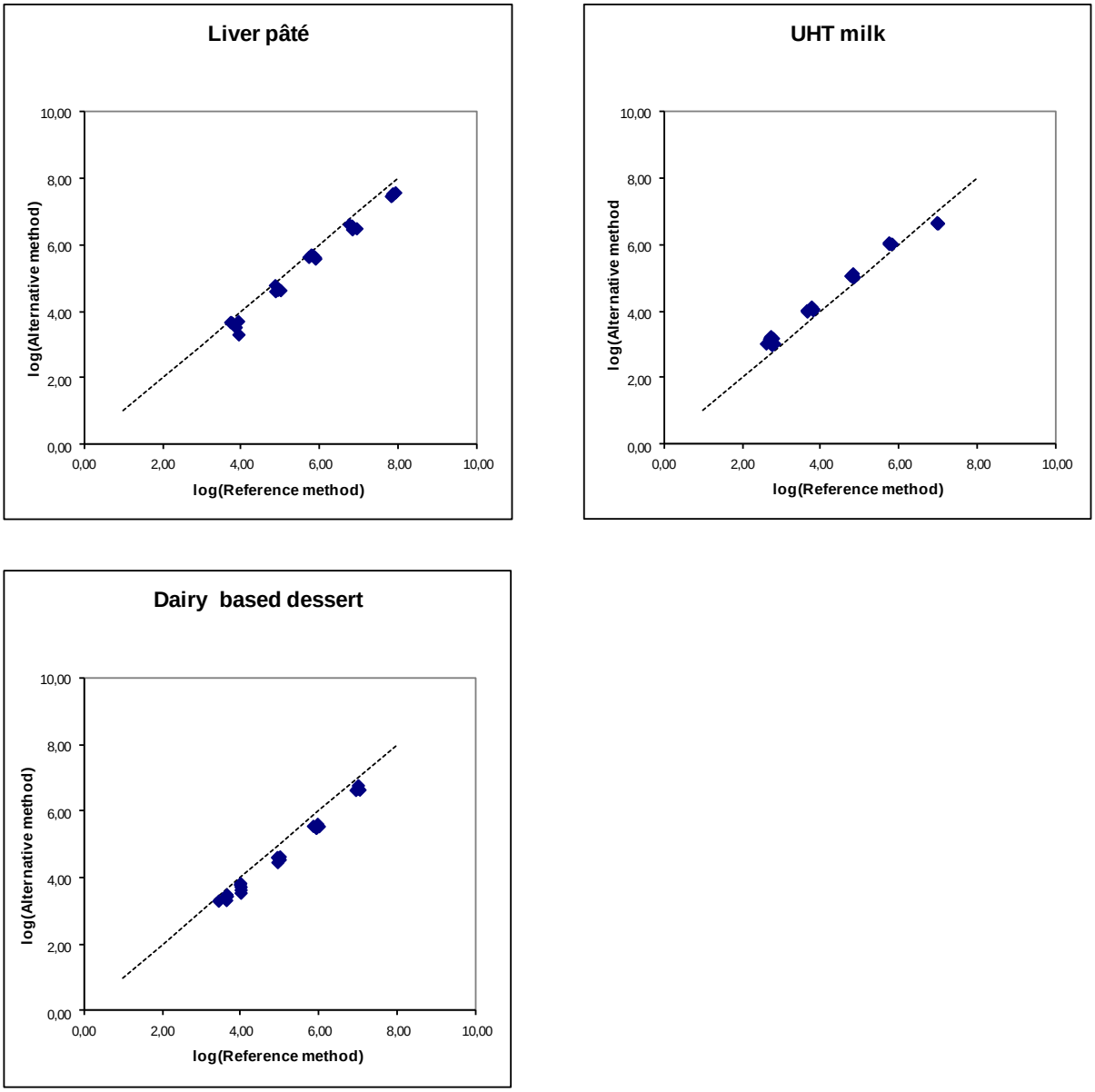
Matrix	Strain	Inoculation levels
Liver pâté	*Escherichia coli* Ad 239	5.10^3 5.10^4 5.10^5 5.10^6 5.10^7
UHT milk	*Bacillus licheniformis* Ad 880	5.10^2 5.10^3 5.10^4 5.10^5 5.10^6
Dairy based dessert	*Bacillus licheniformis* Ad 880	

The raw data are provided in **Appendix 3**.

3.1.1.2 Calculations and interpretations

The bi-dimensional graphs are given in Figure 1.

Figure 1 – Linearity: bi-dimensional graphs



3.1.1.3 Statistical results

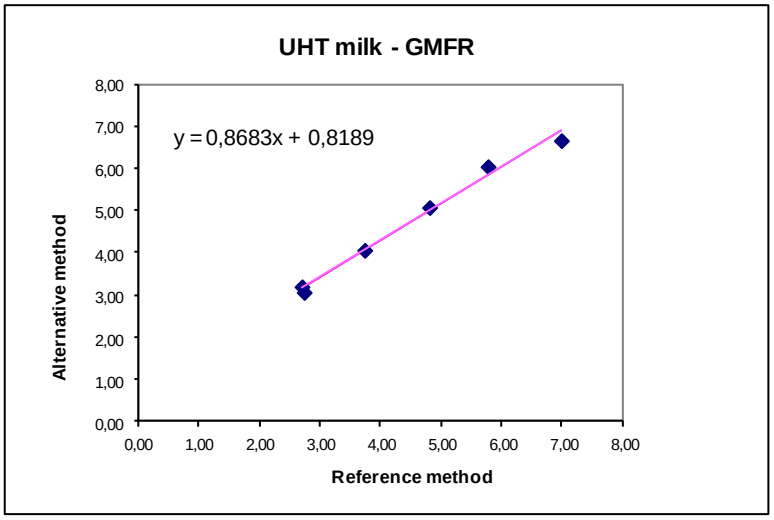
A summary of the results is presented in Table 2.

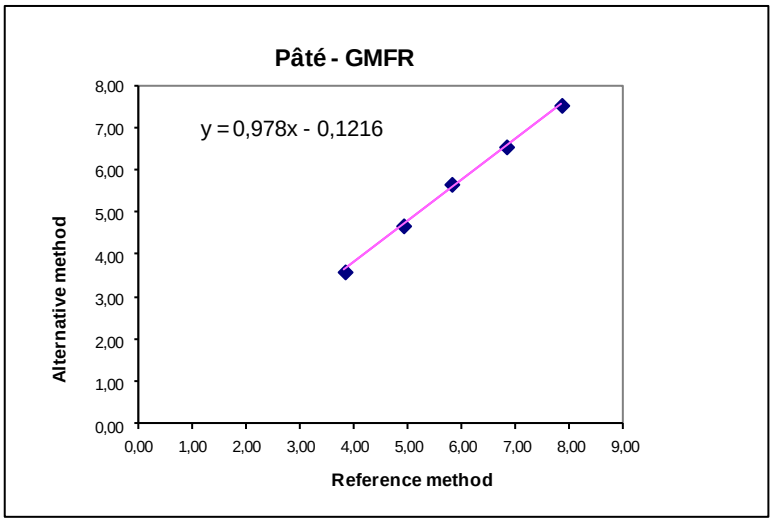
Table 2 – Statistical interpretations

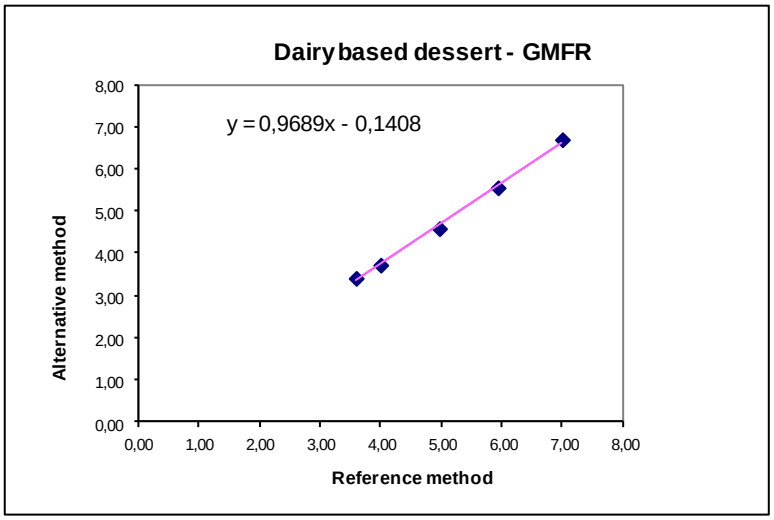
Matrix	R	Selected regression	Rob. F	Critical value	P%	Correlation coefficient	Regression equation
Pâté	0.89	GMFR	12.433	2.120	0	0.999	log Alt. = 0.978 log Ref. – 0.122
UHT milk	1.46	GMFR	178.84	2.120	0	0.995	log Alt. = 0.868 log Ref. + 0.819
Dairy based dessert	1.93	GMFR	15.671	2.120	0	0.999	log Alt. = 0.969 log Ref. – 0.141

The regression lines are provided in Figure 2.

Figure 2 – Linearity: regression lines







3.1.1.4 Conclusion

All the regression lines show satisfying slopes and origin ordinates, except a slight surestimation with the D-Count® TVC method in comparison to the ISO 4833 standard (UHT milk/*Bacillus licheniformis* Ad 880 matrix/strain pair). This is mainly due to the lowest inoculation level; the differences between the compared methods vary from 0.60 to 0.75 log CFU/g for 3 replicates among the 5 tested replicates. For the other inoculation levels, the differences are lower than 0.5 log CFU/g and closed to 0. The results observed for the accuracy part on the UHT milks doesn't confirm this observation. Two hypotheses are possible: artifact linked to the inoculation of a spore-former bacteria or artifact linked to natural contamination of the matrix at a low level.

In order to confirm or not an effect linked to the ability of the strain to produce spores in the inoculums, the same strain was inoculated in the dairy based dessert. The enumeration differences observed between the two methods are all below 0.3 log CFU/g at the low contamination level. The phenomenon is thus probably due to the tested UHT milk which is contaminated with a low level of HRS strains (Highly Heat Resistant Spore), which are able to resist to UHT treatments.

The calculated correlation coefficients are all satisfying, with values closed to or higher than 0.995. The D-Count® TVC method shows a satisfying linearity, in comparison to the ISO 4833 standard.

3.1.2 Relative accuracy

The relative accuracy is the closeness of agreement between a test result and the accepted reference value.

Relative specificity is defined as the degree to which a method is affected (or not) by the other components present in a multi-component sample; that is, it is the ability of the method to measure exactly a given analyte, or its amount, within the sample without interference from non-target components such as matrix effect or background noise.

Relative sensitivity is defined as the ability of the alternative method to detect two different amounts of analyte measured by the reference method within a given matrix over the whole measurement range; that is, it is the minimal quantity variation (increase of the analyte concentration x) which gives a significant variation of the measured signal (response y).

3.1.2.1 Number and nature of samples

The number of samples analysed and exploited per category and analytical protocol is provided in Table 3.

Table 3 - Distribution per tested category and type

Category	Analytical protocol	Number of samples analysed	Number of samples exploited
Meat products	Meats	13	12
Dairy products	UHT milks	13	12
	Dairy based desserts	20 (1)	11
	Total	**46**	**35**

(1) Two samples not included in the scope of the method were analysed (request of the AFNOR Technical Committee): one sample contaminated with molds and one with yeasts. Yeasts were detected in sample n° 4008 (*Pichia Guilliermondii, Rhodorula glutinis* and *Derbarcymones hansenii*) and molds were detected in sample n° 4389.
These 2 samples were not included for statistical interpretation, but showed consistency results with the two methods.

A micro-organisms group particularly wide, defined by the capacity to grow at 30°C in aerobic conditions, was studied in this study. Some targets can be caracterised with a growth temperature: 30°C, 35°C or 37°C for example. Other targets can grow better in anaerobic conditions than in aerobic conditions. This can explain some enumeration differences between the two methods, differences which are below 1 log CFU/g.

3.1.2.2 Results

All the samples were naturally contaminated samples.

The raw data are provided in **Appendix 4**.

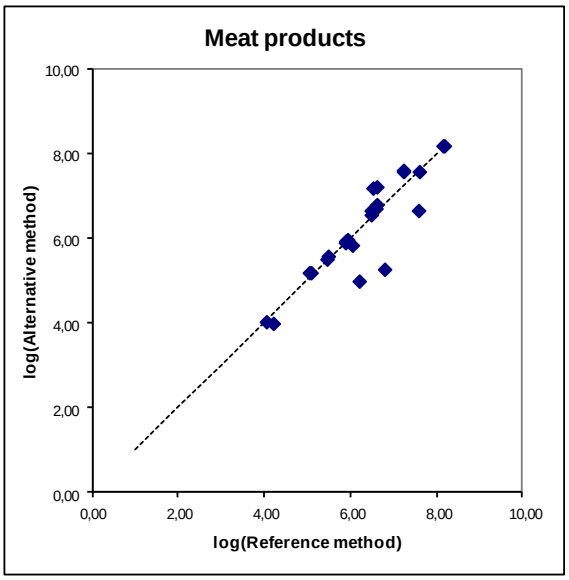
Each sample was analyzed in duplicate with the reference and the alternative methods. The contamination range is presented in Table 4.

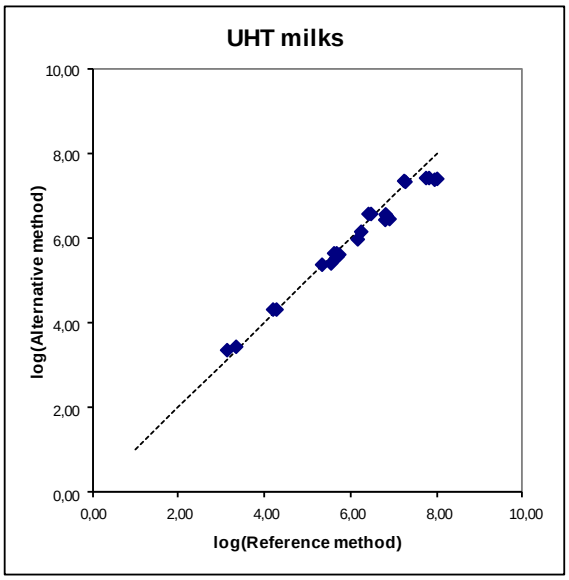
Table 4 – Contamination level

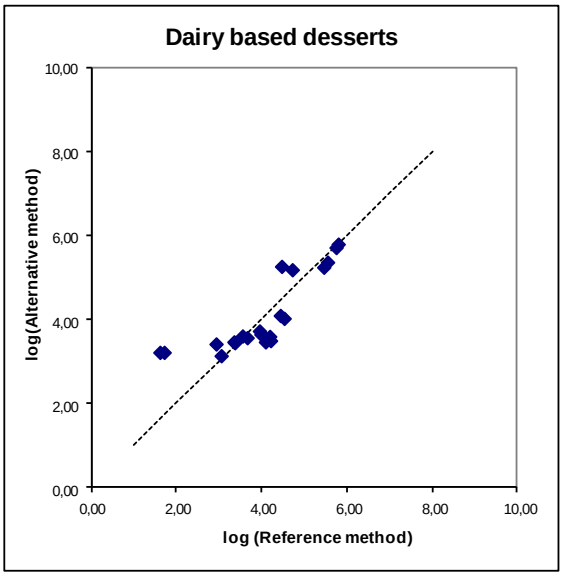
Food category	Analytical protocol	Contamination level (log CFU/g)
Meat products	Meat	4.00 à 8.20
Dairy products	UHT milks	3.11 à 8.00
	Dairy based desserts	1.60 à 5.79

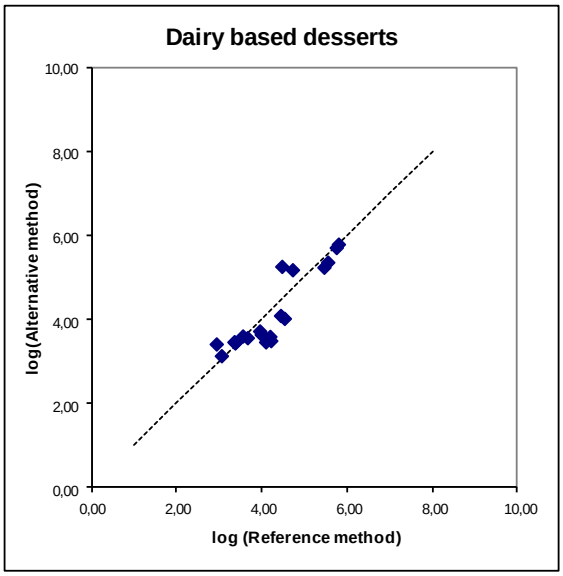
The bi-dimensional graphs are given Figure 3.

Figure 3 – Relative accuracy: bi-dimensional graphs

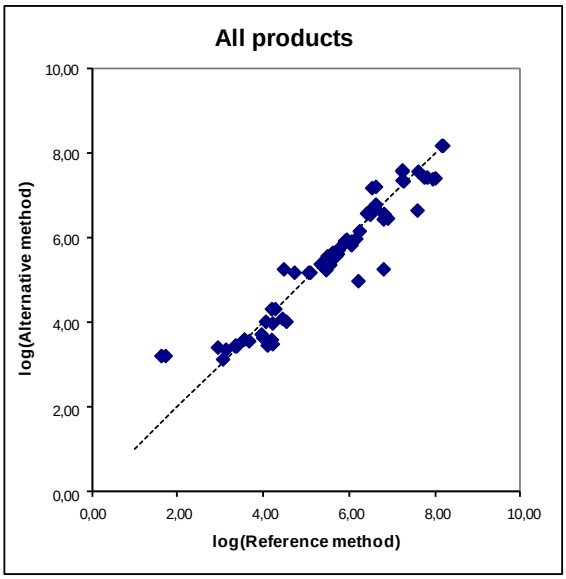








Without Sample n° 5643



3.1.2.3 Statistical interpretation

The results of the statistical interpretation are given in Table 5.

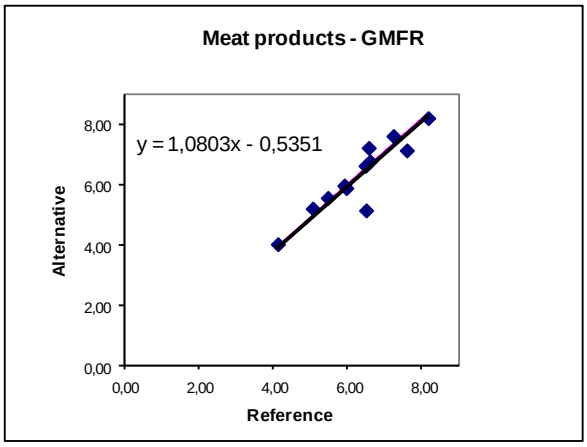
Table 5 – Statistical interpretation

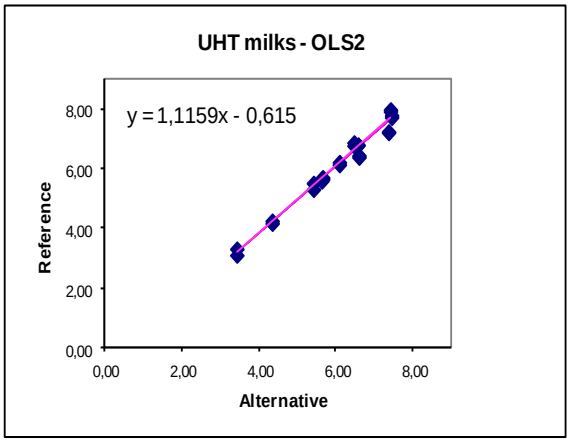
Category	n	R	Regression	a	t(a)	b	t(b)	Critical T	P %	
									Slope	Intercept
Meat products	12	1.43	GMFR	- 0.535	0.579	1.080	0.556	2.228	58	59
UHT milkds	12	0.33	OLS2	- 0.615	2.936	1.116	3.404	2.228	1	0
Dairy based desserts	11	0.89	GMFR	0.874	1.404	0.799	1.341	2.262	19	21
without Sample n° 5643	10	0.89	GMFR	-0.256	0.377	0.157	0.260	2.306	72	80
All products	**35**	**0.57**	**GMFR**	**0.350**	**1.271**	**0.933**	**1.387**	**2.035**	**21**	**17**

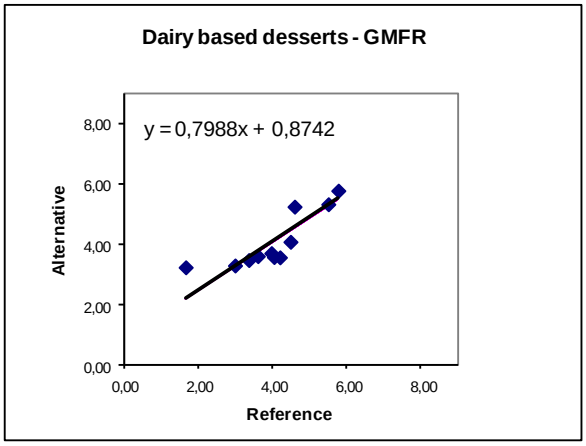
Category	Bias D (median)	Repeatability Alternative method	Repeatability Reference method
Meat products	0.072	0.150	0.103
UHT milks	- 0.023	0.075	0.220
Dairy based desserts	0.005	0.240	0.264
All products	**0.005**	**0.120**	**0.206**

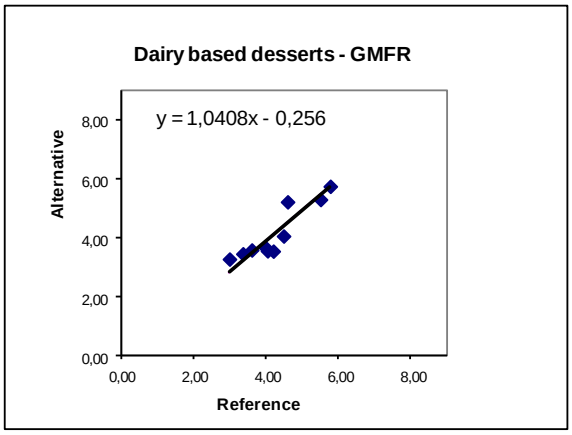
Regression lines (graph and equation representations) for each food category and for all matrices are presented in Figure 4.

Figure 4 – Relative accuracy: regression lines

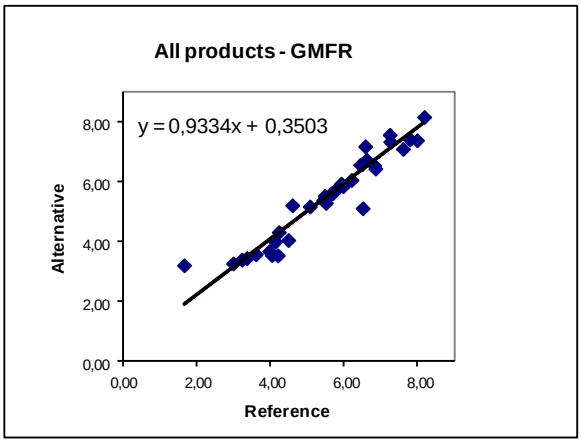








Without sample n° 5643



3.1.2.4 Discussion

Though the study covers only three categories, a statistical for all the products was realised.

The biases between the two methods were low, varying between 0.023 and 0.072 log CFU/g.

The repeatability limits of the D-Count® TVC method and the reference method are similar; they are respectively 0.120 and 0.206 log CFU/g for all products.

Two samples showed an enumeration difference between the two methods, 1.0 and 1.5 log CFU/g:

- sample n° 5649 (cooked ham). The enumerations are higher with the ISO 4833-1 standard;

- sample n° 5643 (vanilla cream). The enumerations are higher with the alternative method. For this sample, the enumeration results are low and have an important impact on the regression line. Indeed, removing these results improves the regression line.

The hypothesis slope close to 1 and ordinate close to 0 are verified with values above 5 % in majority of cases, except for UHT milks. Satisfying results are observed for the slope (1.1) and ordinate (0.6). The repartition of the points on the bisecting line is satisfying.

The D-Count® TVC method shows a satisfying relative accuracy in comparison with the ISO 4833-1 method for the aerobic mesophilic flora enumeration in:

- **Meat products,**
- **UHT milks,**
- **Pasteurised dairy based desserts, ice creams prior to frozen process.**

3.1.3 Detection limit (LOD) and quantification limit (LOQ)

The critical level is the defined as the smallest amount which can be detected (not null), but not quantified as an exact value. Below this value, it cannot be sure that the true value is not null.

The detection limit is defined as being higher than the critical level because it involves a power, the probability 1-β, which has to be well over 50 %, for example 95 %.

The quantification limit is defined as the smallest amount of analyte (that is the lowest actual number of organisms) which can be measured and quantified with defined precision and accuracy under the experimental conditions by the method under validation

3.1.3.1 Protocol

The detection and quantification limits were first determined in pure cultures for the three protocols (meat produces, heat treated milks and dairy based desserts).

Treatments were directly applied on the bacteria suspension for the meat products protocol (enzymatic treatment and centrifugation) and for dairy based desserts (centrifugation).

It was then decided to determine the detection and quantification limits in a sterile matrix.

An *Escherichia coli* strain (ATCC 8739) was inoculated at six levels, with six replicates per level.

3.1.3.2 Results

The raw data are given in **Appendix 5**.

These data are intrinsic to the alternative method and are presented in the following tables:

Table 6 – Meat products protocol

Inoculation level	Number of positive samples	Standard deviation (S0)	Bias X0 (Xoi median)
0	0	/	/
4 000	0	1 689	3 905
8 000	3	3 198	7 640
12 000	6	2 609	12 415

	Calculated values (cfu/ml)
LC	12 917
LOD	18 194
LOQ	39 621

Table 7 – Dairy products protocol

Inoculation level	Number of positive samples	Standard deviation (S0)	Bias X0 (Xoi median)
0	0	/	/
300	0	122	243
500	0	221	624
700	1	349	382
800	2	215	1 040
1 000	6	366	2 217

	Calculated values (cfu/ml)
LC	1 394
LOD	1 749
LOQ	3 188

Table 8 – Dairy based desserts protocol

Inoculation level	Number of positive samples	Standard deviation (S0)	Bias X0 (Xoi median)
0	0	/	/
500	0	178	416
750	1	470	347
1 000	0	236	486
1 250	4	446	1 595
1 500	5	572	1 560
2 000	6	745	1 872

	Calculated values (cfu/ml)
LC	2 332
LOD	3 068
LOQ	6 059

The quantification limits of the D-Count® TVC method are:

- **4.60 Log CFU /g for meat products,**
- **3.50 Log CFU/g for UHT milks,**
- **3.78 Log CFU/g for pasteurized dairy based desserts, ice creams prior to frozen process.**

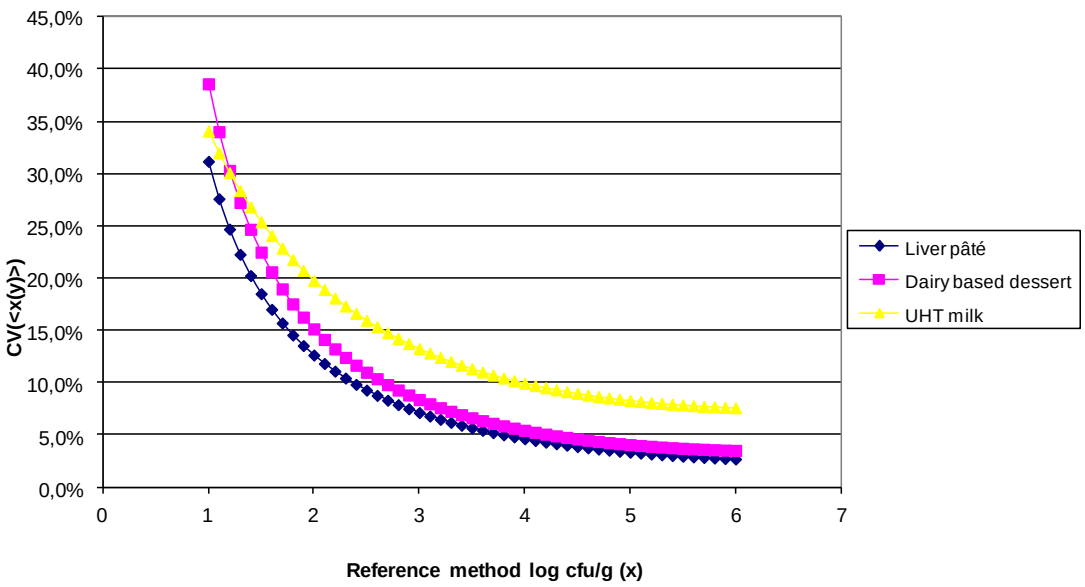
3.1.4 Relative sensitivity

The relative sensitivity is defined as the ability of the alternative method to detect two different amounts of analyte measured by the reference method within a given matrix, at a specified average value, or over the whole measurement range ; that is, it is the minimal quantity variation (increase of the analyte concentration x) which gives a significant variation of the measured signal (response y).

The raw data are given in **Appendix 6**. Data are intrinsic to the method and are obtained from the results of the linearity study.

Sensitivity patterns obtained for tested (matrix/strain) pairs are presented in Figure 5.

Figure 5 – Sensitivity patterns for the tested (matrix/strain) pairs



3.1.5 Practicability

The alternative method practicability was evaluated according to the AFNOR criteria relative to method comparison study.

Packaging and reagent volume	Each type of reagent is in a separate packaging. Depending on the reagents
Storage conditions and shelf-life	Mentioned on the reagent packages
Specific equipment	Equipment usually found in a lab: water centrifuge and the automate
Reagents	The reduction solution can be stored only for 2 hours after reconstitution
Ready to use reagent	All the reagents are ready to use, except the reduction solution.
Training	1 day

Workflow (in minute)

For 1 sample

Steps	Reference method	Alternative method		
		Meat products	UHT milks	Dairy based dessert
Sampling	4	4	0,5	1,5
Dilution and stomaching	2	2	/	/
Sample treatment	/	15	/	10
Automate running	/	15	15	15
Plates preparation	0,5	/	/	/
Inoculation	6	/	/	/
Reading	2.5	0.10	0.10	0.10
Total	15	36.10	15.60	26.60
Time/sample	15	36.10	15.60	26.60

For 20 samples

Steps	Reference method	Alternative method		
		Meat products	UHT milks	Dairy based dessert
Sampling	45	45	7	25
Dilution and stomaching	20	20	/	/
Sample treatment	/	45	/	30
Automate running	/	16	16	16
Plates preparation	15	/	/	/
Inoculation	100	/	/	/
Reading	90	2	2	2
Total	270	128	25	73
Time/sample	13.5	6.4	1.25	3.65

Time to results	Steps	**Reference method**	**Alternative method**
	Sampling	Day 0	Day 0
	Dilution and stomaching	Day 0	Day 0
	Sample treatment	/	Day 0
	Analyses	Day 0	Day 0
	Reading	Day 3	Day 0
Technician background	The same that needed for the reference method		
Common step with the reference method	- No common step for dairy based desserts and UHT milks - Sampling and stomaching steps are common for meat products		
Traceability of the results	Traceability usually done in a microbiology laboratory		
Maintenance	Maintenance of the apparatus described in the utilisation guide		

The D-Count® TVC method allows an enumeration at Day 0, while 3 days are required for the ISO 4833-1 standard.

The alternative method allows reducing time for manipulation when several samples are analysed, optimization of consumables and does not require incubators.

3.2 Inter-laboratory study

3.2.1 Study organisation

Samples were sent to 12 laboratories.

Dairy based dessert samples were inoculated with *Enterobacter* Kobei Ad 706 isolated from milk powder. Inoculation levels targeted were:

- < 10 UFC/g
- 10 000 UFC/g
- 100 000 UFC/g
- 1 000 000 UFC/g.

Each laboratory received 8 samples of 25 g, *i.e.* 2 samples per inoculation level.

Blind coded samples (code is only known by the expert laboratory) were placed in isothermal boxes, which contained cooling blocks, and express-shipped to the different laboratories.

A temperature control flask containing temperature register was added to the package in order to register temperature profile during transport and package delivery.

Samples were shipped in 48h to laboratories of the collaborative study. Sample temperature should be lower or equal to 8°C during transport, and between 0°C - 8°C at arrival.

Collaborative study laboratories and the expert laboratory carried out the analyses with the alternative and reference methods.

In order to evaluate the *Enterobacter* Kobei Ad 706 strain variability during transport, enumerations were performed at different time, *i.e.* inoculation time, after 24 h and 48 h of storage at 2°C.

The collaborative study instructions were sent on April 29, 2014.

3.2.2 Verification of experimental parameters

3.2.2.1 Strain stability during transport

In order to evaluate the *Enterobacter* Kobei Ad 706 strain stability during transport, bacterial count of samples were checked at different times, i.e. inoculation time, after 24 h and 48 h of storage at 4°C.

Six samples (3 contamination levels x 2 samples) were enumerated. The results are reported in table 9.

Table 9 – Mesophilic aerobic count with ISO 4833 and D-Count® TVC methods (in CFU/g)

Day of analysis	Inoculation level	Mesophilic aerobic count (cfu/g)			
		Reference method		Alternative method	
		Replicate1	Replicate2	Replicate1	Replicate2
D0	1	19 000	29 000	12 000	12 000
	2	260 000	190 000	110 000	75 000
	3	1 600 000	1 600 000	640 000	770 000
D1	1	48 000	22 000	9 900	13 000
	2	350 000	310 000	100 000	74 000
	3	2 100 000	2 200 000	870 000	680 000
D2	1	19 000	21 000	7 300	8 100
	2	270 000	250 000	97 000	88 000
	3	2 200 000	2 300 000	830 000	600 000

No evolution of the inoculated strain was observed between Day 0 and Day 2.

3.2.2.2 *Logistic conditions*

Temperature conditions are given below:

Table 10 - Sample temperatures at receipt

Laboratories	Temperature measured by the sensor (°C)	Temperature measured at receipt (°C)	Receipt date and time	
A	3.5	9.0	20/05/2014	15h00
B	4.0	4.5	20/05/2014	11h00
C	4.5	7.5	20/05/2014	10h00
D	4.0	4.5	20/05/2014	14h00
E	5.0	5.0	20/05/2014	12h30
F	3.0	4.0	20/05/2014	13h00
G	**18.5**	**19.4**	20/05/2014	11h15
H	3.0	4.5	20/05/2014	11h00
I	4.0	5.5	20/05/2014	/
J	6.0	6.2	20/05/2014	14h00
K	**15.0**	Not measured	21/05/2014	15h43
L	5.0	5.3	20/05/2014	12h43

- ***Temperature at receipt***

Lab A registered a temperature of 9°C at receipt but the probe measured a temperature of 3.5°C.

Lab G received its package at Day 2 at 19.4°C; this temperature was confirmed by the probe. This Lab didn't proceed to analyses.

Lab K received the samples at Day 2; they decided to not proceed to analyses. The temperature measured by the probe confirmed that it was above the limit.

- ***Temperature during sample storage prior to analysis***

Lab C stored the samples at 8 - 9°C but no impact was observed on the data.

Lab I stored the samples at 8 - 11°C; the enumeration results for this Lab were clearly higher than those expected.

3.2.2.3 Quality Controls and conclusion

Samples were sent to 12 labs:

- Labs G and K didn't proceed to analyses (temperature at receipt above the limit);

- Lab I stored the samples at a higher temperature than that recommended.

Finally, the following labs were retained: A, B, C, D, E, F, H, J and L.

3.2.3 Results analysis

Raw data are provided in **Appendix 7**.

3.2.4 Statistical interpretations and calculations

The calculations were realized according to the amendment number 1 of the ISO 16140 standard (2011):

- ✓ *Accuracy: closeness of agreement between a measurement result and the accepted reference value. Note: Accuracy refers to a combination of trueness and precision*

- ✓ *Trueness: closeness of agreement between the expectation of a measurement result and the accepted reference value. Note: the measure of trueness is usually expressed in terms of bias*

- ✓ *Precision: closeness of agreement between independent measurement results obtained under stipulated conditions. Note: quantitative measures of precision depend critically on the stipulated conditions. Repeatability conditions and reproducibility conditions are particular sets of extreme stipulated conditions*

- ✓ *Repeatability: precision under repeatability conditions*

- ✓ *Repeatability conditions: measurement conditions where independent measurement results are obtained with the same method on identical measurement items in the same laboratory by the same operator using the same equipment within short interval of time.*

- ✓ *Repeatability standard deviation: standard deviation of measurement results obtained under repeatability conditions*

- ✓ *Repeatability limit (r): value less than or equal to which the absolute difference between two measurement results obtained under repeatability conditions is expected to be with a probability of 95%*

- ✓ *Reproducibility: precision under reproducibility conditions*

- ✓ *Reproducibility conditions: measurement conditions where measurement results are obtained with the same method on identical measurement items in different laboratories with different operators using different equipment*

- ✓ *Reproducibility standard deviation: standard deviation of measurement results obtained under reproducibility conditions*

- ✓ *Reproducibility limit (R): value less than or equal to which the absolute difference between two measurement results obtained under reproducibility conditions is expected to be with a probability of 95%*

All the available results are summarized in Tables 11 and 12.

Table 11 – Results synthesis (CFU/g)

Lab.	Level 0				Level 1				Level 2				Level 3			
	Reference method		Alternative method		Reference method		Alternative method		Reference method		Alternative method		Reference method		Alternative method	
A	<10	<10	<1050	<1050	16000	31000	23000	28000	190000	170000	180000	230000	2400000	2300000	2500000	2600000
B	<10	<10	<1050	<1050	21000	27000	51000	62000	260000	120000	690000	250000	1700000	1400000	5200000	3500000
C	<10	<10	<1050	<1050	74000	52000	81000	70000	1100000	510000	1500000	930000	5200000	6700000	12000000	9600000
D	<10	<10	<1050	<1050	22000	18000	34000	980	150000	250000	300000	440000	2200000	2100000	3200000	3300000
E	<10	<10	<1050	<1050	18000	32000	5300	5500	270000	280000	84000	70000	3800000	2200000	950000	780000
F	<10	<10	<1050	<1050	24000	21000	25000	31000	230000	440000	420000	560000	2600000	2400000	4600000	3400000
H	<10	<10	<1050	<1050	21000	40000	38000	64000	220000	220000	510000	310000	2600000	2300000	4400000	5900000
J	<10	<10	2800	<1050	14000	19000	6200	4700	210000	210000	110000	40000	1700000	2100000	250000	190000
L	<10	<10	<1050	<1050	47000	53000	58000	43000	460000	5000000	640000	360000	3700000	4500000	4500000	4000000

Table 12 – Results summary (log CFU/g)

Lab.	Level 0				Level 1				Level 2				Level 3			
	Reference method		Alternative method		Reference method		Alternative method		Reference method		Alternative method		Reference method		Alternative method	
A	<1	<1	<3.02	<3.02	4.204	4.491	4.362	4.447	5.279	5.230	5.255	5.362	6.380	6.362	6.398	6.415
B	<1	<1	<3.02	<3.02	4.322	4.431	4.708	4.792	5.415	5.079	5.839	5.398	6.230	6.146	6.716	6.544
C	<1	<1	<3.02	<3.02	4.869	4.716	4.908	4.845	6.041	5.708	6.176	5.968	6.716	6.826	7.079	6.982
D	<1	<1	<3.02	<3.02	4.342	4.255	4.531	2.991	5.176	5.398	5.477	5.643	6.342	6.322	6.505	6.519
E	<1	<1	<3.02	<3.02	4.255	4.505	3.724	3.740	5.431	5.447	4.924	4.845	6.580	6.342	5.978	5.892
F	<1	<1	<3.02	<3.02	4.380	4.322	4.398	4.491	5.362	5.643	5.623	5.748	6.415	6.380	6.663	6.531
H	<1	<1	<3.02	<3.02	4.322	4.602	4.580	4.806	5.342	5.342	5.708	5.491	6.415	6.362	6.643	6.771
J	<1	<1	3.450	<3.02	4.146	4.279	3.792	3.672	5.322	5.322	5.041	4.602	6.230	6.322	5.398	5.279
L	<1	<1	<3.02	<3.02	4.672	4.724	4.763	4.633	5.663	6.699	5.806	5.556	6.568	6.653	6.653	6.602

3.2.4.1 *Scrutiny of the measurement results for consistency*

In order to identify other measurement results or laboratories that could be inconsistent, two graphical consistency techniques were realized: the robust Mandel's h and k statistics. These graphics are given in **Appendix 8**. All the statistical results are given in **Appendix 9**.

Mandel indicators h and k at 5 % significance highlight some possible inconsistent data:

Table 13

Mandel's values	Number of values above the threshold			
	Reference method		Alternative method	
h > 1 %	Lab L	Level 2	Lab J	Level 3
h > 5 %	Lab C Lab L	Levels 1, 2 and 3 Level 2	Lab E Lab J	Levels 2 and 3 Levels 2 and 3
k> 1 %	Lab L	Level 2	Lab D	Level 1
k > 5 %	Lab E Lab L	Level 3 Level 2	Lab D	Level 1

3.2.4.2 *Comparison of the trueness and precision characteristics of the reference method and alternative methods*

The statistical values are summarized hereafter:

Table 14

Level	Reference method			Alternative method		
	Median	Repeatability standard deviation (Sr)	Reproducibility standard deviation (SR)	Median	Repeatability standard deviation (Sr)	Reproducibility standard deviation (SR)
1	4.377	0.161	0.193	4.445	0.116	0.363
2	5.342	0.166	0.206	5.599	0.200	0.272
3	6.388	0.078	0.178	6.597	0.077	0.234

❒ Bias of the alternative method

In order to estimate the bias of the alternative method with respect to the reference method for each level, Dij and t are calculated as described below:

$$Dij = \bar{Y}\,ij, \mathrm{Alt} - \bar{Y}\,ij, \mathrm{Ref}$$

$$t = \frac{|\mathrm{median}\ i\ (Dij)|}{\sqrt{\pi / (2\ p)}\ \varphi\ Diff}$$

If t is larger than 2, the alternative method is significantly biased with respect to the reference method.

The values obtained for t are given in table 15.

Table 15 – Values obtained for t(d)

Level	Bias D	t (d)	Interpretation
1	0.057	0.50	Non significant bias
2	0.183	2.33	Significant bias
3	0.180	1.37	Non significant bias

According to the statistical tests, the bias is non significant for the inoculation levels 1 and 3. The statistical tests conclude to a significant bias for the inoculation level 2, while a very low bias is observed. Indeed, a value of 0.183 log CFU is fully satisfying.

❒ Comparison of the repeatability standard deviations

If the ratio Srj, Alt / Srj, Ref. of the repeatability standard deviations of the alternative method and the reference method is larger than 2, the precision under repeatability conditions of the alternative method is considered to be lower than that of the reference method. If this ratio is smaller than 0,5, the precision under repeatability conditions of the alternative method is considered to be greater than that of the reference method.

The ratio values are given in table 16.

Table 16

Contamination level	Reference method		Alternative method		Ratio Sr Alt. / Sr Ref.
	Sr Ref.	r Ref.	Sr Alt.	r Alt.	
1	0.161	0.450	0.116	0.324	0.721
2	0.166	0.465	0.200	0.559	1.203
3	0.078	0.217	0.077	0.215	0.991

The ratios of the repeatability standard deviations are comprised below 2 for all the contamination levels; **the precision under repeatability conditions of the alternative method is equivalent to that of the reference method.**

❒ Comparison of the reproducibility standard deviations

If the ratio Srj, Alt / Srj, Ref. of the reproducibility standard deviations of the alternative method and the reference method is larger than 2, the precision under reproducibility conditions of the alternative method is considered to be lower than that of the reference method. If this ratio is smaller than 0,5, the precision under reproducibility conditions of the alternative method is considered to be greater than that of the reference method.

The ratio values are given in table 17.

Table 17

Contamination level	Reference method		Alternative method		Ratio S_R Alt/S_R Ref.
	S_R Ref.	R Ref.	S_R Alt.	R Alt.	
1	0.193	0.541	0.363	1.016	1.877
2	0.206	0.576	0.272	0.762	1.323
3	0.178	0.498	0.234	0.654	1.313

The ratios of the reproducibility standard deviations are comprised below 2 for all the contamination levels, **the precision under reproducibility conditions of the alternative method is equivalent to that of the reference method.**

4 CONCLUSION

The **method comparison study conclusions** are:

- ☒ **The D-Count® TVC method shows linear results in comparison to the ISO 4833 standard.**

- ☒ **In the relative accuracy, the results observed with the D-Count® TVC are globally satisfying.**

- ☒ **The quantification limits determined according to the ISO 16140 standard are 4.60 log CFU/g (meat products), 3.5 log CFU/g (UHT milk) and 3.78 log CFU/g (pasteurized dairy based desserts, ice cream prior to frozen process).**

- ☒ **The D-Count® TVC method allows saving time to results; they are available the day of initializing analyses without any incubation required.**

The **inter-laboratory study conclusions** are:

- ☒ **The biases between the reference and the alternative methods show satisfying values, which are comprised between 0.057 to 0.183 Log CFU/g.**

- ☒ **The repeatability and reproducibility of the alternative method are similar to the repeatability and the reproducibility of the reference method.**

Appendix 1 – Flow diagram of the D-Count® TVC method

Samples preparation

TVC in desserts	TVC in UHT Milk	TVC in meat product
0.5 g sample ↓ Add 4.5 ml of ChemSol A26 ↓ Incubation for 10 min at 37°C ± 2°C ↓ Transfer in a tube 20 ml + ChemFilter 25 ↓ Centrifugation 8 min at 2000 g ↓ Eliminate the supernatant ↓ Anlaysis	0.5 ml sample ↓ Analysis	x g sample ↓ 9 x ml BPW ↓ Stomach for 1 min ↓ 1 ml of stomached sample + 108 µl of lysis reagent ↓ Incubation for 15 min at 37°C ± 2°C ↓ Add 6 ml of ChemSol A7 ↓ Transfer in a tube 20 ml + ChemFilter 25 ↓ Centrifugation 8 min at 2000 g ↓ Eliminate the supernatant ↓ Anlaysis

Appendix 2 – Flow diagram of the ISO 4833-1 method

Non diluted sample (liquid) or sample diluted in BPW (dilution 1/10)

↓

1 ml of each dilution per plate

↓

Pour PCA medium

(+ 1 g skimmed milk powder/l for dairy product analysis)

↓

Incubation 72 h ± 3 h at 30°C ± 1°C

↓

Enumeration

Appendix 3 – Linearity: raw data

N°	Sample	Strain	Inoculation level	Reference method: EN ISO 4833♦				Alternative method: D-Count® TVC	
				Dilution	CFU/ plate	CFU/g	log CFU/g	CFU/g	log CFU/g
4328	Liver pâté	*Escherichia coli* Ad 239	1	100	59	5500	3,74	4900	3,69
				1000	2				
4329				100	88	8500	3,93	2100	3,32
				1000	6				
4330				100	86	8300	3,92	5200	3,72
				1000	5				
4331				100	74	7200	3,86	3500	3,54
				1000	5				
4332				100	56	5200	3,72	4900	3,69
				1000	1				
4333			2	1000	68	74000	4,87	42000	4,62
				10000	13				
4334				1000	105	100000	5,00	45000	4,65
				10000	8				
4335				1000	87	85000	4,93	46000	4,66
				10000	7				
4336				1000	74	72000	4,86	63000	4,80
				10000	5				
4337				1000	95	89000	4,95	48000	4,68
				10000	3				
4338			3	10000	79	780000	5,89	400000	5,60
				100000	7				
4339				10000	63	600000	5,78	520000	5,72
				100000	3				
4340				10000	52	530000	5,72	450000	5,65
				100000	6				
4341				10000	68	660000	5,82	510000	5,71
				100000	5				
4342				10000	77	750000	5,88	440000	5,64
				100000	6				
4343			4	100000	66	6600000	6,82	3900000	6,59
				1000000	7				
4344				100000	55	5600000	6,75	4300000	6,63
				1000000	7				
4345				100000	86	8700000	6,94	3200000	6,51
				1000000	10				
4346				100000	72	6900000	6,84	3500000	6,54
				1000000	4				
4347				100000	69	6800000	6,83	3000000	6,48
				1000000	6				
4348			5	1000000	90	85000000	7,93	39000000	7,59
				10000000	4				
4349				1000000	73	70000000	7,85	33000000	7,52
				10000000	4				
4350				1000000	70	71000000	7,85	32000000	7,51
				10000000	8				
4351				1000000	63	66000000	7,82	30000000	7,48
				10000000	10				
4352				1000000	74	73000000	7,86	36000000	7,56
				10000000	6				

♦ Analyses performed according to the COFRAC accreditation

N°	Produit	Strain	Inoculation level	Reference method: EN ISO 4833♦				Alternative method: D-Count® TVC	
				Dilution	CFU/plate	CFU/g	log CFU/g	CFU/g	log CFU/g
Témoin	UHT milk	/	0	1	0	<1	<0	<1050	<3,02
				10	0				
4383		*Bacillus licheniformis* Ad880	1	1	15	15	1,18	1100	3,04
				10	1				
4384				1	13	12	1,08	<1050	<3,02
				10	0				
4385				1	13	12	1,08	1600	3,20
				10	0				
4386				1	13	14	1,15	<1050	<3,02
				10	2				
4387				1	16	15	1,18	1100	3,04
				10	1				
4378			2	10	68	650	2,81	1460	3,16
				100	3				
4379				10	58	560	2,75	2900	3,46
				100	4				
4380				10	61	600	2,78	1800	3,26
				100	5				
4381				10	47	530	2,72	2100	3,32
				100	11				
4382				10	42	400	2,60	2300	3,36
				100	2				
4145			3	10	46	500	2,70	1700	3,23
				100	9				
4146				10	43	460	2,66	1300	3,11
				100	8				
4147				10	45	480	2,68	1500	3,18
				100	8				
4148				10	50	530	2,72	1600	3,20
				100	8				
4149				10	50	520	2,72	1800	3,26
				100	7				
4150			4	100	42	4400	3,64	11000	4,04
				1000	6				
4151				100	46	4500	3,65	10000	4,00
				1000	4				
4152				100	58	5800	3,76	14000	4,15
				1000	6				
4153				100	59	6500	3,81	12000	4,08
				1000	13				
4154				100	61	6300	3,80	11000	4,04
				1000	8				
4155			5	1000	56	58000	4,76	120000	5,08
				10000	8				
4156				1000	63	60000	4,78	120000	5,08
				10000	3				
4157				1000	65	66000	4,82	140000	5,15
				10000	8				
4158				1000	65	65000	4,81	110000	5,04
				10000	6				
4159				1000	72	71000	4,85	110000	5,04
				10000	6				

♦ Analyses performed according to the COFRAC accreditation

N°	Sample	Strain	Inoculation level	Reference method: EN ISO 4833♦				Alternative method: D-Count® TVC	
				Dilution	CFU/plate	CFU/g	log CFU/g	CFU/g	log CFU/g
4169	UHT milk	*Bacillus licheniformis* Ad880	6	10000	53	550000	5,74	1200000	6,08
				100000	7				
4170				10000	57	610000	5,79	1100000	6,04
				100000	10				
4171				10000	55	550000	5,74	1100000	6,04
				100000	6				
4172				10000	71	660000	5,82	1100000	6,04
				100000	2				
4173				10000	59	570000	5,76	1100000	6,04
				100000	4				
4164			7	100000	92	9300000	6,97	4600000	6,66
				1000000	10				
4165				100000	94	10000000	7,00	4600000	6,66
				1000000	16				
4166				100000	102	10000000	7,00	4600000	6,66
				1000000	10				
4167				100000	95	9400000	6,97	4600000	6,66
				1000000	8				
4168				100000	93	9300000	6,97	4900000	6,69
				1000000	9				

♦ Analyses performed according to the COFRAC accreditation

N°	Sample	Strain	Inoculation level	Reference method: EN ISO 4833♦				Alternative method: D-Count® TVC	
				Dilution	CFU/plate	CFU/g	log CFU/g	CFU/g	log CFU/g
4016	Dairy based dessert	*Bacillus licheniformis* Ad 880	1	10	77	830	2,92	<1050	<3,02
				100	14				
4017				10	105	1100	3,04	<1050	<3,02
				100	11				
4018				10	107	1100	3,04	<1050	<3,02
				100	11				
4019				10	108	1100	3,04	<1050	<3,02
				100	11				
4020				10	90	860	2,93	<1050	<3,02
				100	5				
4041			2	10	>300	4500	3,65	2900	3,46
				100	45				
4042				10	>300	4400	3,64	3200	3,51
				100	44				
4043				10	>300	3900	3,59	2600	3,41
				100	39				
4044				10	>300	4300	3,63	2200	3,34
				100	43				
4045				10	261	2700	3,43	2100	3,32
				100	36				
4021			3	100	94	9600	3,98	6400	3,81
				1000	12				
4022				100	101	9700	3,99	7100	3,85
				1000	6				
4023				100	107	10000	4,00	5500	3,74
				1000	7				
4024				100	106	10000	4,00	4500	3,65
				1000	9				
4025				100	104	10000	4,00	3600	3,56
				1000	6				
4026			4	1000	88	85000	4,93	43000	4,63
				10000	6				
4027				1000	91	90000	4,95	42000	4,62
				10000	8				
4028				1000	83	88000	4,94	30000	4,48
				10000	14				
4029				1000	105	100000	5,00	45000	4,65
				10000	10				
4030				1000	98	100000	5,00	36000	4,56
				10000	12				

♦ Analyses performed according to the COFRAC accreditation

N°	Sample	Strain	Inoculation level	Reference method: EN ISO 4833♦				Alternative method: D-Count® TVC	
				Dilution	UFC/boite	UFC/g	log UFC/g	UFC/g	log UFC/g
4031	Dairy based dessert	*Bacillus licheniformis* Ad 880	5	10000	99	1000000	6,00	360000	5,56
				100000	14				
4032				10000	82	860000	5,93	320000	5,51
				100000	13				
4033				10000	85	910000	5,96	430000	5,63
				100000	15				
4034				10000	78	830000	5,92	330000	5,52
				100000	13				
4035				10000	74	700000	5,85	370000	5,57
				100000	3				
4036			6	100000	86	8800000	6,94	4500000	6,65
				1000000	11				
4037				100000	101	9900000	7,00	4900000	6,69
				1000000	8				
4038				100000	110	11000000	7,04	4600000	6,66
				1000000	8				
4039				100000	97	9800000	6,99	5100000	6,71
				1000000	11				
4040				100000	100	10000000	7,00	6100000	6,79
				1000000	12				

♦ Analyses performed according to the COFRAC accreditation

Appendix 4 – Relative accurarcy: raw data

N': arithmetical average Ne: estimated number !: D-Count TVC estimation

MEAT PRODUCTS																	
N°	Product (*French name*)	Reference method: EN ISO 4833♦							Alternative method:D-Count® TVC								
		Dilution	CFU/plate		CFU/g (rounded-off number)		LOG CFU/g		Raw data		CFU/g (after applying dilution factor for meat products)		CFU/g (rounded-off number)		LOG CFU/g	
			Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2
3422	Carpaccio de bœuf	100000	37	29	4100000	3300000	6,61	6,52	1699573	1648983	16995730	16489830	17000000	16000000	7,23	7,20
		1000000	8	7												
3423	Brochettes de porc marinées à la mexicaine	10000	110	76	1100000	760000	6,04	5,88	71411	80572	714110	805720	710000	810000	5,85	5,91
		100000	15	8												
3424	Côtes de porc marinées à la provençale	100000	>300	>300	140000000	150000000	8,15	8,18	15536959	15653051	155369590	156530510	160000000	160000000	8,20	8,20
		1000000	140	146			N'	N'								
3425	Steak haché de bœuf	1000	117	117	110000	120000	5,04	5,08	15874	15701	158740	157010	160000	160000	5,20	5,20
		10000	9	15												
3426	Pâté de campagne	10000	80	87	770000	850000	5,89	5,93	90451	94874	904510	948740	900000	950000	5,95	5,98
		100000	5	7												
3428	Poitrine crue de porc	1000	286	307	280000	300000	5,45	5,48	32892	38947	328920	389470	330000	390000	5,52	5,59
		10000	22	24												
3429	Escalope de dinde	10000	>300	>300	3900000	4100000	6,59	6,61	529131	643400	5291310	6434000	5300000	6400000	6,72	6,81
		100000	39	41			N'	N'								
3430	Tranche à Bifteck	10000	>300	294	3000000	3000000	6,48	6,48	477070	374141	4770700	3741410	4700000	3700000	6,67	6,57
		100000	30	36			N'									
3431	Côte échine de porc	10000	>300	>300	17000000	17000000	7,23	7,23	4189308	3888998	41893080	38889980	42000000	39000000	7,62	7,59
		100000	170	172			N'	N'								
3432	Steak haché de porc assaisonné	10	84	151	960	1400	2,98	3,15	382	416	3820	4160	<8000	<8000	<3,90	<3,90
		100	21	8												
5647	Chipolatas	100000	>300	>300	38000000	40000000	7,58	7,60	4668311	3911310	46683110	39113100	4700000	39000000	6,67	7,59
		1000000	38	40	N'	N'										
5648	Terrine de lapin	100	105	143	11000	16000	4,04	4,20	1109	1040	11090	10400	11000	10000	4,04	4,00
		1000	14	30												
5649	Jambon cuit	10000	150	>300	1600000	6100000	6,20	6,79	10228	18928	102280	189280	100000	190000	5,00	5,28
		100000	28	61		N'										

♦ Analyses performed according to the COFRAC accreditation

DAIRY PRODUCTS																	
N°	Product (*French name*)	Reference method: EN ISO 4833♦									Alternative method: D-Count® TVC					
		Dilution	CFU/plate		CFU/g		CFU/g (rounded-off number)		LOG CFU/g		Raw data		CFU/g		LOG CFU/g	
			Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2
4006	Chantilly	10	183	121	1936	1364	1900	1400	3,28	3,15	624	624	<1050	<1050	<3,02	<3,02
		100	30	29												
4007	Chantilly	10	87	108	836	1064	840	1100	2,92	3,04	2704	1387	2700	1400	3,43	3,15
		100	5	9												
4008	Flan	1000	35	58	34545	55455	35000	55000	4,54	4,74	23153	34737	23000	35000	4,36	4,54
		10000	3	3	*Yeasts*	*Yeasts*			*Yeasts*	*Yeasts*						
4013	Chantilly	100	157	158	15636	15273	16000	15000	4,20	4,18	3190	4090	3200	4100	3,51	3,61
		1000	15	10												
4014	Chantilly	100	257	>300	26636	32727	27000	33000	4,43	4,52	13026	10748	13000	11000	4,11	4,04
		1000	36	35 (-4:1)												
4389	Semoule au lait et raisins	10	Unreadable (52 molds)	Unreadable(60 molds)	1200	1700	1200	1700	3,08	3,23	2912	2843	2900	2800	3,46	3,45
		100	12	17					Molds	Molds						
5322	Lait UHT 1/2 écrémé	100000	155	150	17000000	17090909	17000000	18000000	7,23	7,26	23513655	22551504	24000000	23000000	7,38	7,36
		1000000	32	38												
5323	Lait UHT 1/2 écrémé	100000	>300	>300	295000000	>300000000	300000000	>300000000	8,48	>8,48	31518469	31851564	32000000	32000000	7,51	7,51
		1000000	295	436												
5324	Lait UHT entier	10000	>300	>300	6300000	6600000	6300000	6600000	6,80	6,82	3866857	3607489	3900000	3600000	6,59	6,56
		100000	63	66												
5325	Lait UHT 1/2 écrémé	100000	>300	>300	87000000	99000000	87000000	99000000	7,94	8,00	25577201	26882729	26000000	27000000	7,41	7,43
		1000000	87	99												
5326	Lait écrémé bio UHT	100000	>300	>300	55000000	65000000	55000000	65000000	7,74	7,81	27755080	27752380	28000000	28000000	7,45	7,45
		1000000	55	65												
5327	Lait 1/2 écrémé bio UHT	10000	>300	>300	6200000	7700000	6200000	7700000	6,79	6,89	2894520	3009084	2900000	3000000	6,46	6,48
		100000	62	77												

♦ Analyses performed according to the COFRAC accreditation

DAIRY PRODUCTS																	
N°	Product (*French name*)	Reference method: EN ISO 4833♦									Alternative method: D-Count® TVC					
		Dilution	CFU/plate		CFU/g		CFU/g (rounded-off number)		LOG CFU/g		Raw data		CFU/g		LOG CFU/g	
			Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2
5328	Lait écrémé UHT	10000	170	141	1736364	1427273	1700000	1400000	6,23	6,15	1525710	1020443	1500000	1000000	6,18	6,00
		100000	21	16												
5329	Lait entier UHT	10000	245	291	2518182	2918182	2500000	2900000	6,40	6,46	4020641	4045266	4000000	4000000	6,60	6,60
		100000	32	30												
5330	Lait 1/2 écrémé UHT bio	1000	>300	>300	460000	520000	460000	520000	5,66	5,72	467339	440977	470000	440000	5,67	5,64
		10000	46	52												
5331	Lait écrémé bio UHT	1000	>300	>300	400000	480000	400000	480000	5,60	5,68	465391	385517	470000	390000	5,67	5,59
		10000	40	48												
5437	Lait UHT 1/2 écrémé	100	12	23	1273	2091	1300	2100	3,11	3,32	2357	2911	2400	2900	3,38	3,46
		1000	2	0												
5438	Lait UHT 1/2 écrémé	100	179	144	18182	15091	18000	15000	4,26	4,18	21557	21982	22000	22000	4,34	4,34
		1000	21	22												
5439	Lait UHT écrémé	1000	203	30(-4)	207273	336363	210000	340000	5,32	5,53	251256	274700	250000	270000	5,40	5,43
		10000	25	7(-5)												
5635	Dessert lacté à la pistache	10	0	0	<10	<10	<10	<10	<1,00	<1,00	0	69	<1050	<1050	<3,02	<3,02
		100	0	0												
5636	Dessert lacté au chocolat blanc	10	0	0	<10	<10	<10	<10	<1,00	<1,00	139	0	<1050	<1050	<3,02	<3,02
		100	0	0												
5637	Dessert lacté à la vanille	10	0	0	<10	<10	<10	<10	<1,00	<1,00	139	0	<1050	<1050	<3,02	<3,02
		100	0	0												
5638	Préparation pour glace	10000	64	54	618182	554545	620000	550000	5,79	5,74	646321	538224	650000	540000	5,81	5,73
		100000	4	7												
5639	Crème légère UHT	10	0	0	<10	<10	<10	<10	<1,00	<1,00	693	277	<1050	<1050	<3,02	<3,02
		100	0	0												
5640	Crème fleurette UHT	10	>300	>300	14400	14800	14000	15000	4,15	4,18	624	624	<1050	<1050	<3,02	<3,02
		100	144	148			N'	N'								

DAIRY PRODUCTS																	
N°	Product (*French name*)	Reference method: EN ISO 4833♦									Alternative method: D-Count® TVC					
		Dilution	CFU/plate		CFU/g		CFU/g (rounded-off number)		LOG CFU/g		Raw data		CFU/g		LOG CFU/g	
			Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2
5641	Crème anglaise UHT	100	96	113	9545	11727	9500	12000	3,98	4,08	4579	2983	4600	3000	3,66	3,48
		1000	9	16												
5642	Crème anglaise UHT	100	35	48	3545	4545	3500	4500	3,54	3,65	4160	3813	4200	3800	3,62	3,58
		1000	4	2												
5643	Crème vanille	10	4	5	40	50	40	50	1,60	1,70	1664	1665	1700	1700	3,23	3,23
		100	1	1			Ne	Ne								
5644	Crème nougatine	10	0	0	<10	<10	<10	<10	<1,00	<1,00	694	416	<1050	<1050	<3,02	<3,02
		100	0	0												
5687	Dessert lacté à la vanille	10000	26	32	281818	345455	280000	350000	5,45	5,54	177979	240946	180000	240000	5,26	5,38
		100000	5	6												
5688	Crème dessert	1000	26	52	29091	50909	29000	51000	4,46	4,71	186437	163955	190000	160000	5,28	5,20
		10000	6	4												
5689	Dessert lacté au chocolat blanc	100	88	99	8727	9636	8700	9600	3,94	3,98	5548	4646	5500	4600	3,74	3,66
		1000	8	7												
5690	Crème fraiche entière UHT	10	226	227	2164	2327	2200	2300	3,34	3,36	2982	2912	3000	2900	3,48	3,46
		100	12	29												

Appendix 5 – Quantification and detection limits: raw data

Meat product protocol (pure culture of the strain)

	T 3000	T 5000	T 10000
cfu/ml *	3128	5088	9960

*:average on 5 enumerations

Inoculation level	Inoculum (cfu/ml)	Replicate (cfu/ml)					
		1	2	3	4	5	6
0	0	0	0	0	0	0	0
3000	3128	3120	1390	0	0	3120	0
5000	5088	2080	4160	3810	1730	3470	1040
10000	9960	1730	3810	8320	8660	4160	6930

Detection limit: 8 000 CFU/ml

UHT milk protocol (pure culture of the strain)

	T 300	T 500	T750	T 1000
cfu/ml *	344	656	470	1250

*:average on 5 enumerations

Inoculation level	Inoculum (cfu/ml)	Replicate (cfu/ml)					
		1	2	3	4	5	6
0	0	0	0	0	0	0	0
300	344	416	208	208	69	347	277
500	656	832	416	624	624	555	1040
700	590	208	416	347	208	1109	693
800	395	1109	901	1040	1525	1040	1040
1000	1250	1941	2215	1802	2564	2218	2772

Detection limit: 1 050 CFU/ml

Dairy based dessert protocol (pure culture of the strain)

	T 300	T 500	T 1000
cfu/ml *	344	656	1250

*:average on 5 enumerations

Inoculation level	Inoculum (cfu/ml)	Replicate (cfu/ml)					
		1	2	3	4	5	6
0	0	0	0	0	69	0	0
300	344	0	277	347	347	0	139
500	656	139	277	832	485	347	208
1000	1250	901	416	1248	1109	1386	971

Detection limit: 1 050 CFU/ml

Meat products protocol (liver pâté)

	T 4000	T 8000	T 12000
cfu/ml *	6200	13000	17000

*:average on 5 enumerations

Inoculation level	Inoculum (cfu/ml)	Replicate (cfu/ml)					
		1	2	3	4	5	6
0	0	0	1390	350	1390	350	35
4000	6200	2080	3810	3120	1730	2770	2080
8000	13000	7280	5550	8320	11440	6930	8320
12000	17000	11100	12830	13180	13180	21500	12830

Detection limit: 8 000 CFU/ml

Dairy based dessert protocol

	T500	T750	T1000	T1250
cfu/ml *	420	580	810	1480
	T1500		**T2000**	
cfu/ml *	2100		2800	

*:average on 5 enumerations

Inoculation level	Inoculum (cfu/ml)	Replicate (cfu/ml)					
		1	2	3	4	5	6
0	0	0	0	0	69	69	0
500	420	624	277	347	624	208	485
750	580	416	555	1456	208	277	277
1000	810	763	208	347	277	624	693
1250	1480	2010	1040	901	1526	1872	1664
1500	2100	2357	1803	2080	1317	832	1248
2000	2800	3050	2357	1386	1040	2218	1525

Detection limit: 1 050 CFU/ml

Appendix 6 – Relative sensitivity: raw data

X	Liver pâté	Dairy based dessert	UHT milk
1	31,2%	38,6%	34,1%
1,1	27,6%	34,0%	32,0%
1,2	24,7%	30,3%	30,1%
1,3	22,3%	27,3%	28,4%
1,4	20,3%	24,7%	26,8%
1,5	18,5%	22,5%	25,4%
1,6	17,0%	20,6%	24,1%
1,7	15,7%	19,0%	22,9%
1,8	14,6%	17,5%	21,8%
1,9	13,6%	16,3%	20,8%
2	12,6%	15,2%	19,8%
2,1	11,8%	14,1%	18,9%
2,2	11,1%	13,2%	18,1%
2,3	10,4%	12,4%	17,3%
2,4	9,8%	11,7%	16,6%
2,5	9,3%	11,0%	16,0%
2,6	8,8%	10,4%	15,4%
2,7	8,3%	9,8%	14,8%
2,8	7,9%	9,3%	14,3%
2,9	7,5%	8,8%	13,8%
3	7,1%	8,4%	13,3%
3,1	6,8%	8,0%	12,8%
3,2	6,5%	7,6%	12,4%
3,3	6,2%	7,3%	12,0%
3,4	5,9%	6,9%	11,7%
3,5	5,7%	6,6%	11,3%
3,6	5,4%	6,4%	11,0%
3,7	5,2%	6,1%	10,7%
3,8	5,0%	5,9%	10,5%
3,9	4,8%	5,6%	10,2%
4	4,6%	5,4%	10,0%
4,1	4,5%	5,3%	9,7%
4,2	4,3%	5,1%	9,5%
4,3	4,2%	4,9%	9,3%
4,4	4,0%	4,8%	9,1%
4,5	3,9%	4,6%	9,0%
4,6	3,8%	4,5%	8,8%
4,7	3,6%	4,4%	8,7%
4,8	3,5%	4,3%	8,5%
4,9	3,4%	4,2%	8,4%
5	3,3%	4,1%	8,3%
5,1	3,3%	4,0%	8,2%
5,2	3,2%	3,9%	8,1%
5,3	3,1%	3,8%	8,0%
5,4	3,0%	3,8%	7,9%
5,5	3,0%	3,7%	7,9%
5,6	2,9%	3,7%	7,8%
5,7	2,8%	3,6%	7,7%
5,8	2,8%	3,6%	7,7%
5,9	2,7%	3,6%	7,6%
6	2,7%	3,5%	7,6%

Appendix 7 – Results obtained by each collaborator laboratory and the expert laboratory

Laboratory	Sample n°	Reference method: ISO 4833				Alternative method: D-Count TVC	
		Dilution	cfu/plate	cfu/g	log cfu/g	cfu/g	log cfu/g
A	2	10	0	<10	<1.00	<1050	<3.02
		100	0				
	6	10	0	<10	<1.00	<1050	<3.02
		100	0				
	1	100	155	16000	4.20	23000	4.36
		1000	25				
	4	1000	32	31000	4.49	28000	4.45
		10000	2				
	3	1000	196	190000	5.28	180000	5.26
		10000	15				
	8	1000	172	170000	5.23	230000	5.36
		10000	10				
	5	10000	244	2400000	6.38	2500000	6.40
		100000	25				
	7	10000	241	2300000	6.36	2600000	6.41
		100000	13				
B	2	10	0	<10	<1.00	<1050	<3.02
		100	0				
	6	10	0	<10	<1.00	<1050	<3.02
		100	0				
	1	100	210	21000	4.32	51000	4.71
		1000	19				
	4	100	264	27000	4.43	62000	4.79
		1000	33				
	3	1000	258	260000	5.41	690000	5.84
		10000	28				
	8	1000	123	120000	5.08	250000	5.40
		10000	14				
	5	10000	158	1700000	6.23	5200000	6.72
		100000	32				
	7	10000	128	1400000	6.15	3500000	6.54
		100000	24				
C	2	10	0	<10	<1.00	<1050	<3.02
		100	0				
	6	10	0	<10	<1.00	<1050	<3.02
		100	0				
	1	100	>300	74000	4.87	81000	4.91
		1000	74				
	4	100	>300	52000	4.72	70000	4.85
		1000	52				
	3	1000	>300	1100000	6.04	1500000	6.18
		10000	110				
	8	1000	>300	510000	5.71	930000	5.97
		10000	51				
	5	10000	>300	5200000	6.72	12000000	7.08
		100000	52				
	7	10000	>300	6700000	6.83	9600000	6.98
		100000	67				

Laboratory	Sample n°	Reference method: ISO 4833				Alternative method: D-Count TVC	
		Dilution	cfu/plate	cfu/g	log cfu/g	cfu/g	log cfu/g
D	2	10	0	<10	<1.00	<1050	<3.02
		100	0				
	6	10	0	<10	<1.00	<1050	<3.02
		100	0				
	1	100	221	22000	4.34	34000	4.53
		1000	20				
	4	100	181	18000	4.26	980	2.99
		1000	16				
	3	1000	151	150000	5.18	300000	5.48
		10000	14				
	8	1000	263	250000	5.40	440000	5.64
		10000	17				
	5	10000	213	2200000	6.34	3200000	6.51
		100000	24				
	7	10000	209	2100000	6.32	3300000	6.52
		100000	19				
E	2	10	0	<10	<1.00	<1050	<3.02
		100	0				
	6	10	0	<10	<1.00	<1050	<3.02
		100	0				
	1	100	168	18000	4.26	5300	3.72
		1000	32				
	4	1000	34	32000	4.51	5500	3.74
		10000	1				
	3	10000	28	270000	5.43	84000	4.92
		100000	2				
	8	10000	29	280000	5.45	70000	4.85
		100000	2				
	5	10000	>300	3800000	6.58	950000	5.98
		100000	38				
	7	10000	218	2200000	6.34	780000	5.89
		100000	27				
F	2	10	0	<10	<1.00	<1050	<3.02
		100	0				
	6	10	0	<10	<1.00	<1050	<3.02
		100	0				
	1	100	246	24000	4.38	25000	4.40
		1000	21				
	4	100	215	21000	4.32	31000	4.49
		1000	20				
	3	1000	228	230000	5.36	420000	5.62
		10000	30				
	8	10000	43	440000	5.64	560000	5.75
		100000	5				
	5	10000	260	2600000	6.41	4600000	6.66
		100000	30				
	7	10000	243	2400000	6.38	3400000	6.53
		100000	24				

Laboratory	Sample n°	Reference method: ISO 4833				Alternative method: D-Count TVC	
		Dilution	cfu/plate	cfu/g	log cfu/g	cfu/g	log cfu/g
H	2	10	0	<10	<1.00	<1050	<3.02
		100	0				
	6	10	0	<10	<1.00	<1050	<3.02
		100	0				
	1	100	202	21000	4.32	38000	4.58
		1000	28				
	4	1000	40	40000	4.60	64000	4.81
		10000	4				
	3	1000	217	220000	5.34	510000	5.71
		10000	27				
	8	1000	216	220000	5.34	310000	5.49
		10000	22				
	5	10000	254	2600000	6.41	4400000	6.64
		100000	27				
	7	10000	225	2300000	6.36	5900000	6.77
		100000	26				
I	2	10	123	1200	3.08	<1050	<3.02
		100	8				
	6	10	0	<10	<1.00	<1050	<3.02
		100	0				
	1	10000	71	730000	5.86	430000	5.63
		100000	9				
	4	10000	153	1600000	6.20	450000	5.65
		100000	25				
	3	100000	130	13000000	7.11	8400000	6.92
		1000000	13				
	8	100000	63	6700000	6.83	4700000	6.67
		1000000	11				
	5	100000	>300	55000000	7.74	29000000	7.46
		1000000	55				
	7	100000	285	30000000	7.48	2600000	6.41
		1000000	43				
J	2	10	0	<10	<1.00	2800	3.45
		100	0				
	6	10	0	<10	<1.00	<1050	<3.02
		100	0				
	1	100	132	14000	4.15	6200	3.79
		1000	20				
	4	100	185	19000	4.28	4700	3.67
		1000	20				
	3	1000	216	210000	5.32	110000	5.04
		10000	16				
	8	1000	210	210000	5.32	40000	4.60
		10000	17				
	5	10000	171	1700000	6.23	250000	5.40
		100000	12				
	7	10000	205	2100000	6.32	190000	5.28
		100000	24				

Laboratory	Sample n°	Reference method: ISO 4833				Alternative method: D-Count TVC	
		Dilution	cfu/plate	cfu/g	log cfu/g	cfu/g	log cfu/g
L	2	10	0	<10	<1.00	<1050	<3.02
		100	0				
	6	10	0	<10	<1.00	<1050	<3.02
		100	0				
	1	1000	49	47000	4.67	58000	4.76
		10000	3				
	4	1000	52	53000	4.72	43000	4.63
		10000	6				
	3	10000	46	460000	5.66	640000	5.81
		100000	40				
	8	10000	>300	5000000	6.70	360000	5.56
		100000	50				
	5	10000	>300	3700000	6.57	4500000	6.65
		100000	37				
	7	10000	>300	4500000	6.65	4000000	6.60
		100000	45				

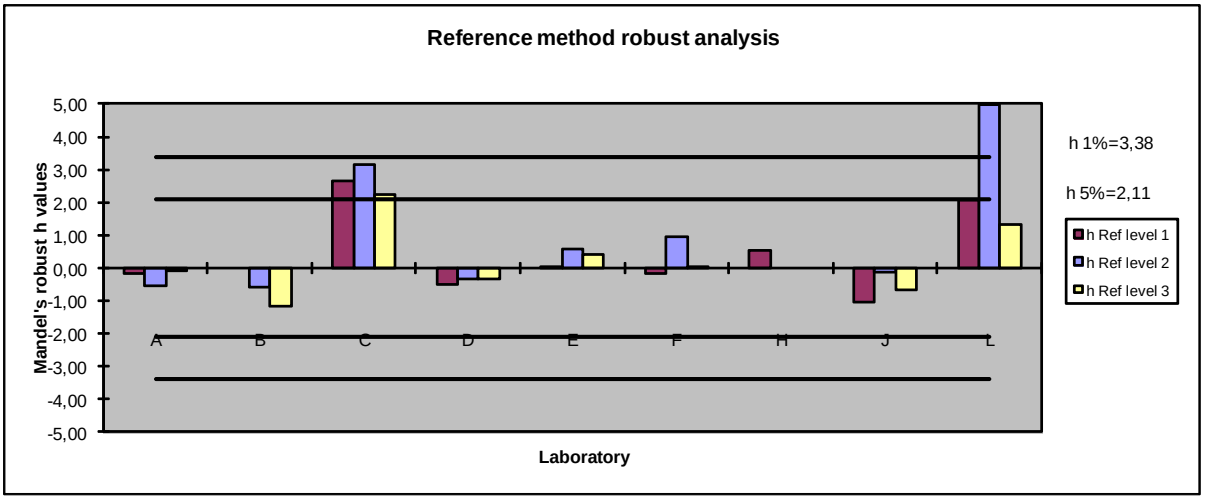
Laboratory	Sample n°	Reference method: ISO 4833♦				Alternative method: D-Count TVC	
		Dilution	cfu/plate	cfu/g	log cfu/g	cfu/g	log cfu/g
N (ADRIA)	2	10	0	<10	<1.00	<1050	<3.02
		100	0				
	6	10	0	<10	<1.00	<1050	<3.02
		100	0				
	1	100	187	19000	4.28	7300	3.86
		1000	23				
	4	100	212	21000	4.32	8100	3.91
		1000	18				
	3	1000	273	270000	5.43	97000	4.99
		10000	22				
	8	10000	23	250000	5.40	88000	4.94
		100000	4				
	5	10000	215	2200000	6.34	830000	5.92
		100000	24				
	7	10000	236	2300000	6.36	600000	5.78
		100000	18				

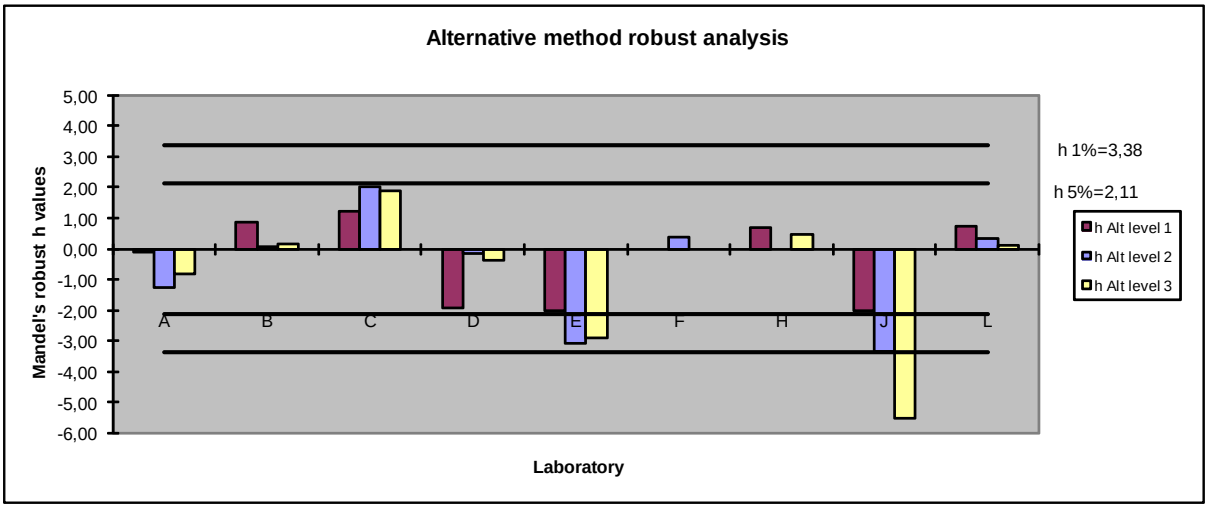
♦ Analyses performed according to the COFRAC accreditation

Appendix 8 – Mandel's graphics

Laboratory	h Ref level 1	h Ref level 2	h Ref level 3	h Alt level 1	h Alt level 2	h Alt level 3	h5%	h1%	h5%	h1%
A	-0.19	-0.52	-0.10	-0.11	-1.25	-0.84	2.11	3.38	-2.11	-3.38
B	0.00	-0.56	-1.18	0.86	0.08	0.14	2.11	3.38	-2.11	-3.38
C	2.66	3.15	2.26	1.22	2.03	1.91	2.11	3.38	-2.11	-3.38
D	-0.50	-0.33	-0.33	-1.93	-0.17	-0.38	2.11	3.38	-2.11	-3.38
E	0.02	0.57	0.43	-2.02	-3.07	-2.91	2.11	3.38	-2.11	-3.38
F	-0.16	0.95	0.05	0.00	0.37	0.00	2.11	3.38	-2.11	-3.38
H	0.55	0.00	0.00	0.70	0.00	0.48	2.11	3.38	-2.11	-3.38
J	-1.05	-0.12	-0.66	-2.02	-3.34	-5.54	2.11	3.38	-2.11	-3.38
L	2.05	4.97	1.31	0.72	0.35	0.13	2.11	3.38	-2.11	-3.38

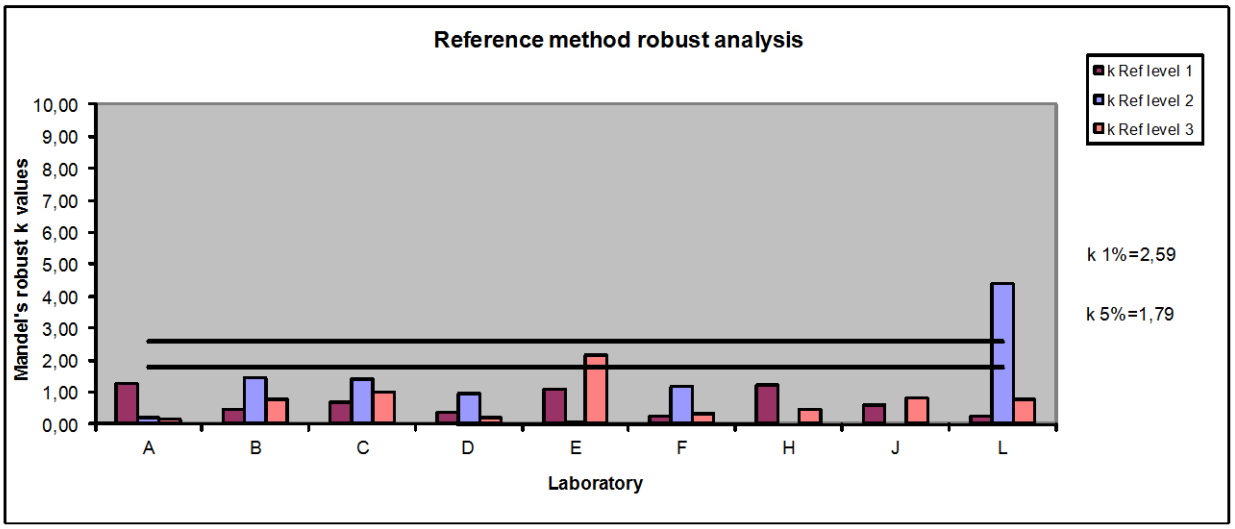
Inter-laboratory consistency

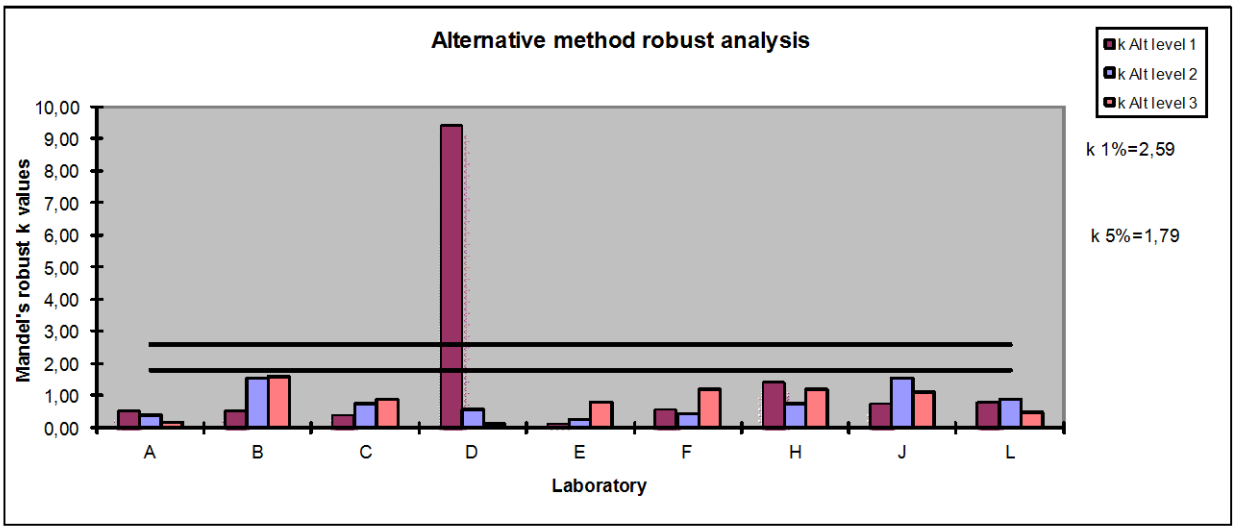




Laboratory	k Ref level 1	k Ref level 2	k Ref level 3	k5%	K1%	k Alt level 1	k Alt level 2	k Alt level 3
A	1,26	0,21	0,17	1,79	2,59	0,52	0,38	0,16
B	0,48	1,43	0,77	1,79	2,59	0,52	1,56	1,58
C	0,67	1,42	1,00	1,79	2,59	0,39	0,73	0,89
D	0,38	0,94	0,18	1,79	2,59	9,40	0,59	0,12
E	1,10	0,07	2,16	1,79	2,59	0,10	0,28	0,79
F	0,26	1,20	0,32	1,79	2,59	0,57	0,44	1,21
H	1,23	0,00	0,49	1,79	2,59	1,38	0,77	1,17
J	0,58	0,00	0,84	1,79	2,59	0,73	1,56	1,10
L	0,23	4,41	0,77	1,79	2,59	0,79	0,88	0,47

Intra-laboratory consistency





Appendix 9 – Statistical results

Level 1

Laboratories number (p) 9

Laboratories	Level 1 Ref		Mean	si	Deviation		h values	k values
A	4,204	4,491	4,348	0,20	-0,144	0,144	-0,186	1,265
B	4,322	4,431	4,377	0,08	-0,055	0,055	0,000	0,481
C	4,869	4,716	4,793	0,11	0,077	-0,077	2,658	0,675
D	4,342	4,255	4,299	0,06	0,044	-0,044	-0,498	0,384
E	4,255	4,505	4,380	0,18	-0,125	0,125	0,022	1,100
F	4,380	4,322	4,351	0,04	0,029	-0,029	-0,163	0,255
H	4,322	4,602	4,462	0,20	-0,140	0,140	0,546	1,232
J	4,146	4,279	4,212	0,09	-0,066	0,066	-1,051	0,584
L	4,672	4,724	4,698	0,04	-0,026	0,026	2,054	0,230

	m	4,376792
	Cn(2p)	2,062
	Cn(p)	1,9228
n=2*p	n	18
	f	10
	l	40
n=p	n	9
	f	5
	l	10
	Qn(2p)	0,055085
	Qn(p)	0,081364
	Qwithin	0,11356
	Qbetween	0,15645
	Sr(repeatability standard deviation)	0,1606
	RSDr(variation coefficient of repeatability)	3,67%
	r(repeatability limit))	0,4497
	SL^2(between laboratories standard deviatio	0,01158
	SR(reproductibility standard deviation)	0,1933
	RSDR(variation coefficient of reproductibilit	0,044
	R(reproductibility limit))	0,5413

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Laboratories	Level 1 Alt		Mean	si	Deviation		h values	k values	D
A	4,362	4,447	4,404	0,06	-0,043	0,043	-0,114	0,521	0,057
B	4,708	4,792	4,750	0,06	-0,042	0,042	0,864	0,518	0,373
C	4,908	4,845	4,877	0,04	0,032	-0,032	1,223	0,387	0,084
D	4,531	2,991	3,761	1,09	0,770	-0,770	-1,933	9,401	-0,537
E	3,724	3,740	3,732	0,01	-0,008	0,008	-2,015	0,098	-0,648
F	4,398	4,491	4,445	0,07	-0,047	0,047	0,000	0,570	0,093
H	4,580	4,806	4,693	0,16	-0,113	0,113	0,703	1,382	0,231
J	3,792	3,672	3,732	0,09	0,060	-0,060	-2,016	0,734	-0,480
L	4,763	4,633	4,698	0,09	0,065	-0,065	0,718	0,793	0,000

	m	4,44465
	Cn(2p)	2,062
	Cn(p)	1,9228
n=2*p	n	18
	f	10
	l	40
n=p	n	9
	f	5
	l	10
	Qn(2p)	0,03974
	Qn(p)	0,183810
	Qwithin	0,08192
	Qbetween	0,35343
	Sr	0,1159
	RSDr	2,61%
	r	0,3244
	SL^2	0,11820
	SR	0,3628
	RSDR	0,082
	R	1,0158

	Accuracy
***median* D**	0,0567
Cp	1,9228
Qn(D)	0,142347
Qdiff	0,273705
t	0,50

Level 2

Laboratories number (p) 9

Laboratories	Level 2 Ref		Mean	si	Deviation		h values	k values
A	5,279	5,230	5,255	0,03	0,024	-0,024	-0,520	0,206
B	5,415	5,079	5,247	0,24	0,168	-0,168	-0,565	1,430
C	6,041	5,708	5,874	0,24	0,167	-0,167	3,151	1,421
D	5,176	5,398	5,287	0,16	-0,111	0,111	-0,328	0,944
E	5,431	5,447	5,439	0,01	-0,008	0,008	0,573	0,067
F	5,362	5,643	5,503	0,20	-0,141	0,141	0,949	1,199
H	5,342	5,342	5,342	0,00	0,000	0,000	0,000	0,000
J	5,322	5,322	5,322	0,00	0,000	0,000	-0,120	0,000
L	5,663	6,699	6,181	0,73	-0,518	0,518	4,965	4,411

	m	5,342423
	Cn(2p)	2,062
	Cn(p)	1,9228
n=2*p	n	18
	f	10
	l	40
n=p	n	9
	f	5
	l	10
	Qn(2p)	0,056972
	Qn(p)	0,0878214
	Qwithin	0,11745
	Qbetween	0,16886
	Sr(repeatability standard deviation)	0,1661
	RSDr(variation coefficient of repeatability)	3,11%
	r(repeatability limit))	0,4651
	SL^2(between laboratories standard deviati	0,01472
	SR(reproductibility standard deviation)	0,2057
	RSDR(variation coefficient of reproductibili	0,039
	R(reproductibility limit))	0,5759

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Laboratories	Level 2 Alt		Mean	si	Deviation		h values	k values	D
A	5,255	5,362	5,309	0,08	-0,053	0,053	-1,251	0,377	0,05
B	5,839	5,398	5,618	0,31	0,220	-0,220	0,081	1,561	0,37
C	6,176	5,968	6,072	0,15	0,104	-0,104	2,033	0,735	0,20
D	5,477	5,643	5,560	0,12	-0,083	0,083	-0,168	0,589	0,27
E	4,924	4,845	4,885	0,06	0,040	-0,040	-3,073	0,280	-0,55
F	5,623	5,748	5,686	0,09	-0,062	0,062	0,371	0,442	0,18
H	5,708	5,491	5,599	0,15	0,108	-0,108	0,000	0,765	0,26
J	5,041	4,602	4,822	0,31	0,220	-0,220	-3,344	1,555	-0,50
L	5,806	5,556	5,681	0,18	0,125	-0,125	0,352	0,885	-0,50

	m	5,59947
	Cn(2p)	2,062
	Cn(p)	1,9228
n=2*p	n	18
	f	10
	l	40
n=p	n	9
	f	5
	l	10
	Qn(2p)	0,068514
	Qn(p)	0,1209543
	Qwithin	0,14124
	Qbetween	0,23257
	Sr	0,1998
	RSDr	3,57%
	r	0,5593
	SL^2	0,03414
	SR	0,2721
	RSDR	0,049
	R	0,7619

	Accuracy
***median* D**	0,1831
Cp	1,9228
Qn(D)	0,098046
Qdiff	0,188522508
t	2,33

Level 3

Laboratories number (p) 9

Laboratories	Level 3 Ref		Mean	si	Deviation		h values	k values
A	6,380	6,362	6,371	0,01	0,009	-0,009	-0,103	0,168
B	6,230	6,146	6,188	0,06	0,042	-0,042	-1,182	0,768
C	6,716	6,826	6,771	0,08	-0,055	0,055	2,260	1,003
D	6,342	6,322	6,332	0,01	0,010	-0,010	-0,331	0,184
E	6,580	6,342	6,461	0,17	0,119	-0,119	0,430	2,162
F	6,415	6,380	6,398	0,02	0,017	-0,017	0,055	0,317
H	6,415	6,362	6,388	0,04	0,027	-0,027	0,000	0,485
J	6,230	6,322	6,276	0,06	-0,046	0,046	-0,662	0,836
L	6,568	6,653	6,611	0,06	-0,043	0,043	1,313	0,774

	m	6,388351
	Cn(2p)	2,062
	Cn(p)	1,9228
n=2*p	n	18
	f	10
	l	40
n=p	n	9
	f	5
	l	10
	Qn(2p)	0,026623
	Qn(p)	0,088046
	Qwithin	0,05488
	Qbetween	0,16929
	Sr(repeatability standard deviation)	0,0776
	RSDr(variation coefficient of repeatability)	1,21%
	r(repeatability limit))	0,2173
	SL^2(between laboratories standard deviatio	0,02565
	SR(reproducibility standard deviation)	0,1780
	RSDR(variation coefficient of reproductibilit	0,028
	R(reproductibility limit))	0,4983

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Laboratories	Level 3 Alt		Mean	si	Deviation		h values	k values	D
A	6,398	6,415	6,406	0,01	-0,009	0,009	-0,839	0,157	0,035
B	6,716	6,544	6,630	0,12	0,086	-0,086	0,145	1,581	0,442
C	7,079	6,982	7,031	0,07	0,048	-0,048	1,908	0,891	0,260
D	6,505	6,519	6,512	0,01	-0,007	0,007	-0,375	0,123	0,180
E	5,978	5,892	5,935	0,06	0,043	-0,043	-2,914	0,787	-0,526
F	6,663	6,531	6,597	0,09	0,066	-0,066	0,000	1,207	0,200
H	6,643	6,771	6,707	0,09	-0,064	0,064	0,484	1,172	0,319
J	5,398	5,279	5,338	0,08	0,060	-0,060	-5,538	1,096	-0,938
L	6,653	6,602	6,628	0,04	0,026	-0,026	0,134	0,470	0,017

	m	6,59712
	Cn(2p)	2,062
	Cn(p)	1,9228
n=2*p	n	18
	f	10
	l	40
n=p	n	9
	f	5
	l	10
	Qn(2p)	0,02637
	Qn(p)	0,1182
	Qwithin	0,05437
	Qbetween	0,22728
	Sr	0,0769
	RSDr	1,17%
	r	0,2153
	SL^2	0,04870
	SR	0,2337
	RSDR	0,035
	R	0,6543

Accuracy

***median* D**	0,1795
Cp	1,9228
Qn(D)	0,1625818
Qdiff	0,312612
t	1,37