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# MICROVAL VALIDATION REPORT

Comparative Study

**MicroVal Project 2021LR97**

# Evaluation report of Bactocount IBC 3.0 for enumeration of somatic cells (ISO 8196-3, ISO 13366-2 and EURL MMP criteria) and total bacterial count (ISO 16297, ISO 21187 and EURL MMP criteria)

**MicroVal study number: 2021LR97**

**Device**: Bactocount IBC 3.0

**MicroVal Expert Laboratory**: ACTALIA Cecalait

**Manufacturer**: Bentley Instruments

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## SUMMARY

Bentley Instruments requested ACTALIA Cecalait to perform a MicroVal evaluation of their BactoCount IBC 3.0 device for the enumeration of somatic cells count (SCC) and total bacterial count (TBC) **in raw cow milk**.

The BactoCount IBC 3.0 can offer simultaneous real-time analysis of SCC and TBC in raw milk with flow cytometer, **but in the frame of this validation, SCC and TBC were tested separately**.

The instrument is equipped with a second laser and two other detectors which were not used in this present validation study (dedicated for other applications).

The instrument has a speed of 200 samples / hour (presence of a rack sampler) and is piloted through its specific software Nexgen, version N° 2.21.

The evaluation protocol was built according to ISO 8196-3 (1) and ISO 13366-2 (2) for SCC and according to ISO 16297 (3) and ISO 21187 (4) for TBC. This report includes only the Method Comparison Study (MCS) of the validation process.

If an interlaboratory study is required, then a new report will be established and will be presented to MicroVal.

The BactoCount IBC 3.0 was not validated by independent laboratory according to International Dairy Federation or International Organization for Standardization. **This study is the first official evaluation performed by an ISO 17025 accredited independent laboratory**. However, reagents used with this device are the same than those used with BactoCount IBC already certified ISO 16140 certificate N°2013 LR 44.

The BactoCount IBC 3.0 was commercialized in the first time in 2019.

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# 1. INTRODUCTION

The BactoCount IBC 3.0 is a fully automatic instrument that uses flow cytometry for the rapid, accurate and highly reliable enumeration of individual bacteria and somatic cells in raw milk. It was developed by the **BENTLEY instruments** company (US) and distributed in France by Bentley Instruments SARL (www.bentleyinstruments.eu).

These enumerations can be performed combined or individually. **In the frame of this validation, SCC and TBC were tested separately**.

## 1.1. Principle of the alternative method

The **BactoCount** is a (fully) **automated flow cytometer** for the rapid enumeration of individual bacteria in raw milk. The raw milk is sampled and dispensed into individual wells located on a carousel with temperature regulated at 50°C. There the raw milk sample **is mixed with an incubation reagent**. The incubation reagent contains a clarification buffer, a proteolytic enzyme, and a **fluorescent marker**. The reagent serves to lyse the somatic cells, to solubilize the fat globules and proteins, to permeabilize the bacterial cell walls and **to stain their DNA**. The fluorescent marker intercalates rapidly and selectively with the bacterial DNA. The mixture is then sonicated twice during the incubation. The sonication process promotes the chemical breakdown of the interfering particles and disrupts the remaining bacteria cells to improve the detection of individual bacteria and reduce the background fluorescence. The cell debris, devoid of nucleic acid, becomes excluded from the analysis.

After the incubation, the mixture is transferred automatically to the **flow cytometer** where the bacteria are aligned and exposed to an intense laser beam which causes them to fluoresce. The fluorescence signal is collected by the optics, filtered, and detected with a photo multiplier. The fluorescence pulses intensity and height are recorded and used as gating parameters. The sorted pulses are then translated into individual bacteria count (IBC) and converted to CFU (reference scale) after applying a conversion equation. An “universal” conversion equation developed on a large database of samples representative of all potential sources of variation in the milk flora (according to ISO 21187|IDF 196) can be installed on the instrument as a startup conversion equation.

The principle is the same for SCC. The somatic cells fluorescence pulses intensity and height are recorded and used as a gating parameter. The sorted pulses are then translated into Somatic Cells Count (SCC) after calibration against a set of SCC reference samples.

**The alternative method protocol** is based on **flow cytometry principle**, where the DNA contents in cells (somatic cells or bacteria) are stained with a fluorescent marker, then detected through fluorescence signal. This signal is then converted into universal unit thanks to the Bentley’s software, NexGen.

Firstly, an incubation reagent is added to the milk:

- **For TBC** in order to clarify the milk matrix, lyse the somatic cells and permeabilize the bacteria and stain their DNA with a fluorescent marker.
- **For SCC** in order to clarify the milk sample and permeabilize the somatic cells and stain their DNA with a fluorescent marker.

The fluorescent marker intercalates rapidly into all the double-stranded nucleic acid. A sonication is needed for the TBC analysis to breakdown the interfering particles and the bacteria colonies into individual bacteria (IBC).

After an incubation period, samples were transferred to the flow cytometer. Then cells are aligned, exposed to an intense laser beam and fluoresce.

The fluorescent signal is collected by the optics, filtered and detected with a photomultiplier. The intensity and height of the fluorescent pulses are recorded and used as gating parameters.

- For TBC, the sorted pulses (IBC) are then converted into Colony-Forming Units (CFU) after the application of a conversion equation on the software NexGen.
- For SCC, the sorted pulses are then converted into somatic cells / ml after the application of a calibration equation (against RM) on the software NexGen.

### 1.2. Scope

Raw cow milk

### 1.3. Restriction of use

None

### 1.4. Reference methods

For accuracy testing (SCC or TBC), the results obtained with the alternative method were compared to the results obtained with the relevant Bentley's device already validated:

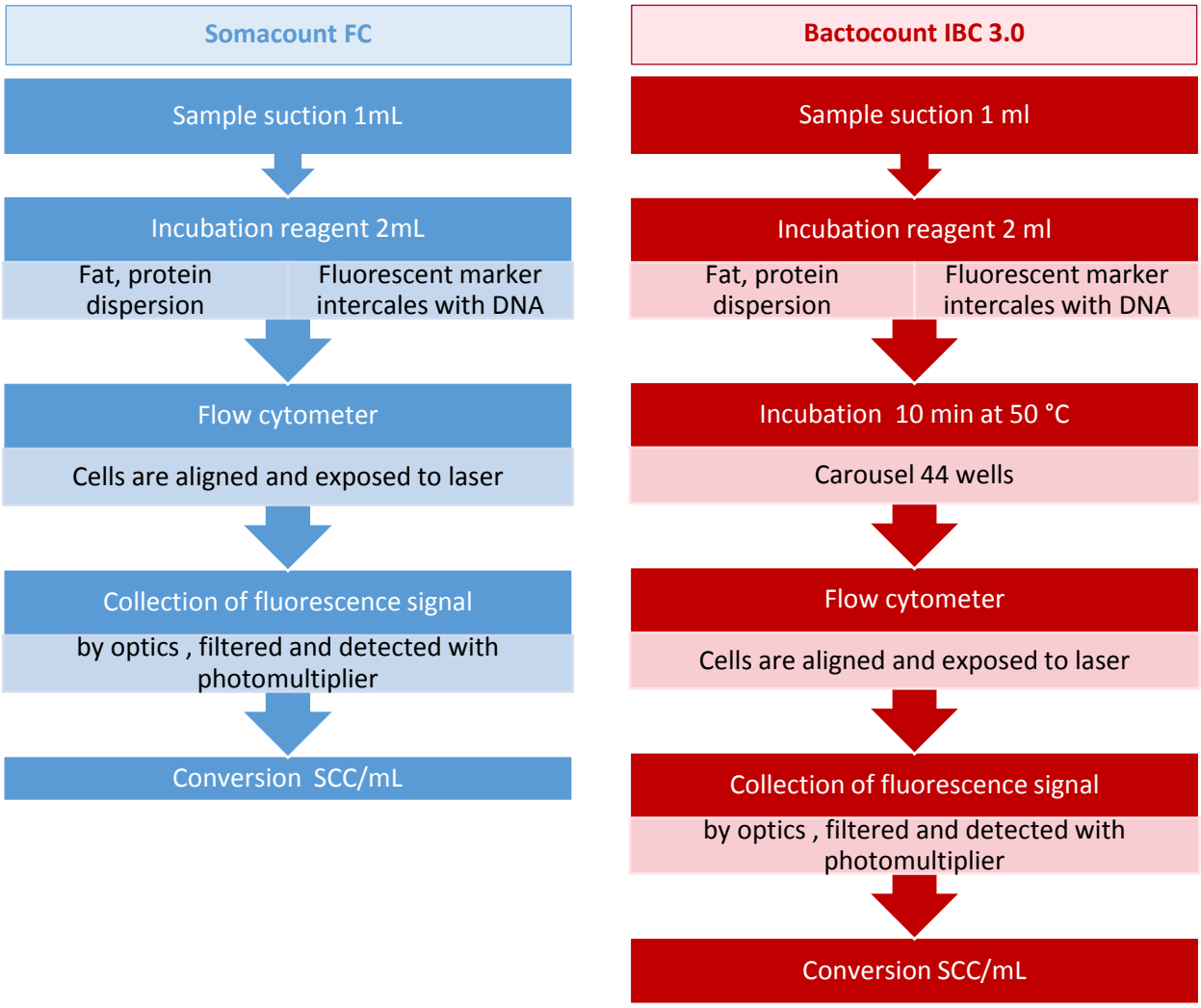
- Somacount FC for SCC (ICAR certified according ISO 8196-3; certificate n°2020/7)
- BactoCount IBC 2.0 for TBC (MicroVal certified; certificate n°2013 LR 44).

### 1.5. Conversion equation (IBC/CFU)

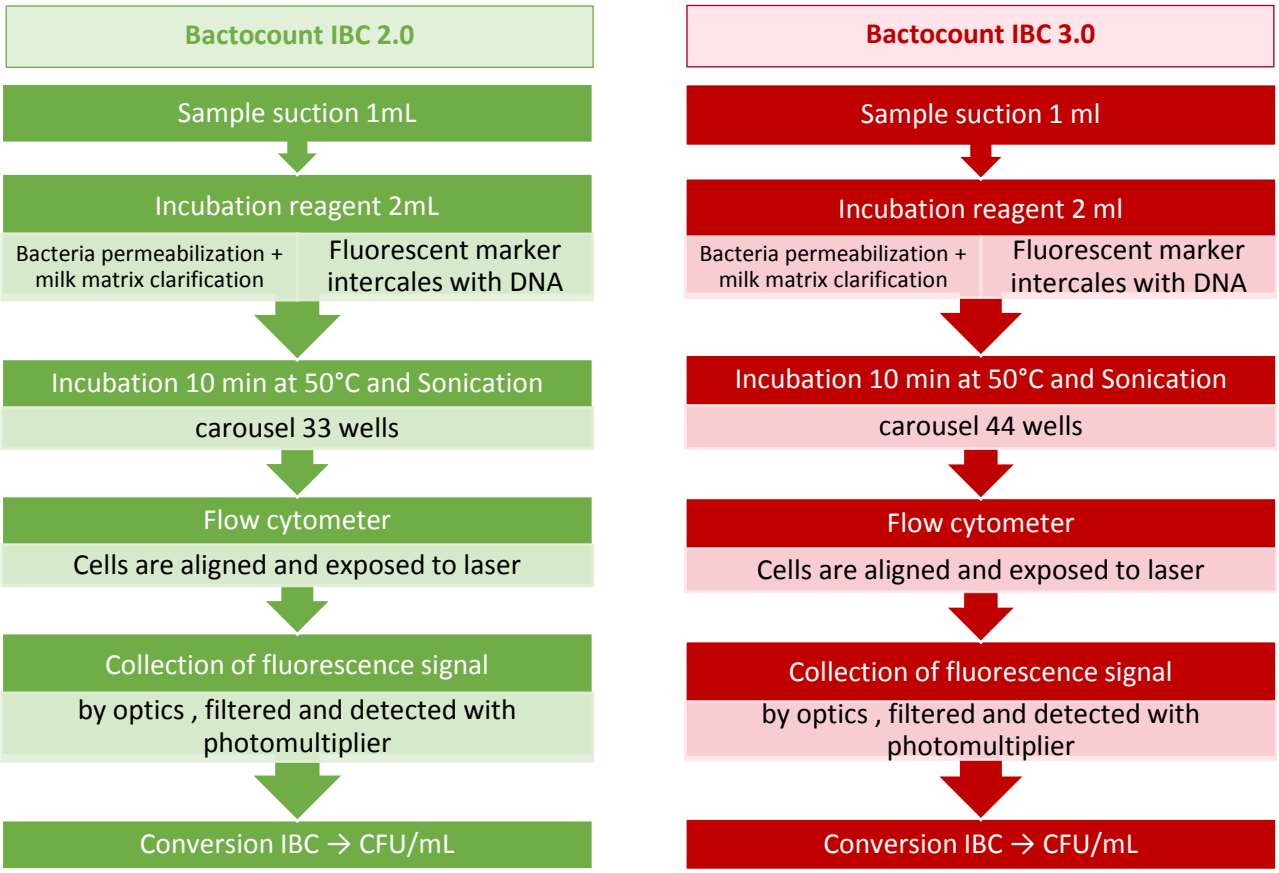
The device to be tested was equipped with an Universal Conversion Equation provided by Bentley Instruments (N° BactoCount U-CE 2013).

## 1.6. Validation procedure

The measurement procedure for the direct comparison of **Bactocount IBC 3.0** and the **Somacount FC** for the **SCC** is schematically presented below.



The measurement procedure for the direct comparison of **Bactocount IBC 3.0** and the **Bactocount IBC 2.0** for **TBC** is schematically presented below.




## 1.7. Safety precautions

Good Laboratory Practices for running food analyses were followed.

# METHOD COMPARISON STUDY
# FOR ENUMERATION OF
# SOMATIC CELLS (SCC)

# 2. METHOD COMPARISON STUDY FOR ENUMERATION OF SOMATIC CELL (SCC)

## 2.1. Materials and equipment used

- Recombined samples with adjusted concentration in SCC (cell concentrate and filtrate from microfiltration);
- Individual raw cow milk samples from milk control;
- Herd raw cow milk from payment for milk quality;
- « Blank milk SCC »: raw cow milk filtrate (SCC concentration near to 0);
- Stock and working solutions for Bactocount IBC 3.0 and Somacount FC, prepared according to manufacturer's instructions;
- RBS 2% solution;
- SCC incubation / dye solution;
- Somatic cells SRM (Standard Reference Material from Actalia Cecalait);
- Refrigerator at 0-4°C;
- Water bath at 40±2°C;
- Standard laboratory glassware and utensils.

To perform the experimental work described in this study, the following was needed:

- Bactocount IBC 3.0;
- Somacount FC (ICAR certified according ISO 8196-3; certificate n°2020/7);
- Instruction and method implementation;
- Statistical expertise.

## 2.2. Preparation of recombined samples

The performance characteristics of the alternative method have been evaluated using **artificially contaminated samples**. Raw cow milk was skimmed and **microfiltered** to obtain **2 suspensions: one with higher (concentrate) and one with lower concentration (filtrate) of somatic cells** (according to ISO 13366:2 § 6.1.2.2). A range of samples was prepared to have specific concentration of somatic cells. Each milk sample was used during the day and was not stored. The milk samples were placed in a water bath at 40±2°C for 20 minutes before the measurement.

## 2.3. Performance characteristics of the alternative method

### 2.3.1. Stability (according to ISO 8196-3 § 5.2.2.1.1)

The **stability of the alternative method** was verified by mimicking routine testing circumstances throughout a working day. To evaluate the stability of the instrument, the standard deviation of repeatability ($s_r$), the standard deviation means ($s_x$), the standard deviation between checks ($s_c$) and the standard deviation of daily reproducibility ($s_{R,daily}$) were determined for different somatic cell count levels (according ISO 8196-3 recommendation)

#### 2.3.1.1. Measurement protocol and calculations

Milk samples were prepared at **four cell count levels**: low, medium 1, medium 2 and high (Table 1).

*Table 1 : Cell count levels of samples used in the stability study of the IBC 3.0 for SCC.*

| Cell count level | Theoretical cell count (x10³ cells/mL) | Cell counts measured with IBC 3.0 (x10³ cells/mL) |
|---|---|---|
| **Low (L)** | 75 | 73 |
| **Medium 1 (M1)** | 500 | 492 |
| **Medium 2 (M2)** | 1 000 | 996 |
| **High (H)** | 1 500 | 1 439 |

Each sample was placed in a water bath (40±2°C) for 20 minutes before measurement.

Samples from each cell count level were measured in triplicate (n=3) with the Bactocount IBC 3.0 in the order: (L – M – H) each 15-20 minutes during a working day with 20 checks in total.

The standard deviation of repeatability ($s_r$), the standard deviation of means ($s_x$), the standard deviation between checks ($s_c$) and the standard deviation of daily reproducibility ($s_{R,daily}$) were calculated according to ISO 8196-3 (1).

- **For every check, j (j=1…..q):**
  - The mean $\bar{x}_j$ was calculated according to:

$$\bar{x}_j = \sum x_{ij} / n$$

with n = number of measurements (n=3) an i = replicate

  - And the standard deviation $s_{rj}$ of replicates according to:

$$s_{rj} = \left[\sum \left(x_{ij} - \bar{x}_j\right)^2 / (n-1)\right]^{1/2}$$

- **For the whole check sequence the following parameters were calculated:**
  - The standard deviation of repeatability $s_r$:

$$s_r = \left(\sum s_{rj}^2 / q\right)^{1/2}$$

with q = number of checks (q = 20)

  - The standard deviation of means $s_{\bar{x}}$:

$$s_{\bar{x}} = \left[\sum \left(\bar{x}_j - \bar{x}\right)^2 / (q-1)\right]^{1/2} = \left\{\left[\sum \bar{x}_j^2 - \frac{\left(\sum \bar{x}_j\right)^2}{q}\right] / (q-1)\right\}^{1/2}$$

with:

$$\bar{x} = \sum \bar{x}_j / q$$

- The standard deviation between checks:

$$s_c = \left(s_{\bar{x}}^2 - s_r^2/n\right)^{1/2}$$

if $s_c < 0$ then $s_c = 0$

- The standard deviation of daily reproducibility:

$$s_{R,daily} = (s_c^2 + s_r^2)^{1/2}$$

The stability of the method response during the sequence of check tests was visualized by plotting the means of the measurement results ($\bar{x}_j$) on the y-axis, versus the check sequence numbers, on the x-axis.

#### 2.3.1.2. Results

A summary of the stability results is given in Table 2. **The standard deviation of repeatability ($s_r$) for each level and for all samples meets the requirements according to ISO 8196-3**, see Table 3. **The standard deviation of daily reproducibility ($s_{R,daily}$) for each level and for all samples meets the requirements according to ISO 8196-3**, see Table 4.

*Table 2: The standard deviation of repeatability ($s_r$), the standard deviation of means ($s_x$), the standard deviation between checks ($s_c$) and the standard deviation of daily reproducibility ($s_{R,daily}$) of the Bactocount IBC 3.0 for enumeration of somatic cells per examined cell count level.*

| Cell count level | $S_r$ (x10³ cells/mL) | $S_x$ (x10³ cells/mL) | $S_c$ (x10³ cells/mL) | $S_{R,daily}$ (x10³ cells/mL) |
|---|---|---|---|---|
| **Low (73 x 10³ cells/mL)** | 4.8 | 2.4 | 0 | 4.8 |
| **Medium 1 (492 x 10³ cells/mL)** | 11.9 | 9.0 | 5.8 | 13.2 |
| **Medium 2 (996 x 10³ cells/mL)** | 18.9 | 13.1 | 7.2 | 20.3 |
| **High (1 439 x 10³ cells/mL)** | 22.3 | 16.3 | 10.0 | 24.4 |

*Table 3: The standard deviation of repeatability ($s_r$) of the Bactocount IBC 3.0 for enumeration of somatic cells calculated per count level and for all samples and acceptability values according to ISO 8196-3.*

| Cell count level | $s_r$ calculated | | $s_r$ acceptability values according to ISO 8196-3 |
|---|---|---|---|
| | x10³ cells/mL | % | % |
| **Low (73 x 10³ cells/mL)** | 4.8 | **6.5%** | **< 8%** |
| **Medium 1 (492 x 10³ cells/mL)** | 11.9 | **2.4%** | **< 4%** |
| **Medium 2 (996 x 10³ cells/mL)** | 18.9 | **1.9%** | **< 4%** |
| **High (1 439 x 10³ cells/mL)** | 22.5 | **1.5%** | **< 2%** |

*Table 4: The standard deviation of daily reproducibility ($s_{R,daily}$) of the Bactocount IBC 3.0 for enumeration of somatic cells calculated per count level and acceptability values according to ISO 8196-3.*

| Cell count level | $S_{R,daily}$ calculated | | $S_{R,daily}$ acceptability values according to ISO 8196-3 |
|---|---|---|---|
| | x10³ cells/mL | % | % |
| **Low (73 x 10³ cells/mL)** | 4.8 | **6.5%** | **< 10%** |
| **Low (492 x 10³ cells/mL)** | 13.2 | **2.7%** | **< 5%** |
| **Medium (996 x 10³ cells/mL)** | 20.3 | **2.0%** | **< 5%** |
| **High (1 439 x 10³ cells/mL)** | 24.4 | **1.7%** | **< 2.5%** |

For the standard deviation between checks ($s_c$) and standard deviation of means ($s_x$), there is no official requirement.

The plot visualizing the stability of the method response during the day is given in Figure 1.

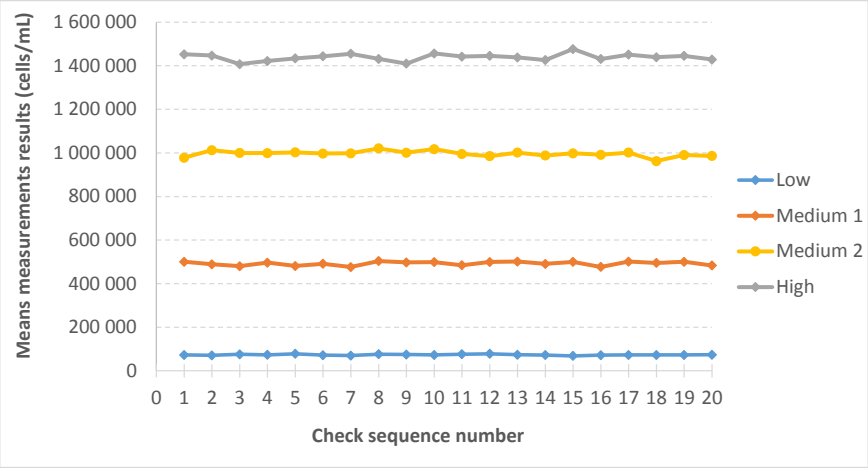



*Figure 1: Bactocount IBC 3.0 stability for enumeration of somatic cells throughout the working day based on the means of the measurement results at four cell count levels.*

#### 2.3.1.3. Conclusion

**The Bactocount IBC 3.0 is stable during the working day for the enumeration of somatic cells. The stability complies with the requirements of ISO 8196-3.**

### 2.3.2. Carry-over effect (according to ISO 8196-3 § 5.2.2.1.2)

Strong differences in somatic cell count levels between two successively analyzed samples may influence the result of a second one. Differences could be caused by incomplete rinsing of the flow system and the measuring cell by liquid circulation and contamination by the stirring device. Automatic correction of results is acceptable within certain limits, provided it can be proven that there is a systematic and constant transfer of a small quantity of material from one measurement to the next. Automated analyzers for liquids often allow automatic correction to compensate for the overall carry-over effect when necessary.

#### 2.3.2.1. Measurement protocol and calculations

Milk samples were prepared at three "**high" cell count levels** by mixing filtrate and concentrate of skimmed raw cow milk. The cell count levels of the "high" samples are given in Table 5 and aligned on recommendations of ISO 8196-3. The "low" samples were unspiked filtrate from skimmed raw cow milk and was also called "**blank milk**".

*Table 5 : Cell count levels of samples used in the stability study with the IBC 3.0 for SCC.*

| Cell count level | Theoretical cell count ( x10$^3$ cells/mL) | Cell counts measured with IBC 3.0 ( x10$^3$ cells/mL) |
|---|---|---|
| **High 1** | 500 | 501 |
| **High 2** | 1 000 | 988 |
| **High 3** | 1 500 | 1 477 |

Each sample was placed in a water bath (40±2°C) for 20 minutes before measurement.

Bactocount IBC 3.0 measurements were performed without carry-over correction factor on 20 sets of samples per cell count level with the following sequence:

$$(L_{H1}, L_{H2}, L_{L1}, L_{L2})_1, (L_{H1}, L_{H2}, L_{L1}, L_{L2})_2 \dots (L_{H1}, L_{H2}, L_{L1}, L_{L2})_{20}$$

thus,

(high milk 1, high milk 2, blank milk 1, blank milk 2)$_1$, (high milk 1, high milk 2, blank milk 1, blank milk 2)$_2$ … (high milk 1, high milk 2, blank milk 1, blank milk 2)$_{20}$

The calculations were performed on raw data without any transformation. **The carry-over (CO) was obtained by applying the following equations**:

$$C_{H/L} = \left(\sum L_{L1} - \sum L_{L2}\right) \times 100 \,/ \left(\sum L_{H2} - \sum L_{L2}\right) = (\overline{L_{L1}} - \overline{L_{L2}}) \times 100 / (\overline{L_{H2}} - \overline{L_{L2}})$$

$$C_{L/H} = \left(\sum L_{H2} - \sum L_{H1}\right) \times 100 \,/ \left(\sum L_{H2} - \sum L_{L2}\right) = (\overline{L_{H2}} - \overline{L_{H1}}) \times 100 / (\overline{L_{H2}} - \overline{L_{L2}})$$

The carry-over effect should not exceed the limit of 2% as required in the ISO 8196-3 and in EURL MMP document (5).

#### 2.3.2.2. Results

For each cell count level, the ratio **$C_{H/L}$ and $C_{L/H}$ were calculated**. The results are given in Table 6.

*Table 6: Calculated ratios $C_{H/L}$ and $C_{L/H}$ per cell count level.*

| Cell count level of the "high" samples | Calculated $C_{H/L}$ % | Calculated $C_{L/H}$ % |
|---|---|---|
| **High 1** (501 x $10^3$ cells/mL) | 0.69% | -0.27% |
| **High 2** (988 x $10^3$ cells/mL) | 0.59% | 0.12% |
| **High 3** (1 477 x $10^3$ cells/mL) | 0.44% | 0.23% |
| **All samples** (989 x $10^3$ cells/mL) | 0.53% | 0.11% |

**The calculated relative carry-over effect for each cell count level and for all samples was smaller than the limit *CO* < 2%.**

#### 2.3.2.3. Conclusion

**The carry-over effect for enumeration of somatic cells with measurements on the Bactocount IBC 3.0 complies with the requirements in ISO 8196-3 and EURL MMP document for each cell count level.**

### 2.3.3. Linearity (according to ISO 8196-3 § 5.2.2.1.3)

According to the classical definition of an indirect method, the instrument signal should result from a characteristic of the component measured and thereby allow the definition of a simple relationship to the component concentration. Linearity expresses the constancy of the ratio between the increase in the concentration of a component and the corresponding increase of the alternative method result. Therefore, linearity of the measurement signal is in most cases essential to maintain a constant sensitivity over the measuring range and to allow easy handling of calibration and fittings. Moreover, it allows in routine (to some extent) measurements beyond the calibration range through linear extrapolation.

#### 2.3.3.1. Measurement protocol and calculations

To evaluate linearity, samples with **different cell count levels distributed over the range of 0 to 2 500 x $10^3$ cells/mL were prepared**. Filtrate of skimmed raw cow milk was spiked with concentrate of skimmed raw cow milk to obtain concentrations covering the working range in routine testing. The samples were measured 4 times in the order of increasing cell count and 4 times in the order of decreasing cell count. Per sample in total, **8 results were collected**.

The **ratio $r_c$ was calculated** as the ratio of the residual range to the signal value range. The calculated cell count levels of the spiked samples were used as the reference values for the calculation.

The means of the replicates per samples (n = 8) were calculated. The mean results were processed by linear regression:

$$y = bx + a$$

$y$ = instrument value (measured value)
$x$ = calculated reference value of the spiked samples.

The residuals, $e_i$, were calculated from the means of replicates and the theoretical reference:

$$e_i = y_i - (bx_i + a)$$

The linearity was visually inspected by plotting the residuals, $e_i$, on the y-axis and the theoretical concentration on the x-axis.

The relative linearity bias was expressed with the ratio $r_c$:

$$r_c = \frac{(e_{max} - e_{min})}{(M_{max} - M_{min})} \times 100$$

where

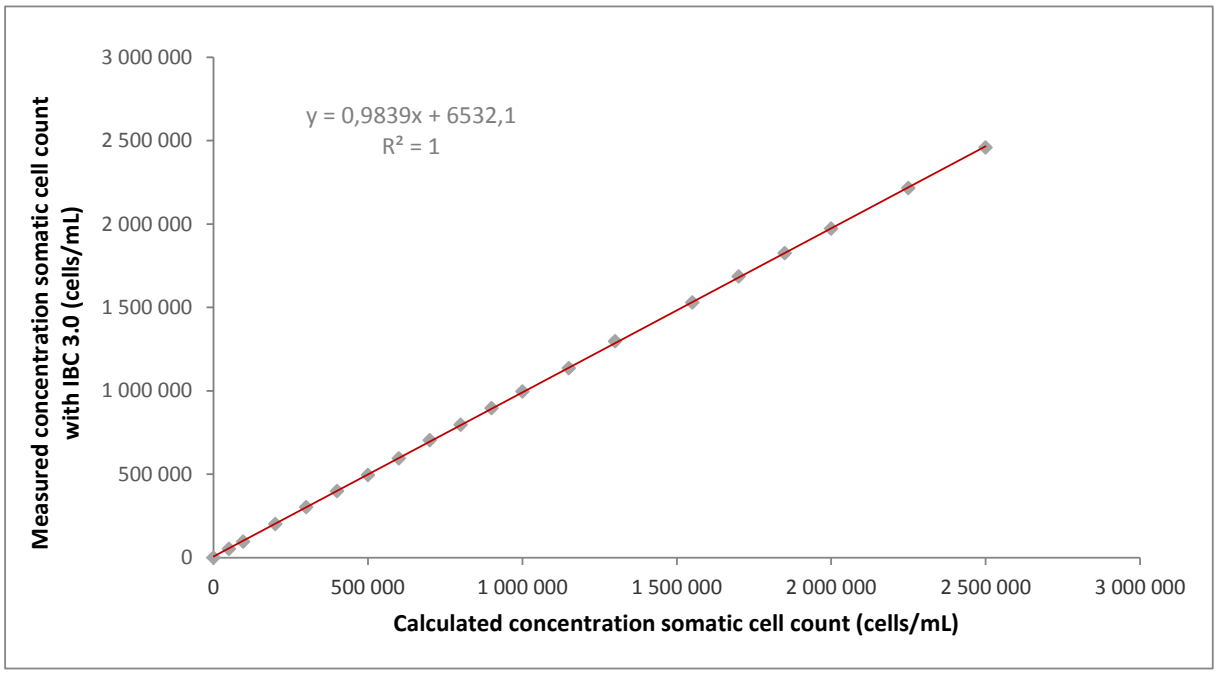
$e_{max}$ is the numerical value of the maximum residual from the regression;
$e_{min}$ is the numerical value of the minimum residual from the regression;
$M_{max}$ is the numerical value of the upper measured value for the samples;
$M_{min}$ is the numerical value of the lower measured value for the samples.

The ratio $r_c$ should be below 2% in order to comply with ISO 8196-3.

#### 2.3.3.2. Results

The results appeared to be linear in the whole testing range up to 2 500 x $10^3$ cells/mL with $r_c$= 0.76%. The results are pictured in Figure 2.

*Figure 2: Linearity of Bactocount IBC 3.0 for enumeration of somatic cells in the testing range up to 2 500 x $10^3$ cells/mL.*




#### 2.3.3.3. Conclusion

**The instrument is linear in the working range and up to 2 500 x $10^3$ cells/mL. The linearity of the Bactocount IBC 3.0 complies with the stated maximum limit value of $r_c \leq 2\%$ in the ISO 8196-3 and EURL MMP document.**

### 2.3.4. Limits of quantification (according to ISO 8196-3 § 5.2.2.1.4)

**Limits of a measurement** with an instrumental method exist at both **extremities** of the analytical range: a lower and an upper limit. The assessment of the measurement limits can be carried out in combination with the evaluation of the linearity. If linearity is not achieved throughout the whole concentration range, then the actual range of application for the method should be evaluated.

The lower limit of quantification is the smallest amount of measurand that can be measured and quantified with a defined coefficient of variation, CV. The lower limit of quantification is defined as multiples of the standard deviation, $\sigma$, of random error observed near to zero (blank).

The upper limit of quantification corresponds to the threshold where the signal deviates significantly from linearity.

#### 2.3.4.1. Measurement protocol and calculations

##### 2.3.4.1.1. Lower limit of quantification, $L_Q$

Filtrate of skimmed raw cow milk was spiked with a **low quantity of concentrate** of the same milk to obtain a mix containing a **low concentration somatic cells**. The obtained milk was used to perform 20 measurements with the Bactocount IBC 3.0. The mean and the standard deviation, $\sigma$, were calculated and the lower limit of quantification, $L_Q$, was determined as:

$$L_Q = average\ blank\ value + 10 \times \sigma$$

##### 2.3.4.1.2. Upper limit of quantification

Upper limit of quantification corresponds to the threshold where the signal or the measurement deviates from linearity. The upper limit of quantification of somatic cells of Bactocount IBC 3.0 was defined on the base of the linearity results.

#### 2.3.4.2. Results

##### 2.3.4.2.1. Lower limit of quantification, $L_Q$

The results for the determination of the lower limit of quantification are shown in Table 7.

**The resulting lower limit of quantification is 10.2x10$^3$ cells/mL**.

*Table 7: Results of lower limit of quantification of somatic cells of the Bactocount IBC 3.0*

| Measurement | Results *(x10³ cells/mL)* |
|---|---|
| **1** | 3 |
| **2** | 2 |
| **3** | 2 |
| **4** | 2 |
| **5** | 1 |
| **6** | 1 |
| **7** | 2 |
| **8** | 4 |
| **9** | 1 |
| **10** | 3 |
| **11** | 3 |
| **12** | 2 |
| **13** | 1 |
| **14** | 2 |
| **15** | 1 |
| **16** | 3 |
| **17** | 1 |
| **18** | 2 |
| **19** | 2 |
| **20** | 2 |
| **21** | 3 |
| **22** | 3 |
| **23** | 2 |
| **24** | 2 |
| **25** | 2 |
| **Mean** | **2.1** |
| $\sigma$ | **0.8** |
| $L_Q$ | **10.2** |

#### 2.3.4.2.2. Upper limit of quantification

The linearity of the method have been tested in the range from 0 to 2 500 x10$^3$ cells/mL. In this range, the method tested is fully linear (see § 2.3.3.2, $r_c$ = 0.76%). The upper limit of quantification of the method is therefore at least 2 500 x10$^3$ cells/mL.

**The upper limit of quantification of Bactocount IBC 3.0 is in accordance with the EURL MMP requirement of > 1 400x10$^3$ cells/mL.**

### 2.3.4.3. Conclusion

**The lower limit of quantification of somatic cells of Bactocount IBC 3.0 is 10 200 cells/mL according to ISO 8196-3. The upper limit of quantification of somatic cells of Bactocount IBC 3.0 is at least 2 500 x10$^3$ cells/mL and complies with EURL requirements.**

## 2.4. Intra laboratory repeatability and accuracy of Bactocount IBC 3.0 for enumeration of somatic cells

The **overall accuracy** is the sum of the **repeatability error**, the **accuracy error** and the **calibration error**.

With raw milk, each part of the overall precision is measured by analysis of milk samples from individual animals and herd milk of the specified animal species. The herd milk samples should be collected in addition to the individual milk samples in order to more accurately measure the amount of variance related to herd effects.

The evaluation should be performed under conditions equivalent to the intended routine use.

The same samples have been analysed in duplicates for repeatability evaluation and for accuracy evaluation (average of the two replicates).

### 2.4.1. Calibration (according to ISO 8196-3 § 5.2.2.2.5.3)

**Calibration of Bactocount IBC 3.0 and Somacount FC** were performed according to the manufacturer's recommendations with somatic cells Standard Reference Material from Actalia Cecalait (traceable to IRMM CRM). The Standard Reference Materials were used to calibrate and check the calibration.

Results of measurement of Standard Reference Materials of 10 milk samples with somatic cells concentration from 0 to 1 800 x$10^3$ cells/mL and the linear regressions of the results obtained with the SomaCount FC and the IBC 3.0 are represented in Figure 3 and Figure 4 respectively.

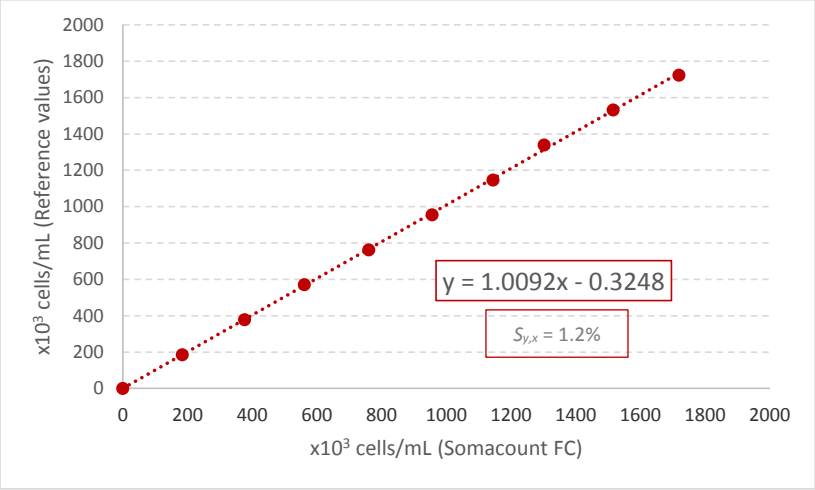



*Figure 3: Linear regression of Somacount FC measurements of SRM*

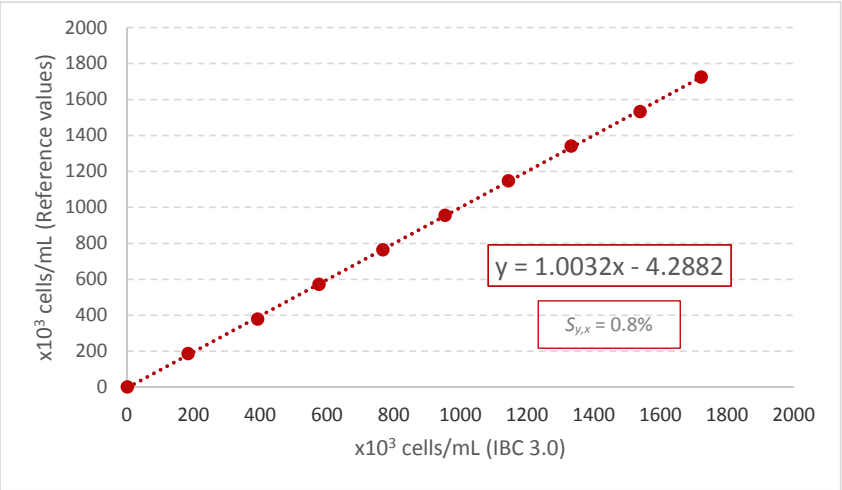



*Figure 4: Linear regression of IBC 3.0 measurements of SRM*

The slope of the linear regression and the relative bias calculated for the two instruments were presented in the Table 8. **All parameters were in accordance with the ISO 8196 requirements for the 2 instruments**.

*Table 8: Slope of the linear regression (b), relative bias ($\bar{d}_{rel}$) and residual standard deviation ($s_{y,x}$) calculated for the two instruments*

| Parameters | Somacount FC | IBC 3.0 | Acceptability values according to ISO 8196-3 |
|---|---|---|---|
| **Slope of the linear regression (*b*)** | **1.009** | **1.003** | **1 ±0.05** |
| **relative bias ($\bar{d}_{rel}$)** | **- 0.87%** | **0.18%** | **± 5%** |
| **Residual standard deviation ($s_{y,x}$)** | **1.2%** | **0.8%** | **-** |

### 2.4.2. Repeatability (according to ISO 8196-3 § 5.2.2.2.4)

**Repeatability** is the primary criterion for determining whether a method produces **stable results in accordance with the user's requirements**. It is the major element of internal quality control. Therefore, each new instrument must meet a maximum limit of repeatability value specified in the applicable International Standard to meet the accreditation criteria.

#### 2.4.2.1. Measurement protocol and calculations

**The standard deviation of repeatability** ($s_r$) of the Bactocount IBC 3.0 was calculated from testing results with 135 individual raw cow milk samples and 67 raw herd bulk cow's milk samples representative for different somatic cell count levels as shown in Table 9. *Note that 1 outlier sample was eliminated by COCHRAN 5% for individual milk samples and 3 for herd milk samples.*

*Table 9: Raw cow milk samples selected for estimation of the repeatability of the Bactocount IBC 3.0 for enumeration of somatic cells; Values in brackets represent the number of samples without COCHRAN 5% elimination.*

| Cell count level (x10³ cells/mL) | Number of individual raw cow's milk samples | Number of herd bulk cow's milk samples | Total samples |
|---|---|---|---|
| **0 – 150** | **102** (102) | **41** (42) | **143** (144) |
| **150 – 300** | **14** (14) | **22** (22) | **36** (36) |
| **300 – 450** | **7** (7) | **4** (5) | **11** (12) |
| **450 – 750** | **8** (8) | **0** (1) | **8** (9) |
| **750 – 1 500** | **4** (5) | **0** (0) | **4** (5) |
| **Total number of samples** | **135** (136) | **67** (70) | **202** (206) |

All raw cow milk samples were measured in duplicate (n=2) with Bactocount IBC 3.0. **The standard deviation of repeatability** ($s_r$) was calculated for each cell count level as:

$$s_r = \left(\sum w_i^2 / 2q\right)^{1/2}0$$

With $i(w_i = |x_{1i} - x_{2i}|)$

The calculations were performed without any transformation.

The repeatability ($r$) is calculated as:

$$r = 2.83 s_r$$

#### 2.4.2.2. Results

**The standard deviation of repeatability ($s_r$)** of Bactocount IBC 3.0 for enumeration of somatic cells was calculated for all the milk samples, for each cell count levels. The results and the acceptability values are given in Table 10. *Note that one outlier sample was eliminated by COCHRAN 5% for individual milk samples and three for herd milk samples.*

*Table 10 : The standard deviation of repeatability (sr) of the Bactocount IBC 3.0 for enumeration of somatic cells calculated per cell count level and acceptability values according to ISO 13366-2 and EURL MMP document; Values in brackets represent the values calculated without COCHRAN 5% elimination.*

| Cell count level | Number of samples | Mean level samples | $s_r$ | | Acceptability values according to 13366-2 |
|---|---|---|---|---|---|
| x10³ cells/mL | - | x10³ cells/mL | x10³ cells/mL | % | % |
| **0 – 150** | **143** (144) | 60 (60) | **5.2** (8.2) | **8.7% (13.7%)** | **6%** |
| **150 - 300** | **36** (36) | 205 (205) | **7.7** (7.7) | **3.7%** (3.7%) | **5%** |
| **300 - 450** | **11** (12) | 355 (352) | **10.2** (18.2) | **2.9%** (5.2%) | **4%** |
| **450 - 750** | **8** (9) | 571 (561) | **10.0** (12.8) | **1.7%** (2.3%) | **3%** |
| **750 – 1 500** | **4** (5) | 960 (936) | **10.3** (18.0) | **1.1%** (1.9%) | **3%** |
| **All** | **202** (206) | 140 (146) | **6.5** (9.6) | **4.6%** (6.6%) | - |

The repeatability ($r$) of Bactocount IBC 3.0 for enumeration of somatic cells was calculated for each cell count levels. The results and the acceptability values are given in Table 11.

*Table 11 : The repeatability (r) of the Bactocount IBC 3.0 for enumeration of somatic cells calculated per cell count level and acceptability values according to ISO 13366-2 and EURL MMP document; Values in brackets represent the values calculated without COCHRAN 5% elimination.*

| Cell count level | Number of samples | Mean level samples | $r$ | Acceptability values according to 13366-2 |
|---|---|---|---|---|
| x10³ cells/mL | - | x10³ cells/mL | x10³ cells/mL | x10³ cells/mL |
| **0 – 150** | **143** (144) | 60 (60) | **15** (23) | **25** |
| **150 – 300** | **36** (36) | 205 (205) | **22** (22) | **42** |
| **300 – 450** | **11** (12) | 355 (352) | **29** (51) | **50** |
| **450 – 750** | **8** (9) | 571 (561) | **28** (36) | **63** |
| **750 – 1 500** | **4** (5) | 960 (936) | **29** (51) | **126** |
| **All** | **202** (206) | 140 (146) | **18** (27) | **-** |

Due to the low mean value (60 x $10^3$ cells/mL) of the first range (0-150 x $10^3$ cells/mL), the Sr% obtained for this range is a little bit higher to the limit , but $r$ value which is the valuable indicator to take into account is in conformity with ISO 13366-2 limits .

The **calculated repeatability (*r*)** for the enumeration of somatic cells by IBC 3.0 is **lower than the limit for all the cell count levels.**

#### 2.4.2.3. Conclusion

**Repeatability ($r$) of the IBC 3.0 for the enumeration of somatic cells complies with the requirement of EURL MMP document and ISO 13366-2 at all cell count levels.**

### 2.4.3. Accuracy (according to ISO 8196-3 § 5.2.2.2.5.2)

The **accuracy** of the alternative method is based on the **residual standard deviation, $s_{yx}$,** of the simple linear regression of the instrumental results obtained in duplicate, $x$, and the reference results obtained in duplicate, $y$.

#### 2.4.3.1. Measurement protocol and calculations

**The residual standard deviation** of the Bactocount IBC 3.0 for enumeration of somatic cells was evaluated at different somatic cell count levels **through comparison with the Somacount FC**. It was calculated with 135 individual raw cow milk samples preserved with bronopol and 67 unpreserved raw herd bulk cow milk samples as shown in Table 12. *Note that one outlier sample was eliminated by COCHRAN 5% for individual milk samples and three for herd milk samples; moreover, one individual milk sample and one herd milk sample were eliminated because difference between methods was greater than 3 times the residual standard deviation.*

All samples were measured in duplicate with the two instruments.

*Table 12: Raw cow's milk samples selected for determination of residual standard deviation of the Bactocount IBC 3.0 for enumeration of somatic cells; Values in brackets represent the number of samples without elimination.*

| Cell count level (x10³ cells/mL) | Number of individual raw cow's milk samples | Number of herd bulk cow's milk samples | Total samples |
|---|---|---|---|
| **0 – 150** | **102** (102) | **40** (42) | **142** (144) |
| **150 – 300** | **14** (14) | **22** (22) | **36** (36) |
| **300 – 450** | **6** (7) | **4** (5) | **10** (12) |
| **450 – 750** | **8** (8) | **0** (1) | **8** (9) |
| **750 – 1 500** | **4** (5) | **0** (0) | **4** (5) |
| **Total number of samples** | **134** (136) | **66** (70) | **200** (206) |

The relationship between results with the evaluated instruments was visually inspected by plotting the results obtained with the Bactocount IBC 3.0 on the x-axis and the results obtained with the Somacount FC on the y-axis.

#### 2.4.3.2. Results

**The residual standard deviation** results and the acceptability values are given in Table 13.

*Table 13: Residual standard deviation ($s_{yx}$) of the Bactocount IBC 3.0 for enumeration of somatic cells and the acceptability values according to ISO 8196-3; Values in brackets represent the number of samples without elimination.*

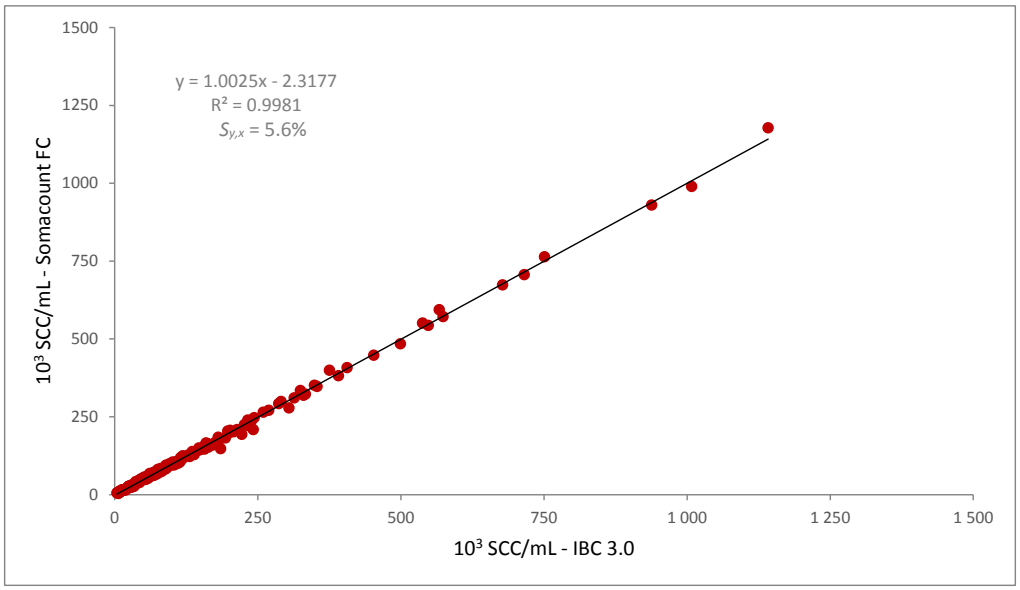
| Cell count level | Mean level samples | $s_{yx}$ | | Acceptability values according to ISO 8196-3 |
|---|---|---|---|---|
| x10³ cells/mL | x10³ cells/mL | x10³ cells/mL | % | % |
| **0 – 150** | 60 (60) | **3.5** (8.1) | **6.1%** (13.7%) | **8%** |
| **150 - 300** | 205 (205) | **10.6** (10.6) | **5.3%** (5.3%) | **8%** |
| **300 - 450** | 355 (352) | **13.2** (39.9) | **3.8%** (11.6%) | **8%** |
| **450 - 750** | 571 (561) | **14.2** (19.5) | **2.5%** (3.5%) | **8%** |
| **750 – 1 500** | 960 (936) | **28.7** (33.8) | **3.0%** (3.6%) | **8%** |
| **All** | 140 (146) | **7.6** (14.7) | **5.6%** (10.2%) | **8%** |

**The accuracy calculated for all cell count levels is lower than the ISO 8196-3 and EURL MMP document requirement.**

The accuracy of Bactocount IBC 3.0 was evaluated against Somacount FC with a linear regression.

The correlation between the evaluated models is visualized in Figure 5.

*Figure 5: Relationship between Bactocount IBC 3.0 and Somacount FC for individual and herd raw cow milk samples*




#### 2.4.3.3. Conclusion

**Accuracy of the IBC 3.0 for the enumeration of somatic cells complies with the requirement of EURL MMP document and ISO 13366-2 at all cell count levels.**

## 2.5. Evaluation of interference on SCC

The effect of milk composition was not evaluated for SCC with the IBC 3.0 in the frame of this validation. **However, data of impact of protein, fat and lactose composition on the equivalence of 2 Bentley's instruments based on the same principle for SCC were available.**

In this study, 98 individual milk samples were used for SCC with Somacount and Bactocount IBC2. Results obtained between the two instruments were compared and correlation between the residual error and the composition of the milk was evaluated (7). Correlations ($r^2$) observed were particularly low: 0.01, 0.006, 0.018 respectively for fat, protein and lactose content despite significant variations in the chemical composition of these milks (21.6 to 68.8 g/L of fat; 26.0 to 46.1 g/L of protein; 41.7 to 50.9g/L of lactose).

This suggested that there was **no effect of the matrix composition on the equivalence of the two instruments for SCC**. Because Somacount instrument is ICAR certified and because the principle of the actual IBC 3.0 instrument for SCC is the same, this suggested that there is no effect of the matrix for SCC with IBC 3.0 either.

## 2.6. Conclusion of the method comparison study for enumeration of somatic cells

Bactocount IBC 3.0 **performance characteristics** for enumeration of somatic cells according to ISO 8196-3 are:

- Bactocount IBC 3.0 functions **stable** through the working day;
- **Carry-over** per cell count level (ISO 8196-3 for each cell count level CO < 2%):
  - Low (501x10$^3$ cells/mL)

    $c_{H/L}$ = 0.69 %

    $c_{L/H}$ = -0.27 %
  - Medium (988x10$^3$ cells/mL)

    $c_{H/L}$ = 0.59 %

    $c_{L/H}$ = 0.12 %
  - High (1 477x10$^3$ cells/mL)

    $c_{H/L}$ = 0.44 %

    $c_{L/H}$ = 0.23 %

- **Linearity**: $r_c$ = 0.76 % (ISO 8196-3 < 2 %)
- **Lower limit of quantification**: $L_Q$ = 10 200 cells/mL
- **Upper limit of quantification**: 2 500x10$^3$ cells/mL

Conclusions of the **overall accuracy evaluation** of Bactocount IBC 3.0 for enumeration of somatic cells according to ISO 8196-3 are:

- **Repeatability** per cell count level:
  - 0 – 150x10$^3$ cells/mL

    $r$ = 15x10$^3$ *(ISO 13366-2: $r$ < 25x10$^3$ cells/mL)*
  - 150 – 300x10$^3$ cells/mL

    $r$ = 22x10$^3$ *(ISO 13366-2: $r$ < 42x10$^3$ cells/mL)*
  - 300 – 450x10$^3$ cells/mL

    $r$ = 29x10$^3$ *(ISO 13366-2: $r$ < 50x10$^3$ cells/mL)*
  - 450 – 750x10$^3$ cells/mL

    $r$ = 28x10$^3$ *(ISO 13366-2: $r$ < 63x10$^3$ cells/mL)*
  - 750 – 1 500x10$^3$ cells/mL

    $r$ = 29x10$^3$ *(ISO 13366-2: $r$ < 126x10$^3$ cells/mL)*

- **Accuracy** per cell count level (ISO 8196-3 for each cell count level $s_{yx,rel}$ < 10%):
  - 0 – 150x10$^3$ cells/mL

    $s_{yx,rel}$ = 6.1%

- 150 – 300x10$^3$ cells/mL

  $s_{yx,rel}$ = 5.3%

- 300 – 450x10$^3$ cells/mL

  $s_{yx,rel}$ = 3.8%

- 450 – 750x10$^3$ cells/mL

  $s_{yx,rel}$ = 2.5%

- 750 – 1 500x10$^3$ cells/mL

  $s_{yx,rel}$ = 3.0%

# METHOD COMPARISON STUDY
# FOR ENUMERATION OF
# TOTAL BACTERIA (TBC)

# 3. METHOD COMPARISON STUDY FOR ENUMERATION OF TOTAL BACTERIA (TBC)

## 3.1. Materials and equipment used

- « Blank milk bacteria »: raw cow milk with bacterial count approximately between 1 000 and 5 000 cfu/mL;
- Culture *of Lactococcus lactis* LC strain (from Actalia Cecalait);
- Herd bulk cow's milk samples;
- Stock and working solutions for Bactocount IBC 3.0 and Bactocount IBC 2.0, prepared according to manufacturer's instructions;
- RBS 2% solution;
- IBC incubation / dye solution;
- IBC standard solutions;
- Refrigerator at 0-4°C;
- Standard laboratory glassware and utensils.

To perform the experimental work described in this study, the following was needed:

- Bactocount IBC 3.0;
- Bactocount IBC 2.0 (MicroVal certified; certificate n°2013 LR 44);
- Instruction and method implementation;
- Statistical expertise.

## 3.2. Preparation of samples

The **performance characteristics** of the **alternative method** were assessed using **artificially contaminated samples**. Raw cow milk was spiked with ***Lactococcus lactis*** **Lc** strain to obtain specific concentration of total bacteria. Each milk sample was used during the day and was not stored. The milk samples were placed between 0 and +4°C before the measurement.

## 3.3. Performance characteristics of the alternative method

### 3.3.1. Stability (according to ISO 8196-3 § 5.2.2.1.1)

The **stability of the alternative method** was verified by mimicking routine testing circumstances throughout a working day. To evaluate the stability of the instrument, the standard deviation of repeatability ($s_r$), the standard deviation of means ($s_x$), the standard deviation between checks ($s_c$) and the standard deviation of daily reproducibility ($s_{R,daily}$) were determined for different bacterial count levels.

#### 3.3.1.1. Measurement protocol and calculations

"Blank" milk samples were spiked with *Lactoccus lactis* Lc culture to obtain **three bacterial count levels**: low, medium and high (Table 14).

*Table 14 : Bacterial count levels of samples used in the stability study with the Bactocount IBC 3.0.*

| Cell count level | Theoretical bacterial count ($Log_{10}$ CFU/mL) | Bacterial counts measured with IBC 3.0 ($Log_{10}$ CFU/mL) |
|---|---|---|
| **Low (L)** | 4.7 | 4.7 |
| **Medium (M)** | 5.2 | 5.2 |
| **High (H)** | 5.5 | 5.5 |

Each sample was stored between 0 and +4°C before measurement.

Samples from each bacterial count level were measured in triplicate (n=3) with the Bactocount IBC 3.0 in the order: (L – M – H) each 15-20 minutes during a working day with 16 checks in total.

The standard deviation of repeatability ($s_r$), the standard deviation of means ($s_x$), the standard deviation between checks ($s_c$) and the standard deviation of daily reproducibility ($s_{R,daily}$) were calculated according to ISO 8196-3:2009. The calculation were performed in units of the alternative method (CFU/mL) after logarithmic transformation of the data.

- **For every check, j (j=1…..q):**
  - The mean $\bar{x}_j$ was calculated according to:

$$\bar{x}_j = \sum x_{ij} / n$$

with n = number of measurements (n=3) an i = replicate

  - And the standard deviation $s_{rj}$ of replicates according to:

$$s_{rj} = \left[\sum \left(x_{ij} - \bar{x}_j\right)^2 / (n-1)\right]^{1/2}$$

- **For the whole check sequence the following parameters were calculated:**
  - The standard deviation of repeatability $s_r$:

$$s_r = \left(\sum s_{rj}^2 / q\right)^{1/2}$$

with q = number of checks (q = 20)

  - The standard deviation of means $s_{\bar{x}}$:

$$s_{\bar{x}} = \left[\sum \left(\bar{x}_j - \bar{x}\right)^2 / (q-1)\right]^{1/2} = \left\{\left[\sum \bar{x}_j^2 - \frac{\left(\sum \bar{x}_j\right)^2}{q}\right] / (q-1)\right\}^{1/2}$$

with:

$$\bar{x} = \sum \bar{x}_j / q$$

- The standard deviation between checks:

$$s_c = \left(s_{\bar{x}}^2 - s_r^2/n\right)^{1/2}$$

if $s_c < 0$ then $s_c = 0$

- The standard deviation of daily reproducibility:

$$s_{R,daily} = (s_c^2 + s_r^2)^{1/2}$$

The stability of the method response during the sequence of check tests was visualized by plotting the means of the measurement results ($\bar{x}_j$) on the y-axis, versus the check sequence numbers, on the x-axis.

#### 3.3.1.2. Results

A summary of the stability results is given in Table 15.

*Table 15: The standard deviation of repeatability ($s_r$), the standard deviation of means ($s_x$), the standard deviation between checks ($s_c$) and the standard deviation of daily reproducibility ($s_{R,daily}$) of the Bactocount IBC 3.0 for enumeration total bacteria per examined cell count level; Results are in $log_{10}$ UFC/mL.*

| Level of contamination ($Log_{10}$ CFU/mL) | $S_r$ | $S_x$ | $S_c$ | $S_{R,daily}$ |
|---|---|---|---|---|
| **Low (4.7)** | 0.03 | 0.01 | 0 | 0.03 |
| **Medium (5.2)** | 0.01 | 0.01 | 0 | 0.01 |
| **High (5.5)** | 0.01 | 0.01 | 0 | 0.01 |

**The standard deviation of repeatability ($s_r$) for each contamination level meets the requirement** according to the EURL MMP document (6) and ISO 16297 of $s_r \leq 0.09$ $log_{10}$ CFU/mL for contamination levels ≥ $2x10^4$ CFU/mL (≥ 4.30 $log_{10}$ CFU/mL).

**The calculated standard deviation of daily reproducibility ($s_{R,daily}$) complies** with the requirement of <0.09 $log_{10}$ CFU/mL at all tested contamination levels.

The small standard deviation between checks ($s_c$) and standard deviation of means ($s_x$) show that the variation of instrument read-outs throughout the day was very small.

The plot visualizing the stability of the method response during the day is given in Figure 6.

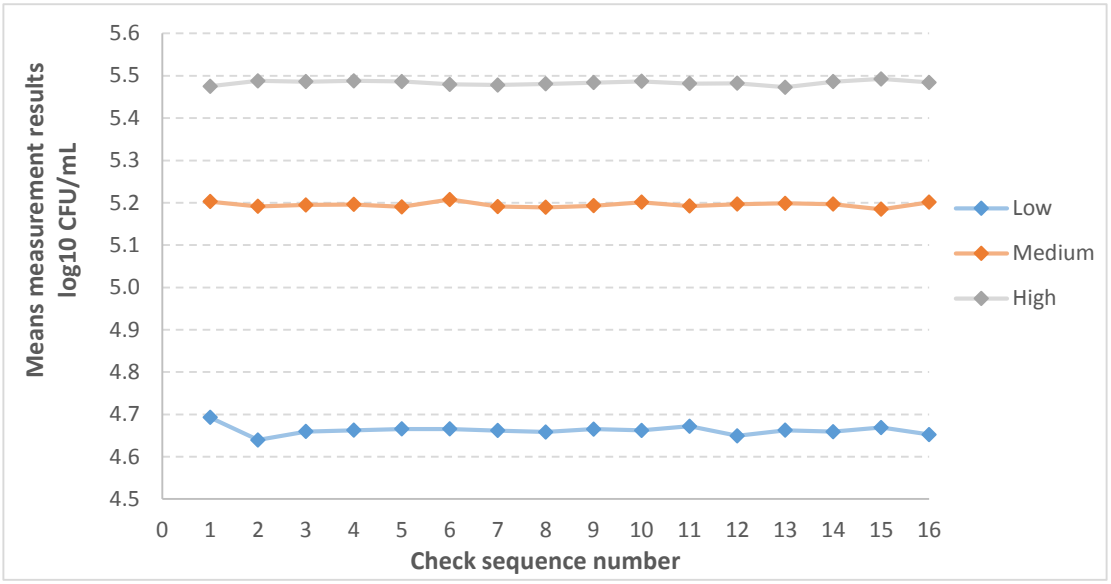



*Figure 6: Bactocount IBC 3.0 stability for enumeration of bacteria throughout the working day based on the means of the measurement results at three bacterial count levels.*

#### 3.3.1.3. Conclusion

**The Bactocount IBC 3.0 is stable during the working day for the enumeration of total bacteria. The stability complies with the requirements of the EURL MMP document and ISO 16297.**

### 3.3.2. Carry-over effect (according to ISO 8196-3 § 5.2.2.1.2 and ISO 16297 § 5.4 )

Strong differences in total bacteria count levels between two successively analyzed samples may influence the result of a second one. Carry-over effect may occur in analytical systems with continuous flow systems. It derives from the transfer of a certain portion of sample to the next or further samples. The overall carry-over effect was assessed without the carry-over correction factor of the instrument.

#### 3.3.2.1. Measurement protocol and calculations

**Milk samples were prepared at four "high" bacterial** count levels by spiking "blank milk" with *Lactococcus lactis* Lc suspsension. The total bacteria count levels of the "high" samples are given in Table 16. The "low" samples were unspiked raw cow milk and were also called "**blank milk**".

*Table 16 : Total bacterial count levels of samples used in the stability study with the Bactocount IBC 3.0.*

| Bacterial level | Theoretical bacterial count ($x10^3$CFU/mL) | Bacterial counts measured with IBC 3.0 ($x10^3$CFU/mL) |
|---|---|---|
| **High 1** | 50 | 46 |
| **High 2** | 150 | 143 |
| **High 3** | 300 | 284 |
| **High 4** | 2 000 | 1 600 |

Each sample was stored between 0 and +4°C before measurement.

Bactocount IBC 3.0 measurements were performed without carry-over correction factor on 20 sets of samples per cell count level with the following sequence:

$(L_{H1}, L_{H2}, L_{L1}, L_{L2})_1, (L_{H1}, L_{H2}, L_{L1}, L_{L2})_2 \dots (L_{H1}, L_{H2}, L_{L1}, L_{L2})_{20}$ or

$(L_H, L_{L1}, L_{L2})_1, (L_H, L_{L1}, L_{L2})_2 \dots (L_H, L_{L1}, L_{L2})_{20}$

The calculations were performed on raw data without any transformation. The relative carry-over (COR) was obtained by applying the following equations:

- The relative carry-over in the i[th] sample set ($COR_i$) was calculated according to:

$$COR_i = \frac{(C_{b1i} - C_{b2i})}{C_{si}} \times 100$$

with

$C_{b1i}$ is the result of the first blank milk in the check $i$

$C_{b2i}$ is the result of the second blank milk in the check $i$

$C_{si}$ is the result of the second high milk in the check $i$

- The relative carry-over ($COR$) was calculated according to:

$$COR = \frac{\sum_i^n C_i}{n}$$

with

$n$ = number of sample sets

The carry-over effect should not exceed the limit of 1% as required in the ISO 16297 standard.

#### 3.3.2.2. Results

For each cell count level, the relative carry-over **COR was calculated**. The results are given in Table 17.

*Table 17: Calculated relative carry-over (COR) for enumeration of total bacterial count obtained with the Bactocount IBC 3.0.*

| Total bacterial count of the "high" samples | Number of sequences | Calculated *COR* % |
|---|---|---|
| **High 1** (46 x 10³ cfu/mL) | 20 | **0.91** |
| **High 2** (143 x 10³ cfu/mL) | 20 | **0.74** |
| **High 3** (284 x 10³ cfu/mL) | 20 | **0.65** |
| **High 4** (1 600 x 10³ cfu/mL) | 20 | **0.45** |
| **Over all** | 80 | **0.69** |

**The calculated relative carry-over effect for each total bacterial count was lower than the limit *COR* < 1%.**

#### 3.3.2.3. Conclusion

**The carry-over effect for enumeration of total bacteria with measurements on the Bactocount IBC 3.0 complies with the requirements in ISO 16297 for each cell count level.**

### 3.3.3. Linearity (according to ISO 8196 § § 5.2.2.1.3 and ISO 16297 § 5.3.3)

**The linearity** is the relationship between the instrument readings and the expected values with incremental additions of the measurand, in this case bacterial cells. This should be linear within the concerned range of bacterial count. Deviations from linearity may stem from non-specific signals and coincidence effects.

#### 3.3.3.1. Measurement protocol and calculations

**The linearity of Bactocount IBC 3.0 for enumeration of total bacteria was evaluated in the range from 5x10$^2$ and 5x10$^6$ CFU/mL. "**Blank milk" was spiked with *Lactococcus lactis* Lc suspension to obtain the defined total bacterial count. The samples were stored between 0 and +4°C before measurement.

The samples were measured with Bactocount IBC 3.0: 2 times in increasing concentration, 2 times in decreasing concentration.

To evaluate the linearity, the raw data were expressed in units of the alternative method (CFU/mL) without any transformation.

The expected value for each sample was calculated as linear regression from the measured values for the low count milk and the high count milk.

A linear regression was applied with the expected values per sample, $C_e$, on the x-axis and the measured values per sample, $C_{meas}$, on the y-axis. From the regression, the residuals were calculated as:

$$\Delta C_{1i} = C_{meas,i} - (aC_{e,i} + b)$$

For visual inspection of the data points, the residuals ($\Delta C_{1i}$), were plotted on the y-axis versus the expected values, $C_e$, on the x-axis. The ratio, $r_L$, was calculated by using the formula:

$$r_L = \frac{(\Delta C_{max} - \Delta C_{min})}{(C_{meas,max} - C_{meas,min})} \times 100$$

where

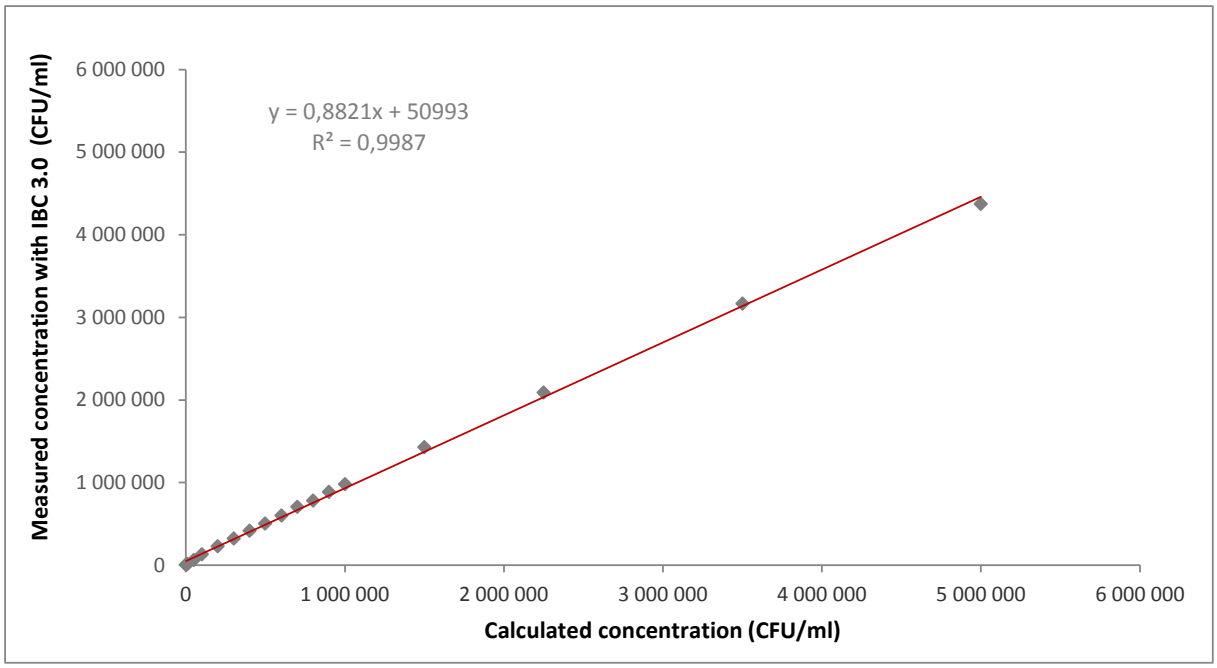
$\Delta C_{max}$ is the numerical value of the maximum residual from the regression;
$\Delta C_{min}$ is the numerical value of the minimum residual from the regression;
$C_{meas,max}$ is the numerical value of the upper measured value for the samples;
$C_{meas,min}$ is the numerical value of the lower measured value for the samples.

The ratio $r_L$ should be below 5% in order to comply with ISO 16297 standard.

#### 3.3.3.2. Results

The results appeared to be **linear in the whole testing range up to 5 000x10$^3$ CFU/mL** with $r_L$= 3.2%. The results are pictured in Figure 7.

*Figure 7: Linearity of Bactocount IBC 3.0 for enumeration of total bacteria in the testing range up to 5 000x10³ CFU/mL.*




#### 3.3.3.3. Conclusion

**The instrument is linear in the tested range up to 5 000x10³ CFU/mL. The linearity of the Bactocount IBC 3.0 complies with the stated maximum limit value of $r_L \leq 5\%$ in the ISO 16297 standard.**

### 3.3.4. Limits of quantification (according to ISO 16297 § 5.3.1 and 5.3.2)

**Limits of a measurement** with an instrumental method exist at **both extremities** of the analytical range: a lower and an upper limit. The assessment of the measurement limits can be carried out in combination with the evaluation of the linearity.

**The lower limit** of quantification is the smallest amount of measurand that can be measured and quantified with a defined coefficient of variation, CV. The lower limit of quantification is defined as multiples of the standard deviation, $s_0$, of random error observed near to zero (blank).

**The upper limit** of quantification corresponds to the threshold where the signal deviates significantly from linearity.

#### 3.3.4.1. Measurement protocol and calculations

##### 3.3.4.1.1. Lower limit of quantification, $L_Q$

Raw cow milk was used to perform 40 measurements. The raw data in units of the alternative method (CFU/mL) were processed without any transformation.

The standard deviation, $s_0$, were calculated and the lower limit of quantification, $L_Q$, was determined as:

$$L_Q = 10 \times s_0$$

#### 3.3.4.1.2. Upper limit of quantification

The upper limit of quantification of total bacteria of Bactocount IBC 3.0 was defined as the highest bacterial count where the instrument still shows a linearity ratio, $r_L \leq 5\%$, the limit value according to ISO 16297.

### 3.3.4.2. Results

#### 3.3.4.2.1. Lower limit of quantification, $L_Q$

The obtained results for determining the lower limit of quantification are shown in Table 18.

*Table 18: Results of lower limit of quantification of total bacteria of the Bactocount IBC 3.0.*

| Measurements | Results *(CFU/mL)* | Measurements | Results *(CFU/mL)* |
|---|---|---|---|
| 1 | **3000** | 21 | **2000** |
| 2 | **2000** | 22 | **1000** |
| 3 | **2000** | 23 | **2000** |
| 4 | **1000** | 24 | **2000** |
| 5 | **2000** | 25 | **2000** |
| 6 | **2000** | 26 | **2000** |
| 7 | **1000** | 27 | **2000** |
| 8 | **1000** | 28 | **2000** |
| 9 | **2000** | 29 | **2000** |
| 10 | **2000** | 30 | **2000** |
| 11 | **2000** | 31 | **2000** |
| 12 | **1000** | 32 | **2000** |
| 13 | **1000** | 33 | **1000** |
| 14 | **2000** | 34 | **1000** |
| 15 | **2000** | 35 | **2000** |
| 16 | **2000** | 36 | **2000** |
| 17 | **1000** | 37 | **1000** |
| 18 | **2000** | 38 | **2000** |
| 19 | **2000** | 39 | **2000** |
| 20 | **3000** | 40 | **2000** |
| **Mean** | 1 800 | | |
| $\sigma$ | 516 | | |
| $L_Q$ | **5 160** | | |

The resulting **lower limit of quantification is 5 160 CFU/mL.**

#### 3.3.4.2.2. Upper limit of quantification

Considering the method is linear in the range up to 5000 x$10^3$ cells/mL (see § 3.3.3.2 : $r_c$ = 3.2%). The upper limit of quantification of the method is at least 5000 x$10^3$ CFU / ml.

#### 3.3.4.3. Conclusion

The **lower limit of quantification** of total bacteria of Bactocount IBC 3.0 **is 5 160 CFU/mL**.

The **upper limit of quantification** of total bacteria of Bactocount IBC 3.0 is at least **5 000x10$^3$ CFU/mL**.

## 3.4. Intra laboratory repeatability and accuracy of Bactocount IBC 3.0 for TBC

The evaluation was performed on **herd cow milk** (milk payment). Precision trails were carried out against Bentley Bactocount IBC 2.0 (MicroVal certified; certificate n°2013 LR 44).

### 3.4.1. Repeatability (according to ISO 16297 § 5.6.2)

Repeatability should be estimated with a large number of measurements in duplicate performed on samples covering the entire measuring range.

#### 3.4.1.1. Measurement protocol and calculations

The standard deviation of repeatability ($s_r$) of the Bactocount IBC 3.0 was calculated from testing results with 250 raw herd bulk cow milk samples representative for different total bacterial count levels as shown in Table 19. *Note that 11 outlier samples were eliminated by COCHRAN 5%.*

*Table 19: Raw cow milk samples selected for estimation of the repeatability of the Bactocount IBC 3.0 for total bacterial count; Values in brackets represent the number of samples without COCHRAN 5% elimination.*

| Bacterial count level ($Log_{10}$ CFU/mL) | Number of herd bulk cow's milk samples |
|---|---|
| **3.7 – 4.7** | **123** (129) |
| **4.7 – 5.7** | **101** (106) |
| **5.7 – 6.7** | **26** (26) |
| **Total number of samples** | **250** (261) |

All raw cow's milk samples were measured in duplicate (n=2) with Bactocount IBC 3.0. The standard deviation of repeatability ($s_r$) was calculated for each cell count level as:

$$s_r = \left(\sum w_i^2 / 2q\right)^{1/2}$$

With $i(w_i = |x_{1i} - x_{2i}|)$

The calculations were performed without any transformation.

#### 3.4.1.2. Results

The **standard deviation of repeatability ($s_r$)** of Bactocount IBC 3.0 for enumeration of total bacteria was calculated for herd bulk cow's milk. The results and the acceptability values are given in Table 20.

*Table 20 : The standard deviation of repeatability (sr) of the Bactocount IBC 3.0 for enumeration total bacteria calculated per bacterial count level and acceptability values according to ISO 16297; Values in brackets represent the values calculated without COCHRAN 5% elimination.*

| Bacterial count level ($Log_{10}$ CFU/mL) | Number of samples | $s_r$ herd bulk cow's milk samples | | $s_r$ acceptability values according to ISO 16297 |
|---|---|---|---|---|
| | | Mean level samples ($Log_{10}$ CFU/mL) | $s_r$ | |
| **< 4.3** | **85** (90) | **4.0** (4.0) | **0.07** (0.14) | **0.12** |
| **≥ 4.3** | **165** (171) | **4.9** (5.0) | **0.05** (0.11) | **0.09** |

#### 3.4.1.3. Conclusion

**The repeatability of the Bactocount IBC 3.0 for total bacterial count complies with the requirement of ISO 16297 and EURL MMP document at all total bacterial count levels.**

### 3.4.2. Accuracy (according to ISO 16297 § 6.4.4)

**The accuracy of the alternative method** is based on the **residual standard deviation, $s_{yx}$,** of the simple linear regression of the instrumental results obtained in duplicate, $x$, and the results obtained with the anchoring method (IBC 2.0 in this study) in duplicate, $y$.

#### 3.4.2.1. Measurement protocol and calculations

The residual standard deviation of the Bactocount IBC 3.0 for enumeration of total bacteria was evaluated at **different total bacterial count levels through comparison with the anchoring method: Bactocount IBC 2.0**. It was calculated with 246 unpreserved raw herd bulk cow milk samples as shown in Table 21. *Note that 11 outlier samples were eliminated by COCHRAN 5%.; moreover, 4 milk samples were eliminated because difference between methods was greater than 3 times the residual standard deviation.*

All samples were measured in duplicate with each instrument.

*Table 21: Raw cow's milk samples selected for determination of residual standard deviation of the Bactocount IBC 3.0 for enumeration of total bacteria; Values in brackets represent the number of samples without elimination.*

| Bacterial count level ($Log_{10}$ cells/mL) | Number of herd bulk cow's milk samples |
|---|---|
| **3.7 – 4.7** | **120** (129) |
| **4.7 – 5.7** | **100** (106) |
| **5.7 – 6.7** | **26** (26) |
| **Total number of samples** | **246** (261) |

The relationship between results with the evaluated instrument was visually inspected by plotting the results obtained with the Bactocount IBC 3.0 on the x-axis and the results obtained with the Bactocount IBC 2.0 on the y-axis.

A linear regression was applied and the standard deviation of individual results $s_{yx}$ was determined.

For each sample, the logarithmic difference between the methods was calculated as:

$$\Delta C_{2i} = C_{alt,i} - C_{anch,i}$$

where:

$\Delta C_{2i}$ = difference between results obtained with the 2 methods for the i$^{th}$ sample
$C_{alt,i}$ = the result of the alternative method for the i$^{th}$ sample
$C_{anch,i}$ = the result of the anchoring method for the i$^{th}$ sample

For each total bacterial count level (interval of 0.5 $\log_{10}$ CFU/mL), following calculate were performed:

- The mean and standard deviation of results of the anchoring method
- The mean of the logarithmic difference, $\overline{\Delta C_{2i}}$
- The standard deviation of logarithmic difference, $s_{\Delta C_{2i}}$
- The limit of logarithmic confidence (95%) as $\overline{\Delta C_{2i}} \pm 1.96 s_{\Delta C_{2i}}$

Results were plotted on a graph as an accuracy profile.

#### 3.4.2.2. Results

The accuracy of Bactocount IBC 3.0 was evaluated against Bactocount IBC 2.0 with a linear regression.

The **standard deviation of individual results $s_{yx}$ was 0.12 $\log_{10}$ CFU/mL** (0.15 without sample elimination) and **complies with the limit of 0.40 $\log_{10}$ CFU/mL** defined in the ISO 16297 and EURL MMP document.

The correlation between the evaluated models is visualized in Figure 8. Moreover, the accuracy profile was determined and presented in Figure 9.

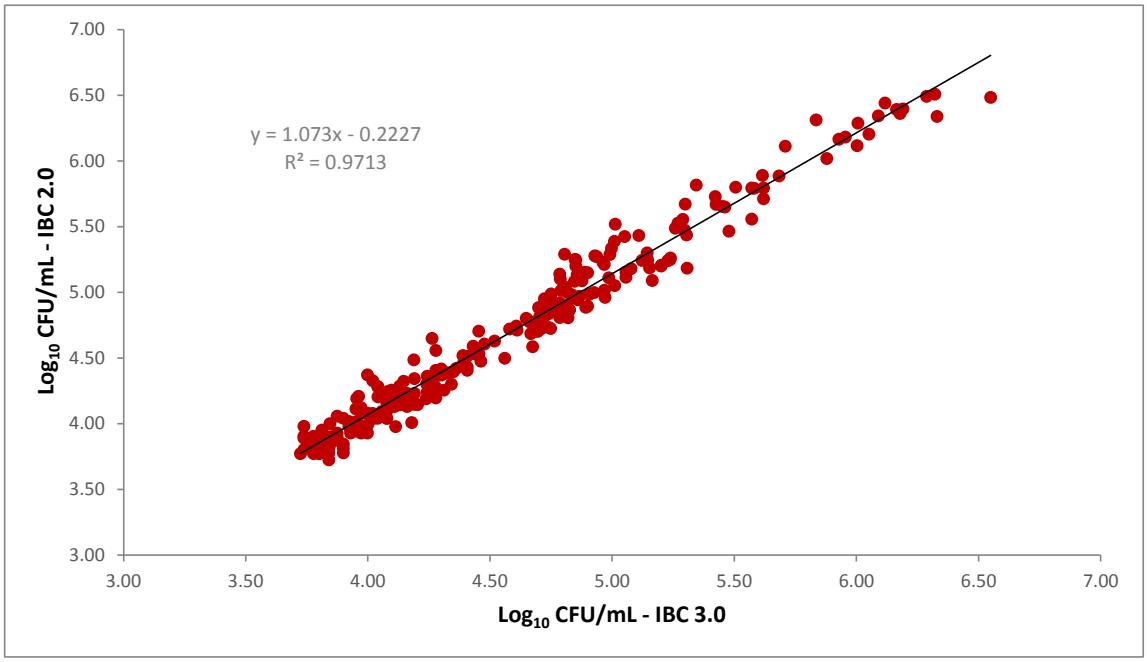



*Figure 8: Relationship between Bactocount IBC 3.0 and Bactocount IBC 2.0 for raw herd bulk cow's milk samples.*

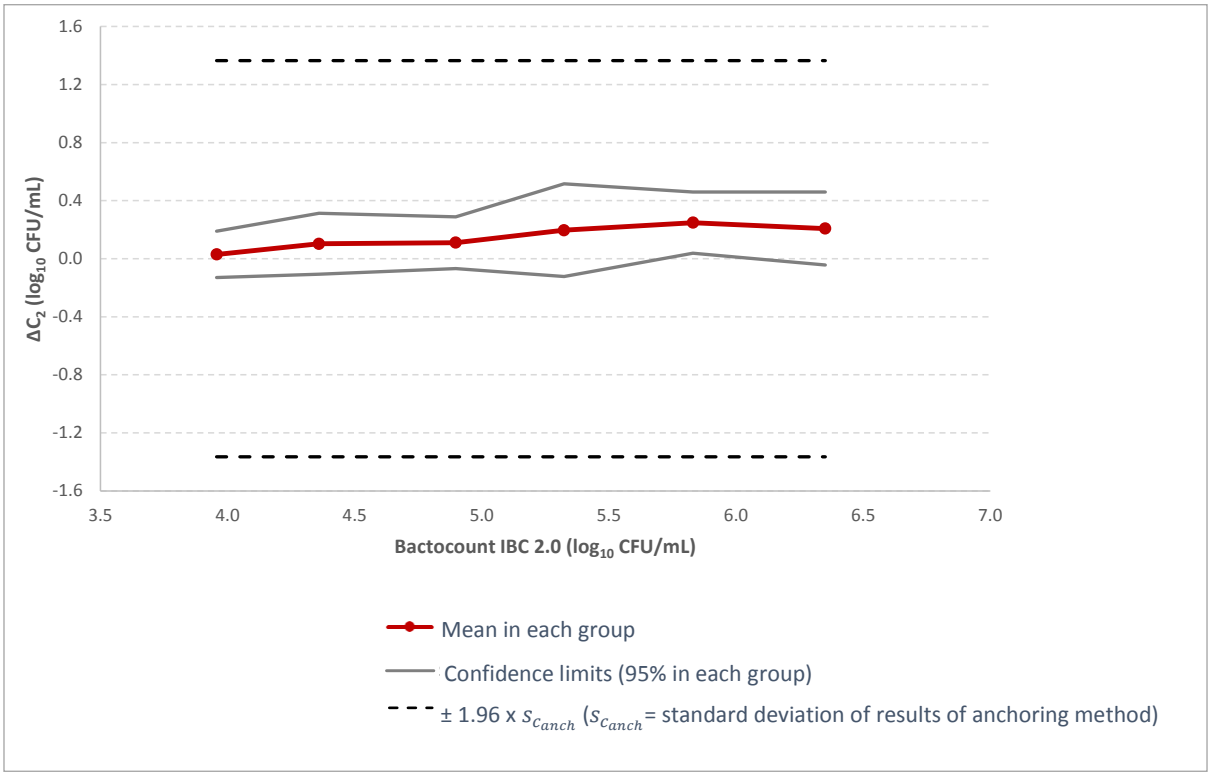



*Figure 9: Accuracy profile of Bactocount IBC 3.0 for enumeration of total bacteria.*

The accuracy was also evaluated using all the results obtained with all the samples measured (Table 22). *Note that 17 outlier samples were eliminated by COCHRAN 5% or because difference between methods was greater than 3 times the residual standard deviation.*

*Table 22: All raw cow's milk samples used for determination of residual standard deviation of the Bactocount IBC 3.0 for enumeration of total bacteria; Values in brackets represent the number of samples without elimination.*

| Bacterial count level ($Log_{10}$ cells/mL) | Number of herd bulk cow's milk samples |
|---|---|
| **3.7 – 4.7** | **184** (193) |
| **4.7 – 5.7** | **100** (106) |
| **5.7 – 6.7** | **24** (26) |
| **Total number of samples** | **308** (325) |

The **standard deviation of individual results $s_{yx}$ was 0.11 $log_{10}$ CFU/mL** (0.14 without sample elimination) and **complies with the limit of 0.40 $log_{10}$ CFU/mL** defined in the ISO 16297 and EURL MMP document and with respective methods reproducibility limits (< 0.16 $log_{10}$).

The correlation between the evaluated models is visualized in Figure 10.

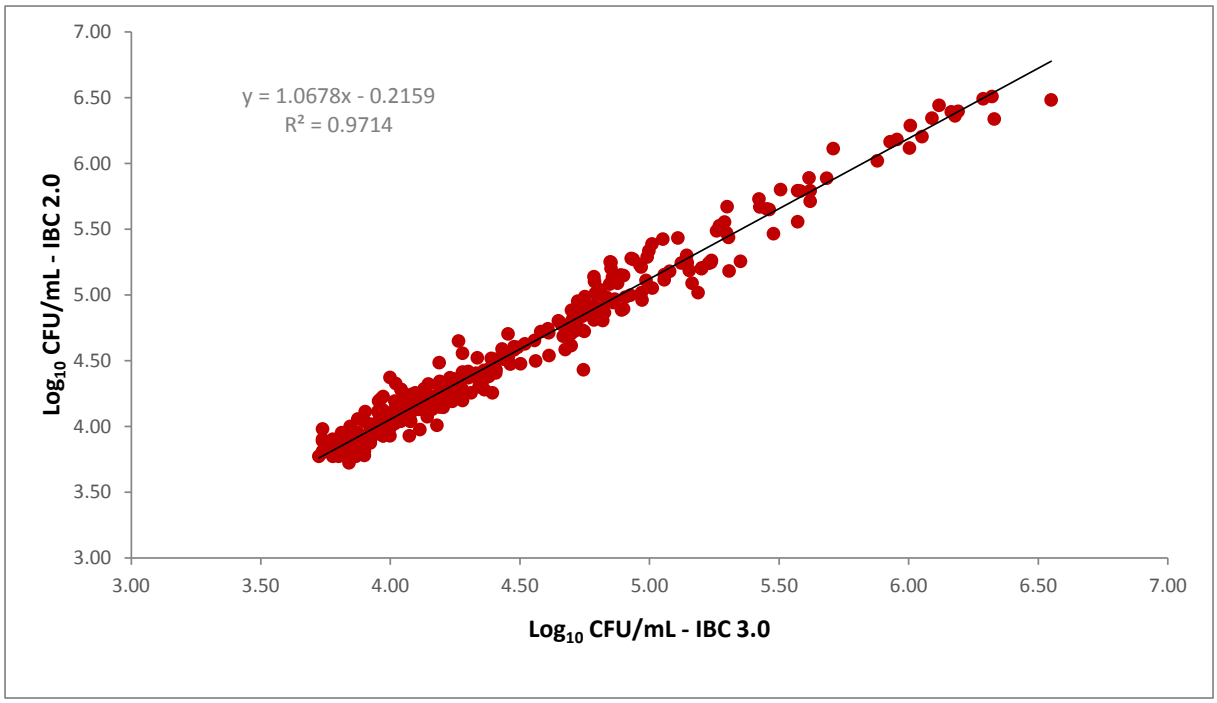



*Figure 10: Relationship between Bactocount IBC 3.0 and Bactocount IBC 2.0 for raw herd bulk cow's milk samples (all samples)*

## 3.5. Evaluation of interference on TBC

**Impact of milk composition on the TBC** was not evaluated for the instrument being evaluated. However, data are available for former instruments for TBC with the same principle than the instrument in evaluation. The evaluated instrument was the BactoCount (ISO 16140 validated) and impact of the matrix on TBC was compared to impact observed with the BactoScan in 2006 (study conducted by AGROSCOPE)(8). Results were analyzed for 398 milk samples. Table 23 is the matrix of Pearson correlation coefficients.

For the Bactocount, the correlation coefficients observed with milk composition were low. The same observation were done with the BactoScan.

*Table 23: Matrix of Pearson correlation coefficients (398 milk samples).*

| | LIBC | LIBCB | QUOTIENT | FETT | PROT | LACT | GP |
|---|---|---|---|---|---|---|---|
| LIBCB | 0.98780 | | | | | | |
| QUOTIENT | -0.12074 | 0.02896 | | | | | |
| FETT | -0.01649 | -0.01919 | -0.00579 | | | | |
| PROT | 0.22200 | 0.22423 | -0.00814 | 0.19452 | | | |
| LACT | -0.23021 | -0.22671 | 0.02590 | 0.05690 | 0.01840 | | |
| GP | -0.03094 | -0.03251 | -0.00357 | -0.14017 | -0.26284 | -0.50808 | |
| LSCC | 0.14542 | 0.15931 | 0.06115 | 0.10227 | 0.16987 | -0.31907 | -0.02870 |
| Number of observations: 398 | | | | | | | |

## 3.6. Conclusion of the method comparison study for TBC

Bactocount IBC 3.0 **performance characteristics** for enumeration of total bacteria according to ISO 16297 are:

- Bactocount IBC 3.0 functions **stable** through the working day;
- **Carry-over** per cell count level (ISO 16297 for each cell count level CO < 1%):
  - Low (46x10$^3$ CFU/mL)
    $COR$ = 0.91 %
  - Medium (143x10$^3$ CFU/mL)
    $COR$ = 0.74 %
  - High 1 (284x10$^3$ CFU/mL)
    $COR$ = 0.65 %
  - High 2 (1 600x10$^3$ CFU/mL)
    $COR$ = 0.45 %
  - All samples
    $COR$ = 0.69 %

- **Linearity**: $L$ = 3.2 % (ISO 16297 < 5 %)
- **Lower limit of quantification**: $L_Q$ = 5 160 CFU/mL
- **Upper limit of quantification**: 5 000x10$^3$ CFU/mL

Conclusions of the **overall accuracy evaluation** of Bactocount IBC 3.0 for enumeration of total bacteria according to ISO 16297 are:

- **Repeatability** per bacterial count level:
  Herd bulk cow's milk samples:
  - < 4.3 $\log_{10}$ CFU/mL
    $s_r$ = 0.07 (ISO 16297: $s_r$ < 0.12 $\log_{10}$)
  - ≥ 4.3 $\log_{10}$ CFU/mL
    $s_r$ = 0.05 (ISO 16297: $s_r$ < 0.09 $\log_{10}$)

- **Accuracy** for all tested samples (ISO 16297 for each cell count level $s_{yx}$ < 0.4):
  Herd bulk cow's milk samples (246 total samples):
  - $s_{yx}$ = 0.12 $\log_{10}$ CFU/mL

  Herd bulk cow's milk samples (308 total samples):
  - $s_{yx}$ = 0.11 $\log_{10}$ CFU/mL

## 4. FINAL CONCLUSION OF THE VALIDATION STUDY

- **SCC**: Performance characteristics of the BactoCount IBC 3.0 for enumeration of somatic cells in raw cow milk comply with the values defined in the ISO 8196-3. The comparison with the anchoring method SomaCount FC for SCC (ICAR certified according ISO 8196-3; certificate n°2020/7) revealed equivalence in terms of enumeration of somatic cells and do comply with the criteria of the EURL MMP document.

- **TBC**: Performance characteristics of the BactoCount IBC 3.0 for total bacterial count in raw cow milk comply with the values defined in the ISO 16297. The comparison with the anchoring method BactoCount IBC 2.0 for TBC (MicroVal certified; certificate n°2013 LR 44) revealed equivalence in terms of enumeration of bacteria and do comply with the criteria of the EURL MMP document.

## 5. REFERENCES

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2. EN ISO 13366-2|IDF 148-1:2006 Milk – Enumeration of somatic cells – Part 2: Guidance on the operation of fluoro-optoelectronic counters.

3. ISO 16297|IDF 161:2013 Milk – Bacterial count – Protocol for the evaluation of alternative methods.

4. ISO 21187:2004 Milk – Quantitative determination of bacteriological quality – Guidance for establishing and verifying a conversion relationship between routine method results and anchor method results.

5. EURL MMP document – Criteria for validation of instrumental (epifluorescent) methods for the enumeration of somatic cells in raw cow's milk, version 2, 21/01/2013.

6. EURL MMP document – Validation criteria of instrumental methods for enumeration of total flora in raw milk, version 2, 21 December 2011.

7. Technical note: Bentley_Effect of matrix on SCC

8. Comparison of the analytical instruments BactoCount and Bacto- Scan FC using raw milk samples. Final report September 2006; Bühlmann, G.; 2006 ; https://docplayer.org/69900419-Comparison-of-the-analytical-instruments-bactocount-and-bacto-scan-fc-using-raw-milk-samples-final-report-september-2006.html