



BactoCount IBC

Evaluation of the BactoCount IBC Analytical Performances



BactoCount IBC

Evaluation of the BactoCount IBC Analytical Performances

1. Instrument Presentation
2. Evaluation of the BactoCount IBC Stability, CECALAIT, France
3. Evaluation of the BactoCount IBC Carry-Over, CECALAIT, France
3. Evaluation of the BactoCount IBC linearity, CECALAIT, France
5. Evaluation of the BactoCount IBC detection limit, CECALAIT, France
6. Evaluation of the BactoCount IBC Repeatability
 1. CECALAIT Evaluation
 2. LIAL FC Evaluation
 3. LDA 39 Evaluation
7. Evaluation of the BactoCount IBC Accuracy
 - 7.1 BactoCount vs Reference Method (IDF 100B:1991)
 1. CECALAIT evaluation
 2. LIAL FC evaluation
 3. LDA 39 evaluation
 4. KRIZEVCI evaluation, Croatia
 - 7.2. BactoCount vs BactoScan
 1. Global Analysis, UK
 2. Krizevci laboratory, Croatia

Conclusions

1. Method Presentation

The BactoCount IBC is a fully automated instrument using flow cytometry (FCM) for the rapid enumeration of individual bacteria in raw milk. The milk is sampled and dispensed in a carousel heated at 45 °C. An incubation reagent made up with a clarification buffer, a proteolytic enzyme, and a fluorescent marker are then added in order to lyse the somatic cells, solubilize the fat globules and proteins, permeabilize the bacteria and stain their DNA. The fluorescence marker intercalates rapidly and selectively into all the bacteria double-stranded nucleic acid. The mixture is then sonicated during the incubation with two ultrasonic probes to help the chemical breakdown of the interfering particles, disrupt the remaining bacteria colonies to improve the detection of individual bacteria and reduce the background fluorescence. The cell debris, devoid of nucleic acid, are excluded from the analysis.

After the incubation period a portion of the incubation mixture is transferred to the flow cytometer where the bacteria are aligned and exposed to an intense laser beam and fluoresce. The fluorescence signal is collected by the optics, filtered, and detected with a photomultiplier. The fluorescence pulses intensity and height are recorded and used as gating parameters. The sorted pulses are then translated into individual bacteria count after instrument calibration.

2. Evaluation of the BactoCount IBC Stability

The instrument stability was evaluated during the CECALAIT evaluation on the BactoCount " -prototype. Three Samples, with a low, medium and high bacteria count and with or without preservative (Azidiol) were analyzed 20 times, in duplicates, with two vials per sample, over half a day to check the stability of the instrument. The repeatability and reproducibility were calculated according to IDF 135 Standard to evaluate the instrument stability. The results, log transformed, are reported in the chart below.

Type of Samples	Mean Value (*1000 CFU/ml)	Sr	GRSRr (%)	SR	GRSDR
Low	23.7 (4.375)	0.0219	5.17	0.0244	5.78
Low + Preservative		0.0247	5.85	0.0276	6.56
Medium	190 (5.279)	0.0206	4.86	0.0582	14.34
Medium + Preservative		0.0304	7.25	0.0636	15.77
High	344 (5.537)	0.0272	6.46	0.0514	12.56
High + Preservative		0.014	3.28	0.0528	12.93
All Levels		0.0232		0.0446	
All Levels + Preservative		0.023		0.048	

Sr and GRSDr: Standard deviation and geometrical standard deviation of repeatability

SR and GRSDR: Standard deviation and geometrical standard deviation of reproducibility

The stability tests performed show an overall standard deviation of reproducibility (SR) of 0.0446 log and a geometrical standard deviation of reproducibility (GRSDR) between 5.8 and 15.8 % that seem to be linked to the count level but independent of the samples preservation mode(with or without preservative). For the samples with low bacteria count, the reproducibility value is of the same order than the repeatability value.

The standard deviation of repeatability and reproducibility were well within the instrument specification with a Sr value < 0.06 Log unit.

3. Evaluation of the BactoCount IBC Carry-Over

The instrument stability was evaluated during the CECALAIT evaluation on the BactoCount " -prototype.

Protocol

The instrument carry-over was evaluated in the automated mode by analyzing 20 samples with low and high bacteria counts as follows : High-High-Low-Low-High-High.... The carry-over was evaluated at 3 different levels.

The carry over coefficient was estimated with the following formula :

$$Tc\% = [(3(Low\ 1 - 3Low\ 2)) / (3High\ 2 - 3Low\ 2)] * 100$$

The Tc% was calculated with the instrument raw CFU values. The results in Log CFU / ml are the reference results performed on the low and high samples.

Low bacteria Count (1000 CFU/ml)			High Bacteria Count (1000 CFU/ml)			Tc%
Inst.	Ref.	Log UFC/ml	Inst.	Ref.	Log UFC/ml	
78	26	4.414	0.8	0.35	2.54	0
949	228	5.358	1.09	0.35	2.54	0.01
1753	430	5.633	0.97	0.35	2.54	0.05

The BactoCount IBC carry-over varies from 0 to 0.05% independently of the contamination level. This carry-over coefficient complies perfectly with the carry-over limit (Tc<1%) generally applied to the automated methods used for milk payment.

4. Evaluation of the BactoCount IBC linearity

The instrument stability was evaluated during the CECALAIT evaluation on the BactoCount " -prototype. The linearity of the instrument was evaluated in the automated mode (repeatability mode, 3 analysis per sample) by analyzing milk samples covering regularly the target range in the ascending and descending order. Three trials were performed to cover different concentration ranges. The results are summarized in the chart below.

	milk type	n	Range (1000*CFU)	Sy,x (Log) Linear Regression	Sy,x (Log) After linearization
Trial 1	milk micro filtrated and recombined	19	17 - 510	0.021	0.0147
Trial 2	milk micro filtrated and recombined	13	0.1 - 219	0.090	0.0221
Trial 3	natural milk	20	37 - 762	0.036	0.0121

The linearity was evaluated by using a linear and a polynomial regression equation with Y as the reference and X as the mean instrumental value. The polynomial equation seems to improve, slightly, the linearity of the instrument and therefore the overall accuracy. The use of a polynomial regression was not necessary on the \$ prototypes and production instruments. The instrument can therefore be calibrated with a simple linear regression.

5. Evaluation of the BactoCount IBC detection limit

The instrument stability was evaluated during the CECALAIT evaluation on the BactoCount " -prototype.

The BactoCount detection limit, the smallest change in concentration that can be measured, was assessed by analyzing in the automated mode (repeatability mode, 3 analysis per sample) 14 milk samples covering the 0 to 5000 CFU range in the ascending and descending order.

A simple linear regression was applied with Y as the reference and X the mean instrumental value. Depending of the formula used, the BactoCount IBC detection limit was between 820 and 1120 CFU / ml well within the method specification.

The BactoCount IBC detection limit is in perfect agreement with the precision required for its use in routine operation.

6. Evaluation of the BactoCount IBC Repeatability

The repeatability standard deviation (Sr) was evaluated in the three independent evaluations according to the IDF 128 standard by analyzing 116 series of 20 bulk milk samples (2320 samples) in duplicates. The CECALAIT, LIAL FC and LDA 39 evaluations gave respectively a repeatability standard deviation(Sr) of 0.044 log (820 samples), 0.052 log (820 samples) and 0.069 log (680 samples) significantly lower than the limit required for milk payment in France (Sr<0.12 log).

Table 1. CECALAIT Evaluation - Repeatability according to IDF 128 Standard

CFU Classes (10³)	n	Mean (Log)	Sr (Log)	GRSDr(%)	RD 95(%)
All levels	820	4.499	0.0445	10.79	32.82
0-50	639	4.335	0.0426	11.03	31.22
50-100	95	4.819	0.0591	14.58	45.78
100-300	60	5.208	0.0464	11.28	34.43
>300	26	5.732	0.0179	4.21	12.09

Table 2. CECALAIT Evaluation :Repeatability - Two consecutive analysis

CFU Classes (10³)	n	Mean (Log)	Sr (Log)	GRSDr(%)	RD 95(%)
All levels	816	4.649	0.0534	13.08	40.57
0-50	508	4.365	0.0514	12.56	38.79
50-100	146	4.832	0.0652	16.2	51.56
100-300	110	5.219	0.0559	13.73	42.82
>300	52	5.711	0.038	9.14	27.42

n : number of samples, GRSDr : Geometrical relative standard deviation of repeatability
RD 95 : 95 % confidence limit for the maximum difference between duplicates

The BactoCount presents an overall Standard Deviation of Repeatability on the 2320 samples of 0.055 Log and is significantly lower than the limit recommended in France for milk payment (Sr < 0.15* Log). The mean repeatability value also complies with the instrument specifications (Sr<0.06 Log).

7. Evaluation of the BactoCount IBC Accuracy

7.1 BactoCount vs IDF 100B:1991 Standard

The BactoCount IBC was evaluated by CECALAIT and two independent payment laboratories (LDA39 and LIAL FC) in France in 2001 to evaluate its analytical performances and overall behavior in routine operation. The evaluations were respectively performed over a 5, 4 and 5-month period without adjusting the instruments' calibration. The BactoCount stability was monitored with a microsphere solution supplied by Bentley Instruments. The instruments were very stable and didn't require any adjustment during the evaluations. The CECALAIT and LDA 39 evaluations were performed on a " -prototype unit, which was not temperature stabilized and secured. The experience acquired during the CECALAIT evaluation was then integrated in the \$-prototypes used for the following evaluations in order to improve the BactoCount analytical performances.

7.1.1. CECALAIT evaluation

In order to have a representative population, 820 samples were collected in two payment laboratories located in two different regions of France. The samples were analyzed in duplicates on the BactoCount IBC and 410 were kept for the evaluation of the instrument accuracy. The selected milk samples were kept between 0 and 2/C during 2 to 4 hours until the samples were ready to be analyzed. The analysis were performed in duplicates on the instrument and were analyzed immediately in duplicates by the reference method. (IDF 100B).

The accuracy evaluation was performed in 9 days over a 4-month period. The milk samples were collected and handled as payment samples (24 to 48 hours in storage tank). The samples were not pre-warmed before analysis. The instrument stability was monitored with the microsphere solution supplied with the instrument. The instrument was not adjusted during the evaluation.

The accuracy was evaluated with the standard deviation of accuracy ($S_{y,x}$) with ($Y = \text{Reference}$, $X = \text{BactoCount}$). A simple linear regression was used to calculate the conversion equation. Only the samples validated by the reference were kept for the evaluation of the instrument accuracy. Two outliers were removed.

The BactoCount IBC gave a Standard Deviation of Accuracy ($S_{y,x}$) of 0.333 Log unit with the samples covering the $10 \cdot 10^3$ - $1000 \cdot 10^3$ CFU ranges.

The slope and bias were significantly different respectively from 1 and 0. Therefore the a conversion equation between IBC and CFU needs to be established before use.

7.1.2. LIAL FC and LDA 39 Evaluations

The BactoCount " and \$ Prototypes were evaluated respectively over 4 and 5 months in routine operation by the LIAL FC (Rioz, France, \$ Prototype) and the LDA 39 (Poligny, France, " Prototype) payment laboratories on samples representative of the laboratories collection area. The tests were performed respectively over 10 days and 16 days from approximately June 2001 to October, 2001. The selected milk samples (LIAL FC, 498; LDA 39, 426) were kept between 0 and 2/C during 2 to 4 hours until the samples were ready to be analyzed. The analyses were performed in duplicates for the reference analyses and only once on the BactoCount as in normal routine operation. The samples were not pre-heated before analysis.

The accuracy was evaluated with the standard deviation of accuracy ($S_{y,x}$) with ($Y = \text{Reference}$, $X = \text{BactoCount}$). A simple linear regression was used to calculate the conversion equation. Only the samples validated by the reference were kept for the evaluation of the instrument accuracy.

The LIAL FC (498 samples)and LDA 39 (426 samples) evaluations gave the same Standard Deviation of Accuracy ($S_{y,x}$) of 0.309 with respectively a correlation coefficient of 0.773 and 0.848 and a mean population of 20,000 cfu and 26,000 cfu. The standard Deviation of Accuracy was respectively of 0.264 log and 0.283 when keeping only the samples with a cfu count over 10,000.

7.1.3. Krizevci laboratory, Croatia

The accuracy of the BactoCount Production Unit was evaluated against the IDF 100B: 1991 on 192 individual milk samples. The samples were analyzed in duplicates first on the BactoCount in the Krizevci Payment and Selection laboratory, and then in the Krizevci Reference laboratory. The samples were preserved with Azidiol and were analyzed cold. The accuracy standard deviation ($S_{y,x}$) against the IDF 100B: 1991 standard was evaluated by performing a simple linear regression between the BactoCount IBC raw data and the reference values and gave a $S_{y,x}$ of 0.2660 log.

7.2 BactoCount vs BactoScan

The agreement between the BactoCount IBC and BactoScan methods were evaluated in two independent laboratories. The results of these evaluations are presented below.

7.2.1. Krizevci Milk Payment and Selection Laboratory, Croatia

439 Individual milk samples were analyzed in parallel by the BactoCount IBC and the BactoScan method in two series of several days in August and December 2001. The samples were brought cold to the Krizevci laboratory in Croatia to be analyzed on the BactoCount IBC in duplicates. The samples were not warmed up before the analyses. The samples were then sent to the reference laboratory in Slovenia to be analyzed on the BactoScan. Azidiol was normally added to each sample after the reference analyses. The time delay between the BactoCount and the BactoScan analyses was between one and three days. The samples were stored in a cold container.

The BactoCount working solutions were readily prepared every day from the stock solutions. The incubation solution was prepared and used over several days. All the reagents were automatically filtrated through 0.2 μ m filters before analysis.

The instruments raw data (*IBC*) were log transformed to evaluate the methods agreement. The methods agreement was evaluated by performing a simple linear regression between the BactoCount and BactoScan raw data. The regressions gave respectively an accuracy standard deviation ($S_{y,x}$) of 0,1588 log and 0,1671 log on the first and second samples series.

7.2.2. Global Analysis Laboratory, UK

The BactoCount evaluation was performed at the Global Analysis laboratory (Mirfield, UK) with a β -prototype over a 3-month period in 2001. 480 bulk milk samples were taken randomly and stored between 0/ C and 4/ C before analysis. The samples didn't contain any preservative. The samples were collected after all the other compositional analyses had been performed and were up to four days old. The samples were not warmed up before analysis.

The samples were analysed only once as routine samples, first on a BactoScan 8000 and then on the BactoCount IBC 150 prototype. The time difference between the analyses on both instruments was less than three hours. Three different BactoScans 8000 were used in this study based on their availability.

The BactoCount IBC stability was monitored with a microsphere solution supplied by Bentley Instruments. The instrument was very stable and didn't require any adjustment over the 3-month period. The standard detection threshold was used during this evaluation. The BactoScan were calibrated every day using the SAITL calibration sample. The BactoCount working solutions were readily prepared every day from the stock solutions. The incubation solution was prepared and used over several days. All the reagents were automatically filtrated through 0.2 μ m filters before analysis.

The methods agreement was evaluated by performing a simple linear regression between the BactoCount and BactoScan raw data. The regression gave an accuracy

standard deviation ($S_{y,x}$) of 0,1870 log.

Conclusions:

The BactoCount Standard Deviation of Accuracy ($S_{y,x}$) against the IDF 100B: 1991 standard was evaluated in four independent evaluations and gave a mean overall $S_{y,x}$ of 0.285 log. The accuracy standard deviation ($S_{y,x}$) was significantly improved on the \$-prototype and production unit with respectively a $S_{y,x}$ of 0.264 log (LIAL FC, \$), and 0.266 log (Croatia, Production Unit) compared to a $S_{y,x}$ of 0.283 log (LDA39, " -prototype), and 0.333 log(CECALAIT, " -prototype).

Three evaluations were performed between the Bentley BactoCount IBC and the Foss BactoScan and indicated a very good agreement with an overall accuracy standard deviation ($S_{y,x}$) of 0.17 log.

The BactoCount IBC " and \$ prototypes stability, carry-over, repeatability, limit of detection, accuracy were extensively evaluated in three independent French laboratories according to the IDF standards. The evaluation reports were submitted to a Technical and Scientific Committee depending of the French Ministry of Agriculture. The BactoCount met all the requirements of the Scientific Committee and was subsequently approved for milk payment in France in July 2001. The accuracy results were significantly improved with the \$ prototype.

The BactoCount IBC accuracy and analytical performances have been extensively evaluated against the IDF 100B: 1991 standard and the BactoScan method. The three methods are in very good agreement. The BactoCount numerous hardware and software features translate into a very accurate, rugged and reliable instrument well suited for the milk payment laboratories.