**FOSS Analytical A/S**
**Foss Alle 1**
**DK-3400 HILLERØD**
**Denmark**

**MICROVAL VALIDATION REPORT**

**Comparative Study**

**MicroVal Project 2016LR65**

# Validation of BacSomatic™ (FOSS) for Total Bacterial Count in Raw Cow's Milk against EURL MMP Criteria

**Confidential**

Qlip B.V.

Authors Vesela Tzeneva

Version: 2

February 2, 2018

**Summary**

BacSomatic™ is a new instrument allowing the simultaneous determination of somatic cell count and total bacterial count in raw milk. However, the instrument can be used as a stand-alone instrument for either somatic cell count or total bacteria count determination as well. This MicroVal validation report presents the results obtained with the newly developed BacSomatic™ (FOSS Analytical A/S) for enumeration of total bacteria. The method comparison study for the validation of BacSomatic™ was performed against the criteria in the EURL MMP document "Criteria for the validation of instrumental (epifluorescent) methods for the determination of total flora in raw milk" from December 2011 (1) and the accuracy of the instrument was evaluated as comparison with the already approved BactoScan™ FC+ (MicroVal certificate 2013LR45). The results of the validation of BacSomatic™ for enumeration of somatic cells are presented in a separate report (MicroVal Project 2016LR64).

**Conclusions from the method comparison study**

BacSomatic™ performance characteristics determined according to ISO 16297 and ISO 16140-2 are:

- BacSomatic™ functions stable through the working day
- Repeatability ($r$): 0,03 – 0,12 $\log_{10}$ IBC/ml
  *(EURL MMP criterion: $r < 0{,}25 \log_{10}$ IBC/mL[1])*
- Carry-over effect, $COR$: 0,03 %
  *(EURL MMP criterion: COR < 1%)*
- Linearity ($r_L$) up to $2.10^6$ cfu/mL: 1,48 %
  *(EURL MMP criterion: $r_L$ < 5 %)*
- Upper limit of quantification: $2{,}64.10^8$ IBC/mL ($1{,}10.10^7$ cfu/mL)
- Lower limit of quantification: $1{,}3.10^4$ IBC/ml ($6.10^3$ cfu/ml)
- The results obtained with BacSomatic™ are not impacted by high contents of fat, protein or somatic cells in the milk.

**Conclusions from the comparison of BacSomatic™ TBC and BactoScan™ FC+**

The bacterial count results obtained with BacSomatic™ are equivalent to those obtained with BactoScan™ FC+ in the range $1.10^4$ – $6{,}5.10^7$ IBC/mL ($4{,}8.10^3$ – $3{,}7.10^6$ cfu/mL).

- Intra-laboratory reproducibility ($R_{intra\text{-}lab}$) per bacterial count level in raw herd bulk cow's milk:
  - Contamination level < 4,30 $\log_{10}$ cfu/mL 0,22 $\log_{10}$ IBC/mL
  - Contamination level ≥ 4,30 $\log_{10}$ cfu/mL 0,19 $\log_{10}$ IBC/mL
  - Over all 0,20 $\log_{10}$ IBC/mL
- The 95 % confidence limit interval of the slope and the intercept included respectively 1 and 0, and the standard error of the results $s_{yx}$ = 0,08 $\log_{10}$ IBC/mL is small, meaning close correlation between the results obtained with BacSomatic™ and BactoScan™ FC+

It is concluded that the results obtained with BacSomatic™ and BactoScan™ FC+ are equivalent.

[1] $r = 2{,}83.s_r = 2{,}83.0{,}09 = 0{,}25 \log_{10}$ IBC/mL

**Final conclusion of the validation study**

The final conclusion of the validation study is:

All results obtained during the method comparison study of BacSomatic™ comply with the criteria of the EURL MMP document. The comparison of results from BacSomatic™ and BactoScan™ FC+ (MicroVal certificate 2013LR45) revealed equivalence in terms of enumeration of bacteria and do comply with the criteria of the EURL MMP document.

**Contents**

## 1. Introduction

The BacSomatic™ from FOSS Analytical A/S is a newly developed low-throughput instrument based on Fossomatic™ FC and BactoScan™ FC/FC+ technology, which were both recently granted with MicroVal certificates. BacSomatic™ flow cytometer is a dedicated instrument for simultaneous determination of somatic cell count and total bacterial count in raw milk. However, the instrument can also be used as a stand-alone for determination of either somatic cell or total bacterial count. This report concerns the validation of BacSomatic™ for total bacterial count in raw cow's milk.

Since independent validation is a critical success factor for the acceptance of the BacSomatic™ as an instrumental method for the enumeration of total bacteria in raw milk in light of EU Regulation No 2074/2005 (2), modified by EU Regulation No 1664/2006 (3), the BacSomatic™ has to be validated against the European criteria published in an EURL MMP document from December 2011 (1). The EURL MMP document for validation of alternative methods refers to performance criteria in ISO 16297 (4) and ISO16140-2 (5).

BacSomatic™ is a downscaled version of BactoScan™ FC+ instruments for determination of total bacterial count in raw milk. The BactoScan™ FC+ was granted with a MicroVal certificate (2013LR45). The hardware and calculation algorithms of both models are highly similar, however the new analyser has some minor differences (using a laser with different wavelengths as a light source) when compared with the BactoScan™ FC+.

The performance characteristics of the BacSomatic™ for total bacterial count are demonstrated during the method comparison study for the matrix raw cow's milk. Its accuracy is demonstrated by comparison with results obtained with the BactoScan™ FC+.

This MicroVal validation report presents the results of an executed method comparison study as prescribed in the EURL MMP document from December 2011 and results of comparison of the two total bacterial counter models.

### 1.1. Principle of the alternative method

The BacSomatic™ is a low-throughput flow cytometer for the rapid enumeration of individual bacteria in raw milk. The working principle of the instrument is based on colouring the bacterial cells with a fluorescent dye - ethidium bromide - after which they are counted electronically.

In the flow cytometer, the mixture of milk and staining solution is surrounded by a sheath liquid and passed through a flow cell. In the flow cell, the stained bacteria are presented one by one to a laser and are exposed to light of a specific wavelength. The design of the flow cell must ensure that single bacteria are separately counted. The bacterial cells emit fluorescent light pulses at a different wavelength, and the pulses are amplified and recorded by a photo detector, multiplied by the working factor and displayed as a Individual Bacterial Count in thousands per milliliter (IBC/mL). The conversion of the IBC/mL to cfu/mL is established with conversion equation as described in ISO 21187 (6). Between each sample the flow system is thoroughly cleaned to reduce the carry-over to a minimum as well as the risk of build-up and clogging inside the analyser.

### 1.2. Scope

Raw cow's milk

### 1.3. Restriction of use

None

### 1.4. Reference method

EN-ISO 4833-1:2013 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 (7).

### 1.5. Comparison instrument

BactoScan™ FC+ with MicroVal certificate number 2013LR45.

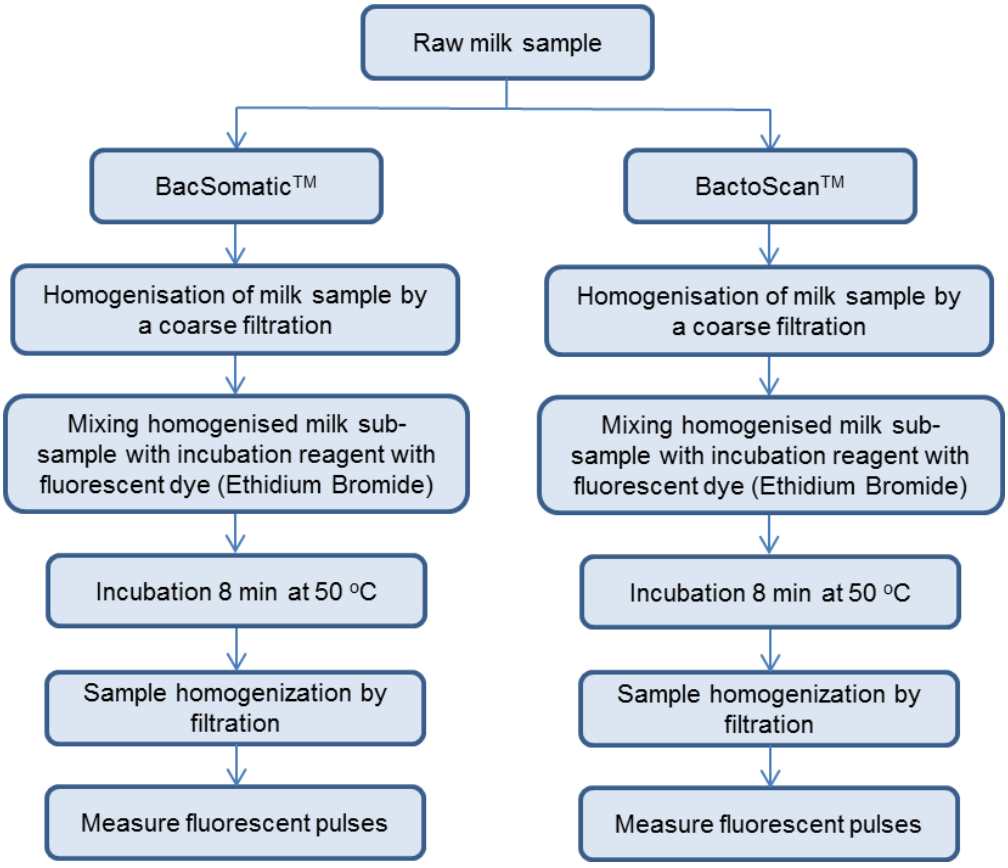
### 1.6. Conversion equation

The conversion of the IBC/mL to cfu/mL units was established based on the Qlip conversion equation:

$$\log_{10} \text{cfu/mL} = 0{,}7596.\log_{10} \text{IBC/µL} + 2{,}9227$$

### 1.7. Validation procedure

The measurement procedure for the direct comparison of BacSomatic™ and BactoScan™ FC+ is schematically presented below, which is illustrative for the mutual resemblance:



### 1.8. Materials and equipment used

- Traditional yoghurt starter culture containing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophiles* (CesKa-stAr C96, CSK, Leeuwarden)
- Sodium azide - preservation mixture with an end concentration in the milk of 0,03%
- 'Blank milk' - raw cow's milk with a bacterial count of approx. $3{,}5.10^3$ cfu/mL and free from growth inhibitory substances
- Stock and working solutions for BactoScan™ FC/FC+ and BacSomatic™, prepared according to manufacturers instructions from supplied consumables:
    - Cleaning solution
    - Buffer solution
    - Rinse solution
    - Incubation/dye solution
    - Sheath solution
    - Bacterial Count Sample
    - Blank solution
- Qlip control samples – preserved commingled raw milk sample with representative total bacterial count for the routine samples
- Herd bulk cow's milk samples
- Refrigerator at 0 – 4 °C
- Flip-top disposable vials
- Pipettes
    - Adjustable pipettes with tips
    - Serological pipettes
- Standard laboratory glassware and utensils

To perform the experimental work described in this test protocol the following was needed:

- BacSomatic™
- BactoScan™ FC+ (MicroVal certificate 2013LR45)
- Instruction and method implementation
- Statistical expertise.

### 1.9. Safety precautions

Good Laboratory Practices for running food analyses were followed.

## 2. Method comparison study

### 2.1. Performance characteristics of the alternative method

#### 2.1.1. 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.

##### 2.1.1.1. Measurement protocol and calculations

Preserved 'blank milk' was spiked with milk starter culture at three levels: low, medium and high. The concentrations of the inoculated samples are given in Table 1.

**Table 1. Inoculation levels of samples used in the stability, repeatability and intra-laboratory reproducibility studies with the BacSomatic™ total bacterial counter**

| Contamination levels | Measured $\log_{10}$ IBC/mL | Converted $\log_{10}$ cfu/mL |
|---|---|---|
| Low | 5,2 | 4,6 |
| Medium | 5,9 | 5,1 |
| High | 6,4 | 5,5 |

The spiked milk samples were left to stabilize for 5 days at 2 ± 2 °C.

Samples from each contamination level were measured in triplicate (n = 3) with the BacSomatic™ in random order during a working day with a duration of 8 hours. During a working day in total 11 series were checked.

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 (8). The calculations were performed in units of the alternative method (IBC/mL) after logarithmic transformation of the data.

For every check, *j* (*j=1….q*), the mean was calculated according to:

$$\bar{x_j} = \sum x_{ij} /n$$

with $n$ = number of measurements (n=3) and $i$ = replicate.

and the standard deviation of replicates according to:

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

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 ($q$ = 11)

the standard deviation of means ($s_{\bar{x}}$)

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

with

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

the standard deviation between checks ($s_c$)

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

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

the standard deviation of daily reproducibility ($s_{R,daily}$)

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

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.

The significance of a possible observed deviation or fluctuation for the standard deviation of means was tested with the *F*- test of a one-way ANOVA with α= 0,05.

#### 2.1.1.2. Results

A summary of the results is given in Table 2.

**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 BacSomatic™ per examined contamination level. Results are in $\log_{10}$ IBC/mL**

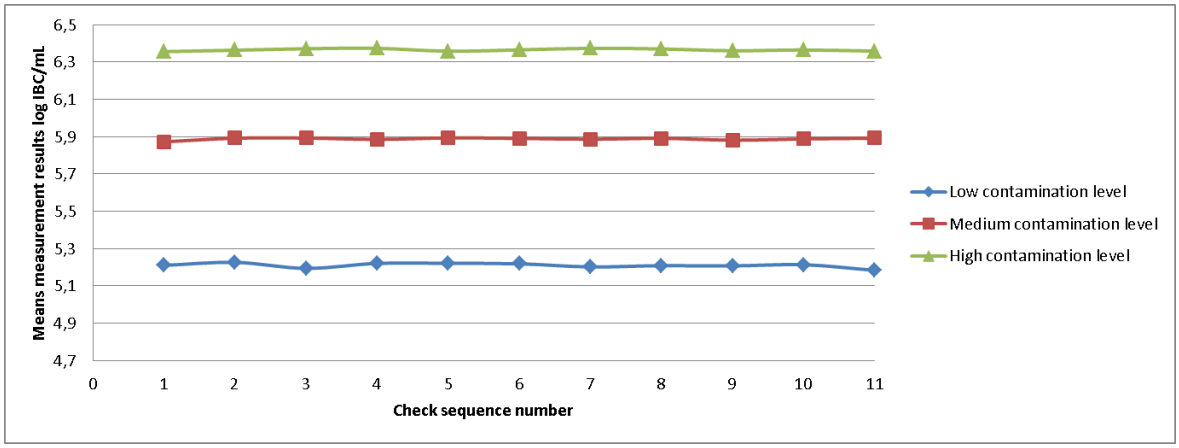
| $\log_{10}$ IBC/mL | Level of contamination | | |
|---|---|---|---|
| | Low | Medium | High |
| standard deviation of repeatability ($s_r$) | 0,02 | 0,01 | 0,01 |
| standard deviation of means ($s_x$) | 0,03 | 0,01 | 0,01 |
| standard deviation between checks ($s_c$) | 0,03 | 0,01 | 0,01 |
| standard deviation of daily reproducibility ($s_{R,daily}$) | 0,03 | 0,02 | 0,02 |

The standard deviation of repeatability ($s_r$) for each contamination level meets the requirement according to the EURL MMP document and ISO 16297 for $s_r \leq 0{,}09$ $\log_{10}$ IBC/mL for contamination levels ≥ $2.10^4$ cfu/mL (≥4,30 $\log_{10}$ cfu/mL).
The calculated $s_r$ complies with the requirement of < 0,09 $\log_{10}$ IBC/mL at all tested contamination levels, with an overall $s_r = 0{,}014$ $\log_{10}$ IBC/mL.

The calculated standard deviation of daily reproducibility ($s_{R,daily}$) complies with the requirement of <0,09 $\log_{10}$ IBC/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 1.

**Figure 1. BacSomatic™ stability for total bacterial count throughout the working day based on the means of the measurement results at three different contamination levels**




The significance of a possible observed deviation or fluctuation during the day was verified with the *F*-test of a one-way ANOVA. The results are given in Table 3.

**Table 3. *F*-test (α=0,05) of a one-way ANOVA for the three different contamination levels each**

| Contamination level | Source of variation | Sum of squares | Degrees of freedom | Mean of squares | F calculated | table value $F_{0,95}$ |
|---|---|---|---|---|---|---|
| Low | Between groups | 0,0048 | 10 | 0,0005 | 2,09 | 2,30 |
| | Within groups | 0,0051 | 22 | 0,0002 | | |
| | Total | 0,0099 | 32 | | | |
| Medium | Between groups | 0,0012 | 10 | 0,0001 | 0,76 | 2,30 |
| | Within groups | 0,0034 | 22 | 0,0002 | | |
| | Total | 0,0046 | 32 | | | |
| High | Between groups | 0,0012 | 10 | 0,0001 | 1,11 | 2,30 |
| | Within groups | 0,0025 | 22 | 0,0001 | | |
| | Total | 0,0037 | 32 | | | |

The calculated $F_{obs}$ values per cell count level were compared with the critical $F_{0,95}$ values. For all evaluated contamination levels $F_{obs} < F_{0,95}$ and no significant shift of instrument response was observed.

#### 2.1.1.3 Conclusion

The BacSomatic™ functioned stable throughout the working day and the stability complies with the requirements of the EURL MMP document (1) and ISO 16297 (4).

### 2.1.2. Repeatability *r* (according to ISO 8196-3 § 5.2.2.1.1)

The repeatability is the absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time.

The repeatability (*r*) is evaluated at different concentration levels.

#### 2.1.2.1. Measurement protocol and calculations

The repeatability (*r*) of BacSomatic™ was calculated based on results from above described stability experiment. For measurement protocol and calculations see clause 2.1.1.1. Additionally the repeatability was calculated on 191 raw herd bulk cow's milk samples representative for different total bacterial count levels as shown in Table 4. From these samples 14 were with elevated fat content, >5%. The results were also used for the evaluation of the intra-laboratory reproducibility ($R_{intra\text{-}lab}$) as described in clause 2.2.

**Table 4. Raw herd bulk cow's milk samples selected for estimation of the performance characteristics of the BacSomatic™. The results of the samples are used for calculation of repeatability (*r*) and intra-laboratory reproducibility ($R_{intra-lab}$)**

| Range ($log_{10}$ cfu/mL) | Number of herd bulk cow's milk |
|---|---|
| 3,75 – 5,0 | 89 |
| 5,0 – 5,7 | 50 |
| 5,7 – 6,0 | 52 |
| Total number samples | 191 |

All raw herd bulk cow's milk samples were measured in duplicate (n = 2) on BacSomatic™. The standard deviation of repeatability ($s_r$) was calculated for each total bacterial count level as described in clause 2.1.1.1. The calculations were performed in units of the alternative method (IBC/mL) after logarithmic transformation of the data.

The repeatability (*r*) is calculated as:

$$r = 2{,}83 . s_r$$

#### 2.1.2.2. Results

The calculated repeatability (*r*) from the stability experiment measured with BacSomatic™ and the acceptability values are presented in Table 5.

**Table 5. The repeatability (*r*) of the BacSomatic™ calculated per total bacterial count level and acceptability values according to ISO 16297 and EURL MMP**

| Contamination levels | *r* calculated $log_{10}$ IBC/mL | *r* ISO 16297 $log_{10}$ IBC/mL |
|---|---|---|
| Low | 0,05 | 0,34 |
| Medium | 0,04 | 0,25 |
| High | 0,03 | 0,25 |

The calculated repeatability (*r*) for raw herd bulk cow's milk samples measured with BacSomatic™ and the acceptability values are summarised in Table 6.

**Table 6. The repeatability (*r*) of the BacSomatic™ calculated per total bacterial count level for raw herd bulk milk samples**

| Contamination levels | *r* calculated $log_{10}$ IBC/mL | *r* ISO 16297 $log_{10}$ IBC/mL |
|---|---|---|
| < 4,30 log cfu/mL | 0,15 | 0,34 |
| ≥ 4,30 log cfu/mL | 0,09 | 0,25 |
| overall | 0,11 | |

The calculated repeatability (*r*) for BacSomatic™ is considerably lower than required by the EURL MMP document and ISO 16297, $r \leq 0{,}25$ $\log_{10}$ IBC/mL[2] for contamination levels ≥ $2.10^4$ cfu/mL (≥ 4,30 $\log_{10}$ cfu/mL), and $r \leq 0{,}34$ $\log_{10}$ IBC/mL[3] for contamination levels < $2.10^4$ cfu/mL (< 4,30 $\log_{10}$ cfu/mL). The calculated overall repeatability ($r_{overall}$= 0,11 $\log_{10}$ IBC/mL) from the measurements of the raw herd bulk cow's milk samples is clearly below the requirement of $r \leq 0{,}25$ $\log_{10}$ IBC/mL.

#### 2.1.2.3. Conclusion

The repeatability (*r*) of the BacSomatic™ for total bacterial count complies with the requirement of EURL MMP document (1) and ISO 16297 (4) at all total bacterial count levels.

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

Carry-over effects may occur in analytical systems with continuous flow systems. It derives from the transfer of a certain portion of sample material from one sample to the next or further sample(s) (4). The overall carry-over effect is assessed without the carry-over correction factor of the instrument.

#### 2.1.3.1. Measurement protocol and calculations

Preserved 'blank milk' was spiked with starter culture to a final concentration of $2{,}9.10^7$ IBC/mL ($2{,}1.10^6$ cfu/mL). The milk was left to stabilize for 5 days at 0-4 °C. These samples are below referred to as "high milk".

BacSomatic™ has 5 incubation chambers in total of which 4 are used for bacterial counting. To evaluate the carry-over effect both overall and per incubation chamber, in total 42 sample sets were measured. Per incubation chamber up to 10 samples sets were measured in the following sequence:

(high milk, $blank_1$, $blank_2)_1$, (high milk, $blank_1$, $blank_2)_2$…. (high milk, $blank_1$, $blank_2)_{12}$;

To evaluate the carry-over effect, raw data were processed in units of the alternative method (IBC/mL) without any transformation.

The relative carry-over, $COR$, is expressed as a percentage with the formulas:

$$COR_i = \frac{C_{b_1 i} - C_{b_2 i}}{C_{si}} \times 100$$

$$COR = \frac{\sum_i COR_i}{n}$$

where

$COR_i$ relative carry-over in the i[th] sample set

$C_{b_1 i}$ result of the first blank sample in the i[th] sample set

[2] $r = 2{,}83.s_r = 2{,}83.0{,}09 = 0{,}25$ $\log_{10}$ IBC/mL

[3] $r = 2{,}83.s_r = 2{,}83.0{,}12 = 0{,}34$ $\log_{10}$ IBC/mL

$C_{b_2 i}$ result of the second blank sample in the i$^{th}$ sample set

$C_{si}$ result of the milk sample in the i$^{th}$ sample set

$n$ number of sample sets

#### 2.1.3.2. Results

The calculated relative effect of carry-over overall and per incubation chamber is given in Table 7.

**Table 7. Overall carry-over effect and carry-over per incubation chamber of BacSomatic™**

| Incubation chamber | Number of sequences | COR (%) |
|---|---|---|
| 1 | 9 | 0,03 |
| 2 | 10 | 0,06 |
| 3 | 10 | 0,01 |
| 4 | 10 | 0,01 |
| over all | 42 | 0,03 |

Both the carry-over effects and the calculated overall carry-over effect were clearly below the required $COR$, < 1 %.

#### 2.1.3.3. Conclusion

The carry-over effect with measurements on the BacSomatic™ complies with the requirements in EURL MMP document (1) and ISO 16297 (4), $COR$ < 1 %.

### 2.1.4. Linearity (according to ISO 16297 §5.2.3)

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 counts (4) (5). Deviations from linearity may stem from non-specific signals and coincidence effects.

#### 2.1.4.1. Measurement protocol and calculations

The linearity of BacSomatic™ was evaluated in the range from $6.10^3$ to $2.10^7$ cfu/mL. Preserved 'blank milk' was inoculated with starter culture in steps of ca. $2.10^5$ cfu/mL in the performance range up to $2.10^6$ cfu/mL, and in steps of ca. $2{,}5.10^6$ cfu/mL in the range $2.10^6$ to $2.10^7$ cfu/mL. The spiked and preserved milk samples were stabilized for 5 days and stored at 2 ± 2 °C for a maximum of 1 month.

The samples were measured 4 times with BacSomatic™ in order of increasing concentration. To evaluate the linearity, the raw data were expressed in units of the alternative method (IBC/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

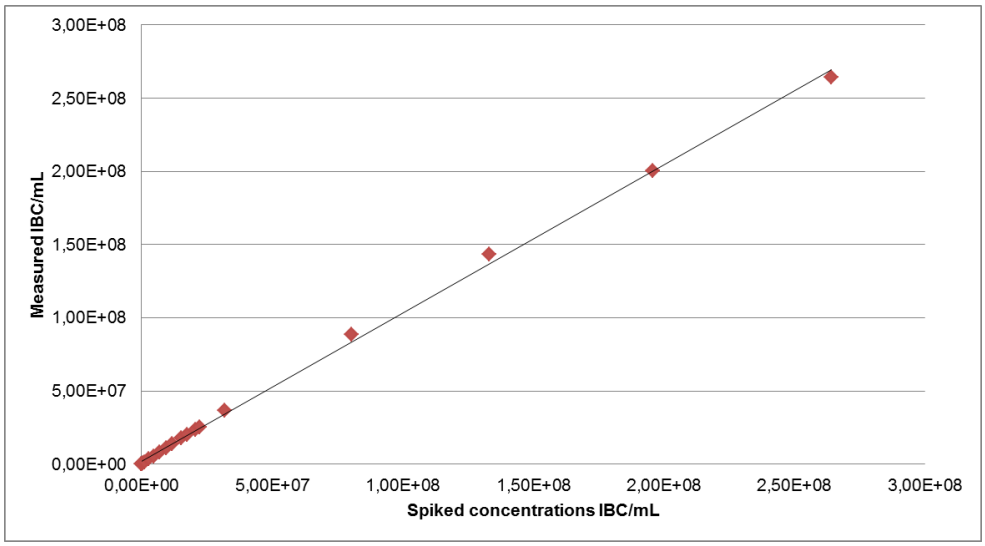
| | |
|---|---|
| $\Delta C_{max}$ | value of the maximum residual from the regression |
| $\Delta C_{min}$ | value of the minimum residual from the regression |
| $C_{meas,max}$ | measured value for the high count milk |
| $C_{meas,min}$ | measured value for the low count milk |

The ratio, $r_L$, should be below 5 % in order to comply with the EURL MMP document and ISO 16297.

#### 2.1.4.2. Results

The results appeared to be linear in the testing range up to 2,64.10$^8$ IBC/mL (1,10.10$^7$ cfu/mL) with $r_L$= 4,49 %. The results are pictured in Figure 2.

**Figure 2. Linearity of BacSomatic™ in the testing range up to 2,64.10$^8$ IBC/mL**




The BacSomatic™ also appeared to be linear ($r_L$= 1,48 %) when more specifically examined in the performance range 6.10$^3$ – 2.10$^6$ cfu/mL.

#### 2.1.4.3. Conclusion

The instrument is linear in the normal working range and in the wider measurement range up to 2,64.$10^8$ IBC/mL (1,10.$10^7$ cfu/mL). In both ranges, the linearity of the BacSomatic™ complies with the stated maximum limit value of $r_L$ ≤ 5 % in the EURL MMP (1) document and ISO 16297 (4).

### 2.1.5. Limits of quantification (according to ISO 16140-2 §6.1.4 and ISO16297 §5.2.2)

Limits of a measurement with an instrumental method exist at both extremities of the analytical range, e.g. a lower limit 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 analyte 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 a random error observed near zero (blank).

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

#### 2.1.5.1. Measurement protocol and calculations

##### *2.1.5.1.1. Lower limit of quantification, $L_Q$*

Preserved 'blank milk' was measured 10 times with with BacSomatic™. The raw data in units of the alternative method (IBC/mL) were processed without any transformation.

The standard deviation, $s_0$, of the measurements was calculated and the lower limit of quantification, $L_Q$, will be determined from:

$$L_Q = 10.s_0$$

##### *2.1.5.1.2. Upper limit of quantification*

The upper limit of quantification of the alternative method was defined as the highest bacterial count where the instrument still shows a linearity ratio, $r_L$, ≤ 5 %, the limit value according to ISO 16297.

#### 2.1.5.2. Results

##### *2.1.5.2.1. Lower limit of quantification, $L_Q$*

The obtained results for determining the lower limit of quantification are shown in Table 8.

**Table 8. Results for lower limit of quantification of the BacSomatic™**

| Measurement | IBC/mL |
|---|---|
| 1 | 7000 |
| 2 | 7000 |
| 3 | 10000 |
| 4 | 9000 |
| 5 | 10000 |
| 6 | 7000 |
| 7 | 8000 |
| 8 | 10000 |
| 9 | 10000 |
| 10 | 8000 |
| mean | 8600 |
| s0 | 1350 |

The resulting lower limit of quantification was $L_Q$ = 13 499 IBC/mL or 6 043 cfu/mL.

*2.1.5.2.2. Upper limit of quantification*

The results appeared to be linear up to $2{,}64.10^8$ IBC/mL ($1{,}10.10^7$ cfu/mL) with $r_L$= 4,49 %. The results are pictured in Figure 2.

### 2.1.5.3. Conclusion

The lower limit of quantification BacSomatic™ is 13 499 IBC/mL (6 043 cfu/mL).

The upper limit of quantification of BacSomatic™ $2{,}64.10^8$ IBC/mL ($1{,}10.10^7$ cfu/mL).

## 2.1.6. Evaluation of factors affecting the results (according to ISO ISO 16297 §5.5.4 and EURL MMP document)

High contents of fat, protein and somatic cell count in the milk could interfere with total bacterial count measurements on the BacSomatic™. The influence of contents of fat, protein and somatic cell count was examined at three relevant levels within the range of the measurand by applying linear regression analysis.

### 2.1.6.1. Measurement protocol and calculations

The total bacterial count in preserved raw cow's milk with ca. 3, 6 and 8 % fat, preserved raw cow's milk with ca. 3,7; 4,7 and 5,6 % protein and preserved raw cow's milk with somatic cell count of about $200.10^3$ cells/mL, $800.10^3$ cells/mL and $1\ 500.10^3$ cells/mL was adjusted at five bacterial concentrations.

The spiked and preserved milk samples were stabilized for 5 days and stored at 2 ± 2 °C for a maximum of 1 month.

Each sample was analysed four times with BacSomatic™.

The means of the replicate measurements per sample (n = 4) were calculated. The possible interference of high contents of fat, protein and somatic cell count on the total bacterial count was assessed by linear regression of the mean results:

$$y = bx + a$$

$y$ = instrument value,

$x$ = calculated value of the spiked samples.

Differences in obtained slopes and intercepts are indicative for interference of high contents of fat, protein and somatic cell count with the total bacterial count. It was required that slopes are within the 95 % confidence limit interval of the calculated slope and intercept for samples with 3 % fat, 3,5 % protein and $200.10^3$ cells/mL or that there is an overlap between the 95 % confidence limit intervals.
The relative linearity bias per fat, protein concentration and somatic cell count was expressed with the ratio $r_L$ and was calculated as described in clause 2.1.4.1.
Additionally 14 raw bulk cow's milk samples with fat content > 5 % were included in the analysis as described in clause 2.2.

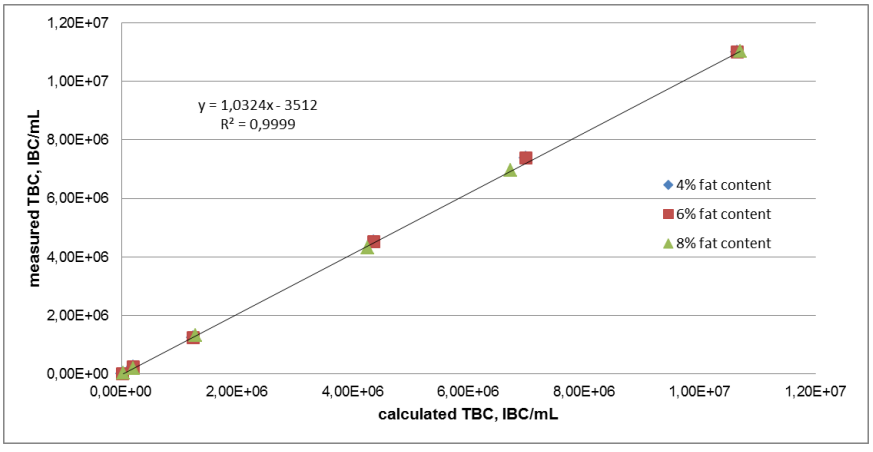
#### 2.1.6.2. Results

The calculated slope (*b*), intercept (*a*), 95% confidence limit interval from linear regression analysis, and linearity ratio ($r_L$) on results obtained with the BacSomatic™ on milk samples with different fat content and different total bacterial counts are given in Table 9 and visualisation of the results is shown in Figure 3.

**Table 9. Calculated slope (*b*), intercept (*a*), 95% confidence limit interval from linear regression analysis, and linearity ratio ($r_L$) on results obtained with the BacSomatic™ on milk samples with different fat content and different bacterial count levels**

| fat | slope (*b*) | | | intercept (*a*) (IBC/mL) | | | $r_L$ |
|---|---|---|---|---|---|---|---|
| concentration | calculated | lowest 95% | highest 95% | calculated | lowest 95% | highest 95% | (%) |
| **3%** | 1,0377 | 1,0184 | 1,0569 | 9368,1883 | -96716,0580 | 115452,4347 | 1,5 |
| **6%** | 1,0274 | 1,0198 | 1,0350 | 1062,6505 | -40090,2034 | 42215,5045 | 1,0 |
| **8%** | 1,0319 | 1,0203 | 1,0436 | -20564,2214 | -84283,1882 | 43154,7453 | 0,9 |

**Figure 3. Linearity of the results obtained with BacSomatic™ on milk samples with increasing fat content and different total bacterial count levels**



The slope and intercept for each fat level was calculated using linear regression. The slopes and the 95 % confidence intervals obtained with milk samples containing 6 % and 8 % fat were compared with the 95 % confidence limit interval of the slope obtained with milk samples containing 3 % fat (Table 4). The slopes obtained with 6 % ($b$ = 1,0274) and 8 % fat ($b$ = 1,0319) were within the 95 % confidence interval obtained for the slope of milk with 3 % fat. The calculated linearity ratio for each fat concentration was $r_L$ < 2 % and the results obtained with BacSomatic™ on milk samples with increasing fat content and different total bacterial count levels appear to be linear up to $1{,}10.10^7$ IBC/mL ($9{,}84.10^5$ cfu/mL).

The intercepts and the 95 % confidence intervals obtained with milk samples containing 6 % and 8 % fat were compared with the 95 % confidence limit interval of the intercept obtained with milk samples containing 3 % fat. The intercepts obtained with milk samples containing 6 % and 8 % fat were within the 95% confidence interval for the intercept obtained with 3 % fat.

The results obtained on raw bulk milk samples with elevated fat content were analysed with linear regression as described in clause 2.2. The variation of these results was within the variation of the results obtained on milk with lower fat content (Figure 6), indicating no interference of the higher fat on the total bacterial count.

It was therefore concluded that milk fat content up to 8 % does not have a relevant influence on the total bacterial count result.

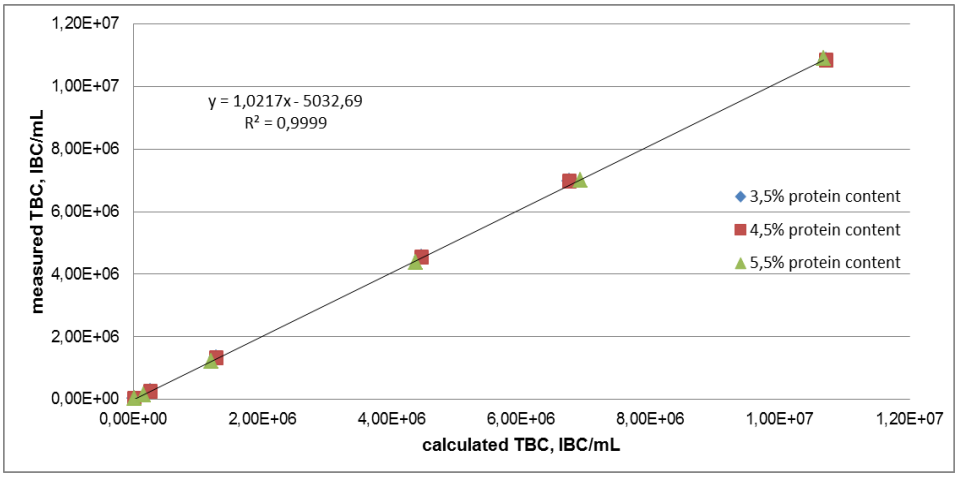
⇨ The total bacterial count results obtained with BacSomatic™ are not relevantly effected by an elevated fat content in the milk up to 8 %.

The calculated slope ($b$), intercept ($a$), 95% confidence limit interval from linear regression analysis, and linearity ratio ($r_L$) on results obtained with the BacSomatic™ on milk samples with different protein content and different total bacterial counts are presented in Table 10 and visualisation of the results is shown in Figure 4.

**Table 10. Calculated slope ($b$), intercept ($a$), 95% confidence limit interval from linear regression analysis, and linearity ratio ($r_L$) on results obtained with the BacSomatic™ on milk samples with different protein content and different bacterial count levels**

| protein | slope ($b$) | | | intercept ($a$) (IBC/mL) | | | $r_L$ |
|---|---|---|---|---|---|---|---|
| concentration | calculated | lowest 95% | highest 95% | calculated | lowest 95% | highest 95% | (%) |
| **3,5%** | 1,0176 | 0,9987 | 1,0365 | 12727,7796 | -91389,0766 | 116844,6358 | 1,6 |
| **4,5%** | 1,0288 | 1,0258 | 1,0318 | -7575,7532 | -23920,9708 | 8769,4643 | 0,2 |
| **5,5%** | 1,0186 | 1,0093 | 1,0279 | -20346,4744 | -71711,5887 | 31018,6399 | 0,7 |

**Figure 4. Linearity of the results obtained with BacSomatic™ on milk samples with increasing protein content and different total bacterial count levels**




The slope for each protein level was calculated using linear regression. The slopes and the 95 % confidence intervals obtained with milk samples containing 4,5 % and 5,5 % protein were compared with the 95 % confidence limit interval of the slope obtained with milk samples containing 3,5 % protein (Table 5). The slopes obtained with 4,5 % ($b$ = 1,0288) and 5,5 % protein contend ($b$ = 1,0186) were within the 95 % confidence interval obtained for the slope of milk with 3,5 % protein.

The intercepts and the 95 % confidence intervals obtained with milk samples containing 4,5 % and 5,5% protein were compared with the 95 % confidence limit interval of the intercept obtained with milk samples containing 3,5 % protein. The intercepts obtained with milk samples containing 4,5 % and 5,5% protein were within the 95% confidence interval for the intercept obtained with 3,5 % protein.

The calculated linearity ratio for each protein concentration was $r_L$ < 2 % and the results obtained with BacSomatic™ on milk samples with increasing protein content and different total bacterial count levels appear to be linear up to $1{,}09.10^7$ IBC/mL ($9{,}75.10^5$ cfu/mL).

It was concluded that milk fat content up to 5,5 % does not have a relevant influence on the total bacterial count result.

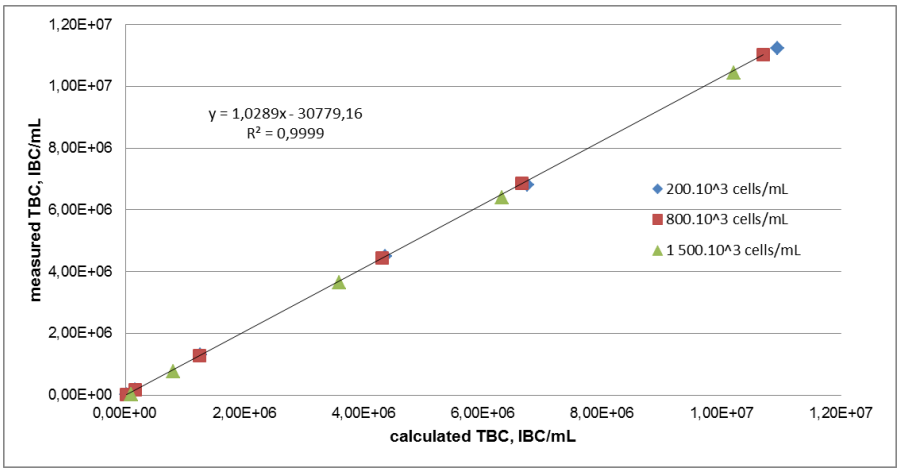
- ⇨ The total bacterial count results obtained with BacSomatic™ are not relevantly effected by an elevated protein content in the milk up to 5,5 %.

The calculated slope ($b$), intercept ($a$), 95% confidence limit interval from linear regression analysis, and linearity ratio ($r_L$) on results obtained with the BacSomatic™ on milk samples with different somatic cell count and different total bacterial counts are given in Table 11 and visualisation of the results is shown in Figure 5.

**Table 11. Calculated slope (*b*), intercept (*a*), 95% confidence limit interval from linear regression analysis, and linearity ratio ($r_L$) on results obtained with the BacSomatic™ on milk samples with different somatic cell count and different bacterial count levels**

| somatic cell count | slope (*b*) | | | intercept (*a*) (IBC/mL) | | | $r_L$ |
|---|---|---|---|---|---|---|---|
| | calculated | lowest 95% | highest 95% | calculated | lowest 95% | highest 95% | (%) |
| 200.10$^3$ cells/mL | 1,0236 | 1,0086 | 1,0385 | -609,8105 | -83774,7907 | 82555,1697 | 1,1 |
| 800.10$^3$ cells/mL | 1,0320 | 1,0311 | 1,0329 | -5671,8263 | -10542,6858 | -800,9668 | 0,5 |
| 1 500.10$^3$ cells/mL | 1,0303 | 1,0240 | 1,0366 | -82497,7105 | -114592,1069 | -50403,3142 | 0,5 |

**Figure 5. Linearity of the results obtained with BacSomatic™ on milk samples with increasing somatic cell count and different total bacterial count levels**




The slope for each somatic cell count level was calculated using linear regression. The slopes and the 95 % confidence intervals obtained with milk samples containing 800.10$^3$ cells/mL and 1 500.10$^3$ cells/mL were compared with the 95 % confidence limit interval of the slope obtained with milk samples containing 200.10$^3$ cells/mL (Table 6). The slopes obtained with 800.10$^3$ cells/mL (*b* = 1,0320) and 1 500.10$^3$ cells/mL (*b* = 1,0303) were within the 95 % confidence interval obtained for the slope of milk with 200.10$^3$ cells/mL.

The intercepts and the 95 % confidence intervals obtained with milk samples containing 800.10$^3$ cells/mL and 1 500.10$^3$ cells/mL were compared with the 95 % confidence limit interval of the intercept obtained with milk samples containing 200.10$^3$ cells/mL. The intercepts obtained with 800.10$^3$ cells/mL and 1 500.10$^3$ cells/mL were within the 95 % confidence interval obtained for the slope of milk with 200.10$^3$ cells/mL.

The calculated linearity ratio for each somatic cell count level was $r_L$ < 2 % and the results obtained with BacSomatic™ on milk samples with increasing somatic cell count and different total bacterial count levels appear to be linear up to 1,04.10$^7$ IBC/mL (9,44.10$^5$ cfu/mL).

It was concluded that milk somatic cell count up to 1 500.10$^3$ cells/mL does not have a relevant influence on the total bacterial count result.

⇨ The total bacterial count results obtained with BacSomatic™ are not relevantly effected by an elevated somatic cell count in the milk up to 1 500.10$^3$ cells/mL.

Additionally the effect of fat, protein and somatic cell count on the total bacterial count were evaluated and reported by Analytical Equivalence di Orlandini Silvia (AEOS) (9). Statistical analysis were performed to check normal distribution of the results with Shapiro test and the standard deviations were compared with Bartlett's and Levene's test (10). In the report it was indicated that that elevated fat, protein and somatic cell count could cause some noise in the results but do not influence the total bacterial count results. The report is provided for MicroVal evaluation.

#### 2.1.6.3. Conclusions

No relevant influence of elevated fat or protein content or somatic cell count of the milk was observed on the total bacterial count results obtained with the BacSomatic™.

### 2.2. Comparison of BacSomatic™ and BactoScan™ FC+

#### 2.2.1. Measurement protocol and calculations

The intra-laboratory reproducibility is the absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by possibly different operators using different instruments at different times (within at most a few hours). The intra-laboratory reproducibility ($R_{intra\text{-}lab}$) of the BacSomatic™ was evaluated at different concentration levels through comparison with the BactoScan™ FC+. $R_{intra\text{-}lab}$ was calculated based on results from 191 raw herd bulk cow's milk samples as shown in Table 4 and Table 12. From these samples 14 were with elevated fat content, > 5 %.

The samples were measured in random order in duplicate with BacSomatic™ and used for calculation of the repeatability ($r$) of the instruments as described in clause 2.1.2. Single measurements of the same samples were performed with a BactoScan™ FC+ instrument. Both instruments were operating in the routine laboratory of Qlip. The time between the measurements on both instruments did not exceed 2 hours. Different laboratory technicians have operated the instruments.

The standard deviation of reproducibility ($s_{R\ intra\text{-}lab}$) was calculated for the whole range and with respect to ≥ $2.10^4$ cfu/mL contamination level. The calculations were performed in units of the alternative method (IBC/mL) after logarithmic transformation. The standard deviation of intra-laboratory reproducibility, $s_{R\ intra\text{-}lab}$, was calculated with the first result from duplicate measurement obtained with the BacSomatic™ and the result obtained with the BactoScan™ FC+ as:

$$s_{R\ intra-lab} = \sqrt{\frac{\sum(x_1 - x_2)^2}{2n}}$$

where

$x_1$ = first result from duplicate measurement obtained with the BacSomatic™

$x_2$ = result obtained with the BactoScan™ FC+

$n$ = number of samples.

The intra-laboratory reproducibility, $R_{intra\text{-}lab}$, was calculated as:

$$R_{intra-lab} = 2{,}83.s_{R,intra-lab}$$

The relationship between results with the evaluated models was visually inspected by plotting the results obtained with the BacSomatic™ on the y-axis and the results obtained with the BactoScan™ FC+ on the x-axis. The standard error ($s_{yx}$) was calculated.
The accuracy of BacSomatic™ against BactoScan™ FC+ was evaluated by linear regression analysis after logarithmic transformation of the results. It was required that the calculated slope and intercept do not significantly differ from these of the identity function (f(x) = x), which means slope = 1 and intercept = 0 are within the 95 % confidence limit interval of the calculated slope and intercept.

### 2.2.2.Results

The results obtained with BacSomatic™ and BactoScan™ FC+ at different contamination levels were compared by calculating the intra-laboratory reproducibility ($R_{intra\text{-}lab}$) (Table 12).

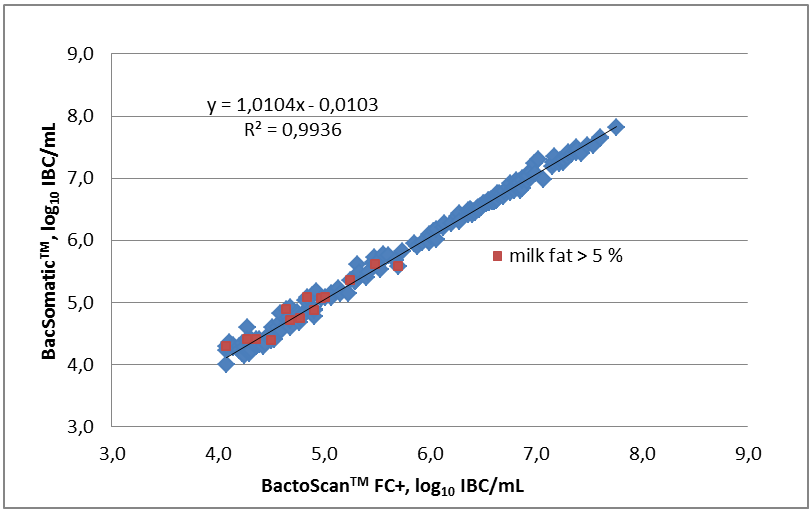
**Table 12. Intra-laboratory reproducibility ($R_{intra\text{-}lab}$) BacSomatic™**

| Contamination levels | Number of samples n | $R_{intra\text{-}lab}$ calculated $\log_{10}$ IBC/mL |
|---|---|---|
| < 4,30 log cfu/mL | 52 | 0,23 |
| ≥ 4,30 log cfu/mL | 139 | 0,19 |
| overall | 191 | 0,20 |

The calculated intra-laboratory reproducibility of BacSomatic™ compared to BactoScan™ FC+ complies even with the most strict ISO 16297 requirement for repeatability (<0,25 $\log_{10}$ IBC/mL) at all tested contamination levels.

The accuracy of BacSomatic™ was evaluated against BactoScan™ FC+ with a linear regression. The correlation between the evaluated models is visualised in Figure 6.

**Figure 6. Correlation of BacSomatic™ and BactoScan™ FC+ for raw herd bulk cow's milk samples**




The slope, intercept and calculated 95 % confidence interval of the regression analysis are shown in Table 13.

**Table 13. Slope, intercept and 95 % confidence interval limits from the linear regression analysis between results obtained with BacSomatic™ and BactoScan ™ FC+**

| | Coefficient | Lowest 95 % | Highest 95 % |
|---|---|---|---|
| **slope** | 1,0104 | 0,9988 | 1,0220 |
| **intercept** | -0,0103 | -0,0787 | 0,0582 |

The 95 % confidence limit interval of the slope and the intercept included respectively 1 and 0, meaning that the relationship between the results obtained with BacSomatic™ and BactoScan™ FC+ were statistically identical at threshold $p < 0,05$. The calculated standard error of the results was small, $s_{yx}$= 0,08 $\log_{10}$ cfu/mL.

The small standard error ($s_{yx}$) and the not significant deviation of the regression line from the identity function demonstrated a close correlation between the results obtained with both instruments and indicate that both instruments can be considered equivalent.

Note: The herd bulk milk samples used in the comparative evaluation of BacSomatic™ were measured simultaneously for total bacterial and somatic cell counts (combi) mode of BacSomatic. The same samples were measured with BactoScan™ FC/FC+ and Fossomatic™ FC, both granted with MicroVal certificates. The simultaneous determination of total bacterial and somatic cell counts demonstrated the performance of BacSomatic™ in its routine modus. The somatic cell count results were used for the MicroVal validation of BacSomatic™ in terms of somatic cell counting (MicroVal Project 2016LR64).

### 2.2.3.Conclusion

The bacterial count results obtained with BacSomatic™ are equivalent to those obtained with BactoScan™ FC/FC+.

## 3. Conclusions

### The method comparison study

BacSomatic™ performance characteristics determined according to ISO 16297 and ISO 16140-2 are:

- BacSomatic™ functions stable through the working day
- Repeatability (*r*): 0,03 – 0,12 $\log_{10}$ IBC/ml
  *(EURL MMP criterion: r < 0,25 $\log_{10}$ IBC/mL)*
- Carry-over effect, $COR$: 0,03 %
  *(EURL MMP criterion: COR < 1%)*
- Linearity ($r_L$) up to $2.10^6$ cfu/mL: 1,48 %
  *(EURL MMP criterion: $r_L$ < 5 %)*
- Upper limit of quantification: $2{,}64.10^8$ IBC/mL ($1{,}10.10^7$ cfu/mL)
- Lower limit of quantification: $1{,}3.10^4$ IBC/ml ($6.10^3$ cfu/ml)
- The results obtained with BacSomatic™ are not impacted by high contents of fat, protein or somatic cells in the milk.

### The comparison of BacSomatic™ and BactoScan™ FC+

The bacterial count results obtained with BacSomatic™ are equivalent to those obtained with BactoScan™ FC+ in the range $1.10^4$ – $6{,}5.10^7$ IBC/mL ($4{,}8.10^3$ – $3{,}7.10^6$ cfu/mL).

- Intra-laboratory reproducibility ($R_{intra\text{-}lab}$) per bacterial count level in raw herd bulk cow's milk:
  - Contamination level < 4,30 $\log_{10}$ cfu/mL 0,22 $\log_{10}$ IBC/mL
  - Contamination level ≥ 4,30 $\log_{10}$ cfu/mL 0,19 $\log_{10}$ IBC/mL
  - Over all 0,20 $\log_{10}$ IBC/mL
- The 95 % confidence limit interval of the slope and the intercept included respectively 1 and 0, and the standard error of the results $s_{yx}$=0,08 $\log_{10}$ IBC/mL is small, meaning close correlation between the results obtained with BacSomatic™ and BactoScan™ FC+

It is concluded that the results obtained with BacSomatic™ and BactoScan™ FC+ are equivalent.

### Final conclusion of the validation study

The final conclusion of the validation study is:

All results obtained during the method comparison study of BacSomatic™ comply with the criteria of the EURL MMP document. The direct comparison of results from BacSomatic™ and BactoScan™ FC+ (MicroVal certificate 2013LR45) revealed equivalence in terms of enumeration of bacteria and do comply with the criteria of the EURL MMP document.

## 4. References

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

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

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6. 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.

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9. Orlandini, S. (2018) BacSomatic TBC report- Influence of Fat, Protein and Somatic Cell count on TBC standard deviation. AEOS Report.

10. Hatchavanich, D. (2014) A comparison of type I error and power of BArtlette`s test, Levene`s test and O'Brien's test for homogeneity of variance test. Southeast-Asian J. of Science, 3:2, 181-194.