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**MICROVAL VALIDATION REPORT**

**Comparative Study**

**MicroVal Project 2016LR62**

# Validation of Fossomatic™ 7 (FOSS) for enumeration of somatic cells in raw cow's milk against EURL MMP criteria

**Confidential**

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Version: 2

September 15, 2017

**Summary**

This MicroVal validation report presents the results of the comparative study of the newly developed Fossomatic™ 7 (FOSS Analytical A/S) with the already approved Fossomatic™ FC (MicroVal certificate 2015LR55) against the criteria in the EURL MMP document from January 2013 (1).

**Conclusions of the method's comparison study**

Fossomatic™ 7 performance characteristics determined according to ISO 8196-3 and ISO 13366-2 are:

- Fossomatic™ 7 functions stable through the working day
- Repeatability (*r*) per cell count level:
  - Low (ca. 90.10$^3$ cells/mL) 11 % *(ISO 13366-2: ≤ 17 %)*
  - Medium (ca. 508.10$^3$ cells/mL) 5 % *(ISO 13366-2: ≤ 11 %)*
  - High (ca. 1 520.10$^3$ cells/mL) 3 % *(ISO 13366-2: ≤ 8 %)*
- Carry-over per cell count level (*ISO 13366-2: for each cell count level CO < 2 %)*
  - Low (ca. 500.10$^3$ cells/mL) $C_{H/L}$ = 0,14 %; $C_{L/H}$ = 0,48 %
  - Medium (ca. 1 000.10$^3$ cells/mL) $C_{H/L}$ = 0,07 %; $C_{L/H}$ = 0,14 %
  - High (ca. 3 000.10$^3$ cells/mL) $C_{H/L}$ = 0,05 %; $C_{L/H}$ = 0,32 %
- Linearity ($r_C$): 1,8 % *(ISO 13366-2: $r_C$ ≤ 2 %)*
- Lower limit of quantification ($L_Q$): 17.10$^3$ cells/mL
- Upper limit of quantification: 10 000.10$^3$ cells/mL
- High fat (up to 8 %) and protein (up to 5,5 %) content of the milk do not relevantly influence the somatic cell count results with the Fossomatic™ 7.

**Conclusions of the comparison of Fossomatic™ 7 and Fossomatic™ FC**

The results obtained from the comparison of Fossomatic™ 7 and Fossomatic™ FC are:

- Intra-laboratory reproducibility ($R_{intra\text{-}lab}$) per cell count level in raw herd bulk cow's milk:
  - Low (50-200.10$^3$ cells/mL) 11 % *(ISO 13366-2: ≤ 19 %)*
  - Medium (201-400.10$^3$ cells/mL) 9 % *(ISO 13366-2: ≤ 19 %)*
  - High medium (401-650.10$^3$ cells/mL) 9 % *(ISO 13366-2: ≤ 14 %)*
  - Low high (651-1 000.10$^3$ cells/mL) 7 % *(ISO 13366-2: ≤ 14 %)*
  - High (1 000-1 500.10$^3$ cells/mL) 10 % *(ISO 13366-2: ≤ 11 %)*

- Intra-laboratory reproducibility ($R_{intra\text{-}lab}$) per cell count level in individual raw cow's milk:
    - Low (50-200.$10^3$ cells/mL) 13 % *(ISO 13366-2: ≤ 19 %)*
    - Medium (201-400.$10^3$ cells/mL) 4 % *(ISO 13366-2: ≤ 19 %)*
    - High medium (401-650.$10^3$ cells/mL) 12 % *(ISO 13366-2: ≤ 14 %)*
    - Low high (651-1 000.$10^3$ cells/mL) 11 % *(ISO 13366-2: ≤ 14 %)*
    - High (1 000-1 500.$10^3$ cells/mL) 7 % *(ISO 13366-2: ≤ 11 %)*

- Standard error ($s_{yx}$) of the results is small and demonstrates a close relationship between the results obtained with Fossomatic™ 7 and Fossomatic™ FC. Standard error ($s_{yx}$) is:
    - for individual raw cow's milk $s_{yx}$= 5,8 %
    - for raw herd bulk cow's milk $s_{yx}$= 4,1 %

- The performed statistical tests (t-test and F-test) demonstrated that the results obtained with Fossomatic™ 7 and Fossomatic™ FC are not significantly different.

- It is concluded that the results obtained with Fossomatic™ 7 and Fossomatic™ FC are equivalent for all cell count levels.

**Final conclusion validation study**

The final conclusion of the validation study is:

- The Method Comparison Study of Fossomatic™ 7 (FOSS Analytical A/S) and the direct comparison with Fossomatic™ FC (MicroVal certificate 2015LR55) show that the results obtained with both instruments are equivalent and comply with the criteria of the EURL MMP document.

**CONTENTS**

## 1. Introduction

The Fossomatic™ 7 flow cytometer from FOSS Analytical A/S is a dedicated instrument for high-throughput enumeration of somatic cells in raw milk.

Since independent validation is a critical success factor for the acceptance of the Fossomatic™ 7 as an instrumental method for the enumeration of somatic cells in raw milk in light of EU Regulation No 2074/2005, modified by EU Regulation No 1664/2006, the Fossomatic™ 7 has to be validated against the European criteria published in an EURL MMP document from January 2013 (1). The EURL MMP document for validation of alternative methods refers to performance criteria in ISO 8196-3 (2) and ISO 13366-2 (3).

Fossomatic™ 7 is a new generation of Fossomatic™ FC instruments for somatic cell counting in raw milk. The Fossomatic™ FC was recently granted with a MicroVal certificate. The hardware and calculation algorithms of both models are highly similar, however the new analyser has some minor differences (using a laser as a light source instead of a halogen lamp) when compared with the Fossomatic™ FC.

The performance characteristics of the Fossomatic™ 7 are demonstrated during the methods' comparison study for the matrix raw cow's milk. Its accuracy is demonstrated by comparison with results obtained with the Fossomatic™ FC.

This MicroVal validation report presents the results of an executed methods' comparison study as prescribed in the EURL MMP document from January 2013 and results of comparison of two Fossomatic models.

### 1.1. Principle of the alternative method

The Fossomatic™ 7 is a fully automated flow cytometer for the rapid enumeration of somatic cells in raw milk. The working principle of the instrument is based on colouring the somatic 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 somatic cells are exposed to light of a specific wavelength. The 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 somatic cell count in thousands per milliliter. The design of the flow cell must ensure that single cells are separately counted.

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

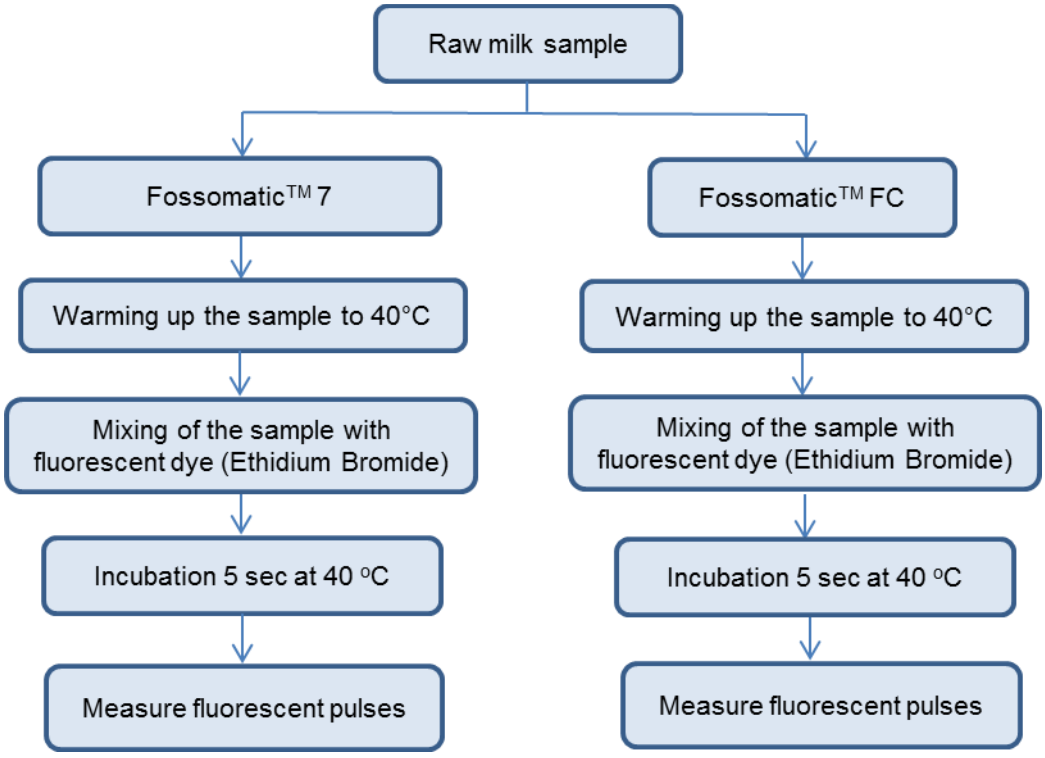
ISO 13366-1:2008 Milk - Enumeration of somatic cells - Part 1: Microscopic method (Reference method) (4).

### 1.5. Comparison instrument

Fossomatic™ FC with MicroVal certificate number 2015LR55.

### 1.6. Validation procedure

The measurement procedure with both instruments is schematically presented below, which is illustrative for the mutual resemblance:




### 1.7. Materials and equipment used

- Milk leucocyte suspension, prepared by creaming of raw herd bulk milk with a cell count of about $200.10^3$ cells/mL and subsequent centrifugation of the cream layer. The procedure for leucocyte isolation from milk has been developed in a collaboration of Cornell University (USA) and ASIA-LSL (Italy) in 2011/2012 (5). This procedure is also advocated by the EU Joint Research Centre for the development of a certified reference material.

- Preservation mixture with an end concentration in the milk of 0,02 % m/m sodium azide and 0,005% m/m bronopol
- 'Blank milk' – semi skimmed UHT milk with 1 mL/L polypropylene glycol 2000 (Baker) and 0,04 %m/m bronopol
- Stock and working solutions for Fossomatic™ 7 and Fossomatic™ FC, prepared according to manufacturers' instructions from supplied consumables:
    - Cleaning solution
    - Buffer solution
    - Rinse solution
    - Incubation/dye solution
    - Blank solution
    - Fossomatic 7DC Detergent product number 60045445
- Pilot samples - preserved commingled raw milk samples with representative somatic cell count for the routine samples
- Calibration samples - a series of preserved milk samples in ascending order of adjusted somatic cell count in the range $200.10^3$/mL – $2\,000.10^3$/mL, which is used in the calibration of Fossomatic™ FC. The concentrations were adjusted with the leucocyte suspension. Samples were stored at 2 - 8 $^{o}$C for a maximum of 3 months.
- Individual raw cow's milk samples and raw herd bulk cow's milk samples
- 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:

- Fossomatic™ 7
- Fossomatic™ FC (MicroVal certificate number 2015LR55)
- Instruction and method implementation
- Statistical expertise.

### 1.8. Safety precautions

Good Laboratory Practices for running food analyses were followed.

## 2. Methods' 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 somatic cell count levels.

##### 2.1.1.1. Measurement protocol and calculations

Preserved 'blank milk' was spiked with milk leucocyte suspension at three cell count levels: low, medium and high. The corresponding cell count ranges are given in Table 1.

**Table 1. Cell count levels of samples used in the stability, repeatability and intra-laboratory reproducibility studies with the Fossomatic™ 7 instrument**

| Cell count levels | Cell counts measured with Fossomatic™ 7 (.$10^3$ cells/mL) |
|---|---|
| **Low** | 90 |
| **Medium** | 508 |
| **High** | 1 520 |

The spiked milk samples were stored at 2 ± 2 °C for a maximum of 1 month.

Samples from each cell count level were measured in triplicate (n=3) with the Fossomatic™ 7 in random order each 20 min during a working day with 20 checks in total. Routine individual raw cow's milk samples were run in between.

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 (2). The calculations were performed without any transformation.

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 ($q$ = 20)

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. The raw data and calculations are in Annex 1.

**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 Fossomatic™ 7 per examined cell count level**

| Cell count levels | $s_r$ (.10$^3$ cells/mL) | $s_x$ (.10$^3$ cells/mL) | $s_c$ (.10$^3$ cells/mL) | $s_{R,daily}$ (.10$^3$ cells/mL) |
|---|---|---|---|---|
| low (90.10$^3$ cells/mL) | 3,6 | 5,8 | 5,4 | 6,5 |
| medium (510.10$^3$ cells/mL) | 8,1 | 17,7 | 17,0 | 18,9 |
| high (1 520.10$^3$ cells/mL) | 14,9 | 29,3 | 28,0 | 31,8 |

The standard deviation of repeatability ($s_r$) for each cell count level meets the requirement according to the EURL MMP document and ISO 13366-2, see Table 3.

**Table 3. The standard deviation of repeatability ($s_r$) of the Fossomatic™ 7 calculated per cell count level and acceptability values according to ISO 13366-2**

| Cell count levels | $s_r$, calculated | $s_r$ acceptibility values according ISO 13366-2* |
|---|---|---|
| | (.10$^3$ cells/mL) | |
| low (90.10$^3$ cells/mL) | 3,6 | 5,4 |
| medium (508.10$^3$ cells/mL) | 8,1 | 20,3 |
| high (1 520.10$^3$ cells/mL) | 14,9 | 45,6 |
| | (%) | |
| low (90.10$^3$ cells/mL) | 4 | 6 |
| medium (508.10$^3$ cells/mL) | 2 | 4 |
| high (1 520.10$^3$ cells/mL) | 1 | 3 |

*the acceptability values presented in .10$^3$ cells/mL are calculated on the basis of the measured cell count levels and following Table 2 in ISO 13366-2.

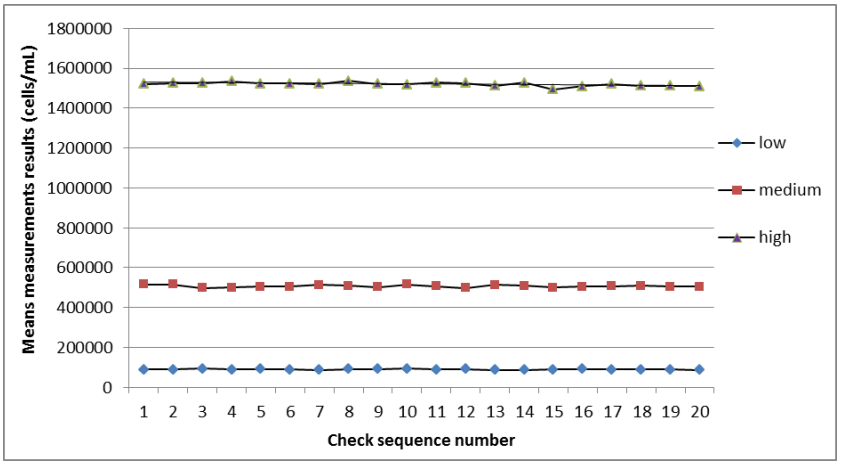
For the standard deviation of daily reproducibility ($s_{R,daily}$), standard deviation between checks ($s_c$) and standard deviation of means ($s_x$) there are no official requirements.

The calculated standard deviation of daily reproducibility ($s_{R,daily}$) for each cell count level was small. Even for medium and high cell count levels it complies with the requirements for standard deviation of repeatability.

The small standard deviation between checks ($s_c$) and standard deviation of means ($s_x$) demonstrate that the variation of instruments 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. Fossomatic™ 7 stability throughout the working day based on the means of the measurement results at three cell count 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 4.

**Table 4. *F*- test (α=0,05) of a one-way ANOVA per cell count level**

| Cell count level | Source of variation | Sum of squares | Degrees of freeedom | Mean of squares | *F* calculated | table value $F_{0,95}$ |
|---|---|---|---|---|---|---|
| Low | Between groups | $2,0.10^8$ | 19 | $1,1.10^7$ | | |
| | Within groups | $5,3.10^8$ | 40 | $1,3.10^7$ | | |
| | | | | | 0,80 | 1,85 |
| | Total | $7,3.10^8$ | 59 | | | |
| Medium | Between groups | $1,9.10^9$ | 19 | $1,0.10^8$ | | |
| | Within groups | $2,6.10^9$ | 40 | $0,7.10^8$ | | |
| | | | | | 1,50 | 1,85 |
| | Total | $4,5.10^9$ | 59 | | | |
| High | Between groups | $5,2.10^9$ | 19 | $2,7.10^8$ | | |
| | Within groups | $8,9.10^9$ | 40 | $2,2.10^8$ | | |
| | | | | | 1,22 | 1,85 |
| | Total | $1,4.10^{10}$ | 59 | | | |

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

#### 2.1.1.3. Conclusion

The Fossomatic™ 7 flow cytometer functions stable throughout the working day and the stability complies with the requirements of the EURL MMP document and ISO 13366-2.

### 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 Fossomatic™ 7 was calculated from the stability experiment. For measurement protocol and calculations see clause 2.1.1.1. Additionally the repeatability was calculated on 220 individual raw cow's milk samples and 179 raw herd bulk cow's milk samples representative for different somatic cell count levels as shown in Table 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 5. Raw cow's milk samples selected for estimation of the performance characteristics of the Fossomatic™ 7**

| Cell count levels ($.10^3$ cells/mL) | Number of individual cow's milk samples | Number of herd bulk cow's milk samples |
|---|---|---|
| 50 - 200 | 33 | 49 |
| 201 - 400 | 48 | 32 |
| 401 - 650 | 42 | 37 |
| 651 - 1 000 | 49 | 12 |
| 1 000 - 1 500 | 48 | 49 |
| Total number of samples | 220 | 179 |

The raw cow's milk samples were measured in duplicate (n= 2) with Fossomatic™ 7. The standard deviation of repeatability ($s_r$) was calculated for the individual raw cow's milk and raw herd bulk cow's milk separately and for each cell count level as described in clause 2.1.1.1. The calculations were performed without any transformation.

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 Fossomatic™ 7 instrument and the acceptability values are given in Table 6.

**Table 6. The repeatability (*r*) of the Fossomatic™ 7 calculated per cell count level and acceptability values according to ISO 13366-2**

| Cell count levels | *r*, calculated ($2{,}83 s_r$) | *r* acceptibility values according ISO 13366-2* |
|---|---|---|
| | (.$10^3$ cells/mL) | |
| low (90.$10^3$ cells/mL) | 10,3 | 15,3 |
| medium (508.$10^3$ cells/mL) | 23,0 | 57,4 |
| high (1 520.$10^3$ cells/mL) | 42,2 | 129,0 |
| | (%) | |
| low (90.$10^3$ cells/mL) | 11 | 17 |
| medium (508.$10^3$ cells/mL) | 5 | 11 |
| high (1 520.$10^3$ cells/mL) | 3 | 8 |

*the acceptability values presented in .$10^3$ cells/mL are calculated on the basis of the measured cell count levels and following Table 2 in ISO 13366-2.

The calculated repeatability (*r*) for individual raw cow's milk and raw herd bulk cow's milk samples measured with Fossomatic™ 7 instrument and the acceptability values are given Table 7.

**Table 7. The repeatability (*r*) of the Fossomatic™ 7 calculated per cell count level for individual raw cow's milk and bulk herd milk samples and acceptability values according to ISO 13366-2**

| Cell count levels | *r*, individual cow's milk | *r*, herd bulk cow's milk | *r* acceptability values ISO 13366-2 |
|---|---|---|---|
| | (.$10^3$ cells/mL) | | |
| Low (50 - 200.$10^3$ cells/mL) | 10,0 | 12,8 | 25,0 |
| Medium (201 - 400.$10^3$ cells/mL) | 17,5 | 18,7 | 42,0 |
| High medium (401 - 650.$10^3$ cells/mL) | 25,8 | 20,5 | 50,0 |
| Low high (650 - 1 000.$10^3$ cells/mL) | 31,0 | 26,8 | 63,0 |
| High (1 000 - 1 500.$10^3$ cells/mL) | 61,2 | 35,0 | 126,0 |
| | (%) | | |
| Low (50 - 200.$10^3$ cells/mL) | 7 | 9 | 17 |
| Medium (201 - 400.$10^3$ cells/mL) | 6 | 6 | 14 |
| High medium (401 - 650.$10^3$ cells/mL) | 6 | 5 | 11 |
| Low high (650 - 1 000.$10^3$ cells/mL) | 4 | 4 | 8 |
| High (1 000 - 1 500.$10^3$ cells/mL) | 4 | 2 | 8 |

*the acceptability values presented in .$10^3$ cells/mL are calculated on the basis of the measured cell count levels and following Table 2 in ISO 13366-2.

#### 2.1.2.3. Conclusion

The repeatability (*r*) of the Fossomatic™ 7 complies with the requirement of EURL and ISO 13366-2 at all cell count levels.

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

Strong differences in somatic cell count levels between two successively analysed samples may influence the result of the second.

Differences can 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 analysers for liquids often allow automatic correction to compensate for the overall carry-over effect when necessary.

#### 2.1.3.1. Measurement protocol and calculations

Preserved 'blank milk' was spiked with milk leucocyte suspension at three cell count levels: low, medium and high. The cell count levels of the samples are given in Table 8.

**Table 8. Cell count levels of samples used in the carry-over assessment of Fossomatic™ 7**

| Cell count levels | Theoretical cell counts levels ($.10^3$ cells/mL) | Cell counts levels measured with Fossomatic™ 7 ($.10^3$ cells/mL) |
|---|---|---|
| **Low** | 500 | 509 |
| **Medium** | 1 000 | 1 036 |
| **High** | 3 000 | 2 951 |

The spiked milk samples were stored at 2 ± 2 °C for a maximum of 1 month.

Fossomatic™ 7 measurements were performed without carry-over correction factor on 20 sets of samples per cell count level with the following sequence:

$$(L_{L_1}, L_{L_2}, L_{H_1}, L_{H_2})_1, (L_{L_1}, L_{L_2}, L_{H_1}, L_{H_2})_2 \ldots (L_{L_1}, L_{L_2}, L_{H_1}, L_{H_2})_{20}.$$

thus,

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

The calculations were performed on raw data without any transformation. The COR ($C$) was obtained by applying the following equations:

$$C_{H/L} = \frac{(\sum L_{L_1} - \sum L_{L_2}) \times 100}{(\sum L_{H_2} - \sum L_{L_2})} = (\overline{L_{L_1}} - \overline{L_{L_2}}) \times 100/(\overline{L_{H_2}} - \overline{L_{L_2}})$$

$$C_{L/H} = \frac{\left(\sum L_{H_2} - \sum L_{H_1}\right) \times 100}{\left(\sum L_{H_2} - \sum L_{L_2}\right)} = \left(\overline{L_{H_2}} - \overline{L_{H_1}}\right) \times 100 / \left(\overline{L_{H_2}} - \overline{L_{L_2}}\right)$$

The carry over effect should not exceed the limit of 2 % as required in the EURL MMP document.

#### 2.1.3.2. Results

For each cell count level the ratios $C_{H/L}$ and $C_{L/H}$ were calculated. The results are given in Table **9**. The carry-over results per cell count level are given in **Fout! Verwijzingsbron niet gevonden.**.

**Table 9. Calculated ratios $C_{H/L}$ and $C_{L/H}$ per cell count level**

| Cell count levels | Calculated $C_{H/L}$ (%) | Calculated $C_{L/H}$ (%) |
|---|---|---|
| **Low** | 0,14 | 0,48 |
| **Medium** | 0,07 | 0,14 |
| **High** | 0,05 | 0,32 |

The calculated relative carry-over effect for each cell count level was smaller than the limit $COR$ < 2 %.

#### 2.1.3.3. Conclusion

The carry-over effect with measurements on the Fossomatic™ 7 complies with the requirements in EURL MMP document, $COR$ < 2 % for each cell count level.

### 2.1.4. Linearity (according to ISO 8196-3 §5.2.2.1.3 and ISO 13366-2 §6.2.2)

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.

#### 2.1.4.1. Measurement protocol and calculations

To evaluate linearity, two sets of samples with cell count levels distributed over the range of 0 – 10 000.$10^3$ cells/mL were prepared. Preserved 'blank milk' was spiked with milk leucocyte suspension in steps of 150.$10^3$ cells/mL in the range 0 – 2 000.$10^3$ cells/mL, covering the working range in routine testing, in steps of 500.$10^3$ cells/mL in the range 2 000.$10^3$ - 5 000.$10^3$ cells/mL and in steps of 1 000.$10^3$ cells/mL in the range 5 000.$10^3$ - 10 000.$10^3$ cells/mL. The samples in the first set were measured 4 times in order of increasing cell count and in the second set 4 times in 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[1]. The calculated cell count levels of the spiked samples were used as the reference values for the calculations.

The means of the replicates per sample (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 concentrations 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 set of samples concerned;

$M_{\min}$ is the numerical value of the lower measured value for the set of samples concerned.

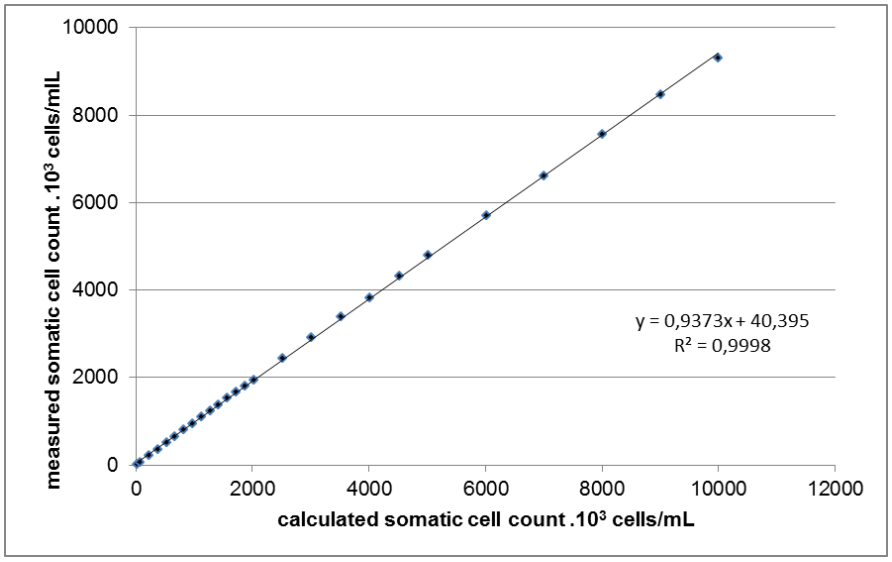
The ratio, $r_C$, should be below 2% in order to comply with the EURL MMP document and ISO 13366-2.

[1] The ratio $r_C$ is calculated by using the formula described in ISO 13366-2. The symbols are as in the original formula and deviate from these used in ISO 8196-3.

#### 2.1.4.2. Results

The results appeared to be linear in the whole testing range up to 10 000.10$^3$ cells/mL with $r_C$= 1,8 %. The results are pictured in Figure 2 and the underlying data are shown in **Fout! Verwijzingsbron niet gevonden.**.

**Figure 2. Linearity of Fossomatic™ 7 in the testing range up to 10 000.10$^3$ cells/mL**




The Fossomatic™ 7 also appeared to be linear ($r_C$= 0,8 %) when more specifically examined in the performance range 100 – 1 500.10$^3$ cells/mL.

#### 2.1.4.3. Conclusions

The instrument is linear in the normal working range and in the wider measurement range up to 10 000.10$^3$ cells/mL. In both ranges, the linearity of the Fossomatic™ 7 complies with the stated maximum limit value of $r_C$ ≤ 2 % in the EURL document and ISO 13366-2.

### 2.1.5. Limits of quantification (according to ISO 8196-3 §5.2.2.1.5 and §5.2.2.1.6)

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 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 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, $L_Q$*

Semi skimmed UHT milk was measured 20 times with Fossomatic™ 7. The mean and standard deviation, $\sigma$, of the measurements were calculated and the lower limit of quantification, $L_{Q,}$ was calculated as:

$$L_Q = mean + 10\sigma$$

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

The upper limit of quantification is the highest possible reading of the method without interference of methodological limitations. The upper limit of quantification of the alternative method is the ratio, $r_C$, exceeding the 2 % limit value according to EURL MMP document and ISO 13366-2.

The upper limit of quantification of Fossomatic™ 7 was determined as linearity of the instrument in the range above the working range. For measurement protocol and calculations see clause 2.1.4.

#### 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 10.

**Table 10. Results lower limit of quantification of the Fossomatic™ 7**

| Measurement | Result ($.10^3$ cells/mL) |
|---|---|
| **1** | 5 |
| **2** | 6 |
| **3** | 6 |
| **4** | 6 |
| **5** | 9 |
| **6** | 6 |
| **7** | 7 |
| **8** | 6 |
| **9** | 7 |
| **10** | 7 |
| **11** | 8 |
| **12** | 7 |
| **13** | 8 |
| **14** | 8 |
| **15** | 7 |
| **16** | 6 |
| **17** | 7 |
| **18** | 8 |
| **19** | 8 |
| **20** | 7 |
| **Mean** | 7,0 |
| **σ** | 1,0 |
| **$L_Q$** | 16,9 |

The resulting lower limit of quantification is $17.10^3$ cells/mL.

*2.1.5.2.2. Upper limit of quantification*

The results appeared to be linear in the range up to 10 000.$10^3$ cells/mL with $r_C$= 1,8 %. The relevant data with the resulting upper limit of quantification are shown in Annex 3. The results are pictured in Figure 2.

The upper limit of quantification of Fossomatic™ 7 complies with the EURL MMP requirement.

#### 2.1.5.3. Conclusion

The lower limit of quantification of Fossomatic™ 7 is 17.$10^3$ cells/mL.

The upper limit of quantification of Fossomatic™ 7 is 10 000.$10^3$ cells/mL.

### 2.1.6. Evaluation of factors affecting the results (according to ISO 13366-2 §10.2 and EURL MMP document)

High fat and protein content could interfere in somatic cell count measurements with the Fossomatic™ 7. The influence of fat and protein content was examined at three relevant fat and protein levels within the range of the measurand.

The effect of increasing fat and protein content on the somatic cell counts was evaluated by linear regression.

#### 2.1.6.1. Measurement protocol and calculations

The somatic cell count in preserved raw cow's milk with 3, 6 and 8 % fat and preserved raw cow's milk with 3,5, 4,5 and 5,5 % protein was adjusted at five cell count levels. The cell count levels are given in **Fout! Verwijzingsbron niet gevonden.** and **Fout! Verwijzingsbron niet gevonden.**.

The spiked milk samples were stored at 2 ± 2 °C for a maximum of 1 month.

Each sample was analysed four times with Fossomatic™ 7.

The means of the replicate measurements per sample (n= 4) were calculated. The possible interference of high fat and protein content on somatic cell counting was assessed by linear regression of the mean instrument values at each component concentration level against the calculated values:

$$y = bx + a$$

$y$ = instrument value,

$x$ = calculated value of the spiked samples.

Differences in obtained slopes are indicative for interference of high fat and protein content with the somatic cell count. It was required that slopes are within the 95 % confidence limit interval of the calculated slope for samples with 3 % fat or 3,5 % protein or that there is an overlap between the 95% confidence intervals.

The relative linearity bias per fat and protein concentration was expressed with the ratio $r_C$ and was calculated as described in clause 2.1.4.1.

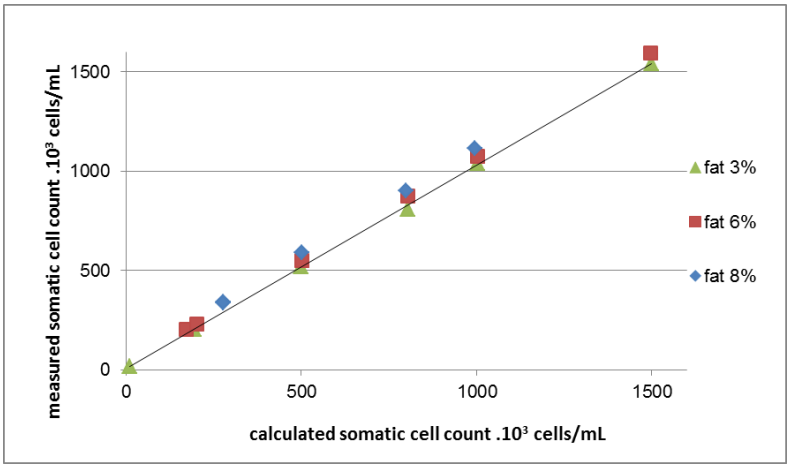
### 2.1.6.2. Results

The calculated slope (*b*), intercept (*a*), 95 % confidence limit interval from linear regression analysis, and linearity ratio ($r_C$) on results obtained with the Fossomatic™ 7 on milk samples with different fat content and different somatic cell count levels are given in Table 11, raw data in **Fout! Verwijzingsbron niet gevonden.** and visualisation of the results is shown in Figure 3 and **Fout! Verwijzingsbron niet gevonden.**.

**Table 11. Calculated slope (*b*), intercept (*a*), 95% confidence limit interval from linear regression analysis, and linearity ratio ($r_C$) on results obtained with the Fossomatic™ 7 on milk samples with different fat content and different somatic cell count levels**

| fat concentration | slope (*b*) | | | intercept (*a*) (.10³ cells/mL) | $r_c$ (%) |
|---|---|---|---|---|---|
| | calculated | lowest 95% | largest 95% | | |
| **3%** | 1,024 | 1,000 | 1,047 | 3 | 1,6 |
| **6%** | 1,048 | 1,037 | 1,060 | 24 | 0,9 |
| **8%** | 1,057 | 1,030 | 1,084 | 51 | 1,6 |

**Figure 3. Linearity of the results obtained with Fossomatic™ 7 on milk samples with increasing fat content and different somatic cell count levels**




The slope for each fat concentration level was calculated using linear regression. The slopes and the 95 % confidence limit 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 11). The slopes obtained with 6 % (*b* = 1,048) and 8 % fat (*b* = 1,057) were slightly higher than the largest limit of the 95 % confidence interval obtained for the slope of milk with 3 % fat (*b* = 1,047), however the calculated 95 % confidence intervals of the three slopes were largely overlapping.

The calculated linearity ratio for each fat concentration is $r_C$ < 2 % and the results obtained with Fossomatic™ 7 on milk samples with increasing fat content and different somatic cell count levels appear to be linear up to 1 500.$10^3$ cells/mL.
Although a slight slope deviation was observed in milk with high fat content, it was concluded that milk fat content up to 8 % does not have a relevant influence on the somatic cell count result.

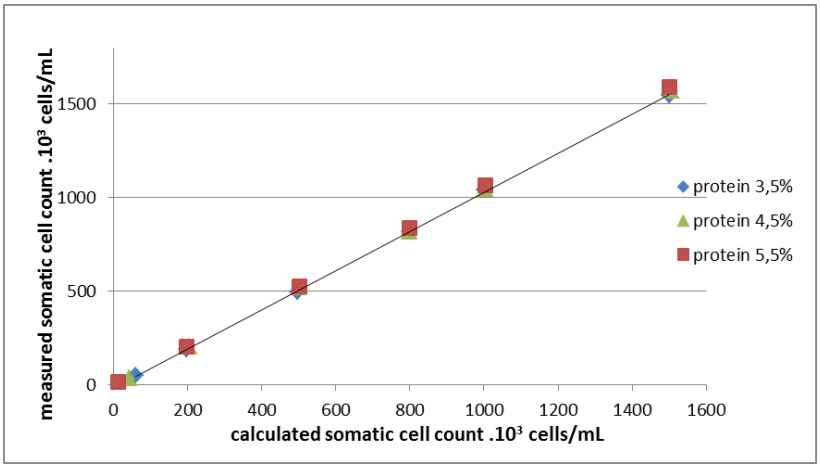
⇨ The somatic cell count results obtained with Fossomatic™ 7 are not relevantly effected by elevated fat content in the milk up to 8%.

The calculated slope (*b*), intercept (*a*), 95 % confidence limit interval from linear regression analysis, and linearity ratio ($r_C$) on results obtained with Fossomatic™ 7 on milk samples with different protein content and different somatic cell count levels are given in Table 12, raw data in **Fout! Verwijzingsbron niet gevonden.** and visualisation of the results is shown in Figure 4 and **Fout! Verwijzingsbron niet gevonden.**.

**Table 12. Calculated slope (*b*), intercept (*a*), 95% confidence limit interval from linear regression analysis, and linearity ratio ($r_C$) on results obtained with the Fossomatic™ 7 on milk samples with different protein content and different somatic cell count levels**

| protein concentration | slope (*b*) | | | intercept (*a*) (.$10^3$ cells/mL) | $r_c$ (%) |
|---|---|---|---|---|---|
| | calculated | lowest 95% | largest 95% | | |
| **3,5%** | 1,044 | 1,027 | 1,060 | -18 | 1,2 |
| **4,5%** | 1,050 | 1,037 | 1,064 | -11 | 1,0 |
| **5,5%** | 1,061 | 1,051 | 1,071 | -4 | 0,6 |

**Figure 4. Linearity of the results obtained with the Fossomatic™ 7 on milk samples with different protein content and different somatic cell count levels**



The slope for each protein concentration level was calculated using linear regression. The slopes and the 95 % confidence limit 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 12). The slope obtained with milk samples containing 4,5 % protein was within the 95 % confidence interval for the slope obtained with 3,5 % protein. The slope ($b$ = 1,061) obtained with milk samples containing 5,5 % protein was slightly higher than the upper limit of the 95 % confidence interval for the slope obtained with 3,5 % protein ($b$ = 1,060). There was a large overlap of the 95 % confidence interval with both slopes and the calculated linearity ratio for each protein concentration was $r_C$ < 2 %. There was no relevant influence of the protein content on the somatic cell count.

⇨ The somatic cell count results obtained with Fossomatic™ 7 are not relevantly effected by an elevated protein content in the milk up to 5,5 %.

#### 2.1.6.3. Conclusions

No relevant influence of elevated fat and protein content of the milk was observed on the somatic cell count results obtained with the Fossomatic™ 7.

### 2.2. Comparison of Fossomatic™ 7 and Fossomatic™ 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 Fossomatic™ 7 was evaluated at different somatic cell count levels through comparison with the Fossomatic™ FC. $R_{intra\text{-}lab}$ was calculated with 220 individual raw cow's milk samples and 179 raw herd bulk cow's milk samples as shown in Table 5.

The samples were measured in random order in duplicate with Fossomatic™ 7 and were used as well for the calculation of the repeatability ($r$) of the instruments as described in clause 2.1.2. Single measurements of the same samples were performed with Fossomatic™ FC. 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 individual raw cow's milk and raw herd bulk cow's milk separately and for each cell count level. The calculations were performed without any 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 Fossomatic™ 7 and result obtained with the Fossomatic™ 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 Fossomatic™ 7

$x_2$ - result obtained with the Fossomatic™ 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 Fossomatic™ 7 on the y-axis and the results obtained with the Fossomatic™ FC on the x-axis. The standard error ($s_{yx}$) was calculated.

The differences of the results obtained with both models was evaluated per cell count level by applying a t-test and the significance of a possible observed deviation in both models was verified with the F-test of a one-way ANOVA with α= 0,05.

### 2.2.2.Results

The intra-laboratory reproducibility results and the acceptability values are given in Table 13.

**Table 13. Intra-laboratory reproducibility *R*$_{intra}$ and the acceptability values according to ISO 13366-2**

| Cell count levels | *R* $_{intra}$ individual cow's milk | *R* $_{intra}$ herd bulk cow's milk | *R* $_{intra}$ acceptability values ISO 13366-2 |
|---|---|---|---|
| | (.10$^3$ cells/mL) | | |
| **Low (50 - 200.10$^3$ cells/mL)** | 19,0 | 16,0 | 29,0 |
| **Medium (201 - 400.10$^3$ cells/mL)** | 40,8 | 26,6 | 50,0 |
| **High medium (401 - 650.10$^3$ cells/mL)** | 53,1 | 40,7 | 63,0 |
| **Low high (650 - 1 000.10$^3$ cells/mL)** | 85,1 | 52,9 | 84,0 |
| **High (1 000 - 1 500.10$^3$ cells/mL)** | 107,8 | 152,0 | 168,0 |
| | (%) | | |
| **Low (50 - 200.10$^3$ cells/mL)** | 13 | 11 | 19 |
| **Medium (201 - 400.10$^3$ cells/mL)** | 14 | 9 | 17 |
| **High medium (401 - 650.10$^3$ cells/mL)** | 12 | 9 | 14 |
| **Low high (650 - 1 000.10$^3$ cells/mL)** | 11 | 7 | 11 |
| **High (1 000 - 1 500.10$^3$ cells/mL)** | 7 | 10 | 11 |

For each cell count level for individual cow´s milk and herd bulk cow´s milk samples the calculated intra-laboratory reproducibility of Fossomatic™ 7 complies with the ISO 13366-2 acceptability values.
The accuracy of Fossomatic™ 7 was evaluated against the results obtained with Fossomatic™ FC. The results for raw milk samples were analysed with linear regression.
The relationship between the evaluated models is visualised in Figure 5 and Figure 6.

**Figure 5. Relationship between Fossomatic™ 7 and Fossomatic™ FC for individual raw cow's milk samples**

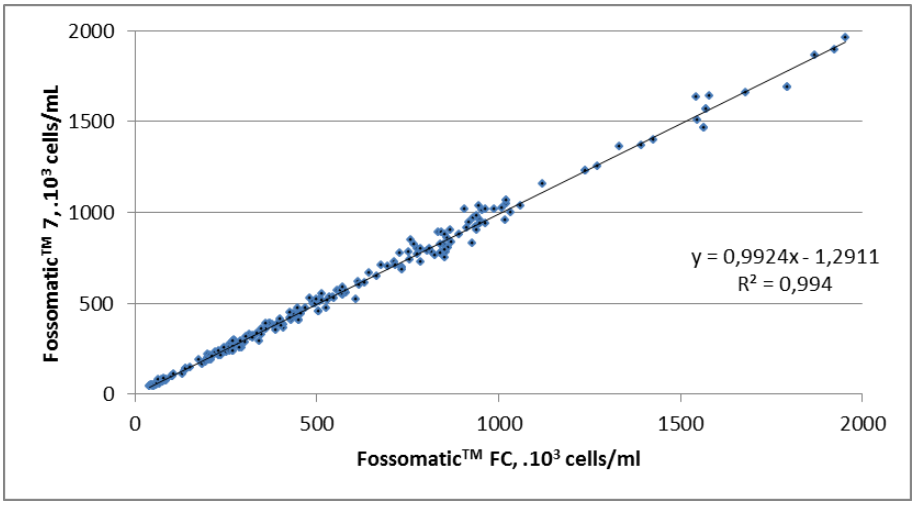
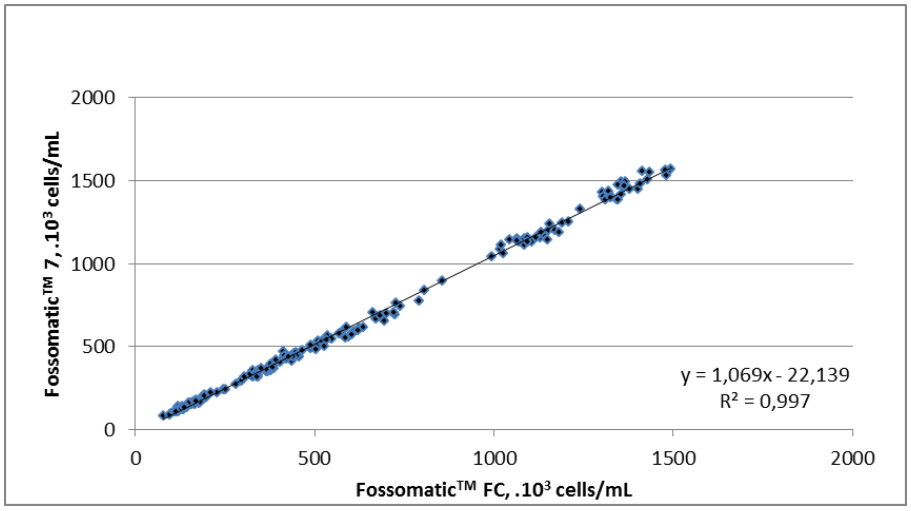



**Figure 6. Relationship between Fossomatic™ 7 and Fossomatic™ for raw herd bulk cow's milk samples**




The calculated standard error of the results was $s_{yx}$= 5,8 % for individual raw cow's milk samples and $s_{yx}$= 4,1 % for raw herd bulk cow's milk samples. The small standard errors ($s_{yx}$) demonstrate a close

relationship between the results obtained with both instruments and indicate that these can be considered equivalent.

The results of the performed t- and F tests are shown in Table 14.

**Table 14. The calculated t-values and F-values per cell count level for differences in somatic cell counts in individual raw cow's milk and bulk herd milk samples as obtained with the Fossomatic™ 7 and the Fossomatic™ FC and the critical $t_{table}$ and $F_{table}$ values. The critical $t_{table}$=2,0 for all somatic cell count levels**

| SCC range .10³ cells/mL | Fossomatic™ 7 - Fossomatic™ FC | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | individual cow's milk samples | | | | | bulk cow's milk samples | | | | |
| | *n* | mean bias (*d*) | $t_{calc}$* | $F_{calc}$ | $F_{table}$ | *n* | mean bias (*d*) | $t_{calc}$* | $F_{calc}$ | $F_{table}$ |
| **50 - 200** | 32 | -4,5 | 3,0 | 1,1 | 1,8 | 49 | -2,1 | 1,9 | 1,0 | 1,6 |
| **201 - 400** | 47 | -5,5 | 1,9 | 1,0 | 1,6 | 32 | -3,1 | 1,3 | 1,1 | 1,8 |
| **401 - 650** | 43 | -7,8 | 2,0 | 1,0 | 1,7 | 37 | 0,7 | 0,2 | 1,2 | 1,7 |
| **651 - 1000** | 48 | -9,6 | 1,6 | 1,0 | 1,6 | 12 | 4,6 | 0,6 | 1,5 | 2,8 |
| **1000 - 2000** | 51 | -8,2 | 1,1 | 1,2 | 1,6 | 49 | 70 | 15 | 1,3 | 1,6 |

*$t_{tab}$=2,0

The calculated t-values were smaller than the $t_{table}$= 2,0 for most cell count levels for individual and herd bulk cow's milk samples. For individual raw cow's milk samples with a somatic cell count level in the range 50-200.10³ cells/mL the calculated t-value was higher than table value. This result was verified by the calculated mean bias between the results obtained with Fossomatic™ 7 and Fossomatic™ FC, which indicates the differences between two measurements. At this somatic cell count level the calculated mean bias (*d*= 4,5) was more than six times lower than the acceptability value of intra laboratory reproducibility ($R_{intra}$= 29,0) indicated in ISO 13366-2 and it can be accepted as numerically negligible.

For herd bulk milk samples in the range 1 000 – 2 000 .10³ cells /mL the calculated t-value was much higher than the $t_{table}$. At this somatic cell count level the calculated mean bias (*d*= 70,0) was about 2,5 times lower than the acceptability value of intra laboratory reproducibility ($R_{intra}$= 168,0) indicated in ISO 13366-2 and it can be considered as not relevant. Moreover, in practice raw herd bulk cow's milk with such high somatic cell count is encountered in very low frequency. The samples with somatic cell count > 500.10³ cells/mL were prepared by mixing herd bulk milk with milk leucocyte suspension.

The calculated F-values for all levels and for individual raw cow's milk samples as well as herd bulk cow's samples were lower than the critical $F_{table}$ values. The calculated deviation in both instruments was not significantly different.

### 2.2.3. Conclusion

The results obtained with Fossomatic™ 7 are equivalent to the results obtained with Fossomatic™ FC at all cell count levels.

## 3. Conclusions of the comparison study

Fossomatic™ 7 performance characteristics determined according to ISO 8196-3 and ISO 13366-2 are:

- Fossomatic™ 7 functions stable through the working day
- Repeatability (*r*) per cell count level:
  - Low (ca. 90.10$^3$ cells/mL) 11 % *(ISO 13366-2: ≤ 17 %)*
  - Medium (ca. 508.10$^3$ cells/mL) 5 % *(ISO 13366-2: ≤ 11 %)*
  - High (ca. 1 520.10$^3$ cells/mL) 3 % *(ISO 13366-2: ≤ 8 %)*
- Carry-over per cell count level (*ISO 13366-2: for each cell count level CO < 2 %)*
  - Low (ca. 500.10$^3$ cells/mL) $C_{H/L}$ = 0,14 %; $C_{L/H}$ = 0,48 %
  - Medium (ca. 1 000.10$^3$ cells/mL) $C_{H/L}$ = 0,07 %; $C_{L/H}$ = 0,14 %
  - High (ca. 3 000.10$^3$ cells/mL) $C_{H/L}$ = 0,05 %; $C_{L/H}$ = 0,32 %
- Linearity ($r_C$): 1,8 % *(ISO 13366-2: $r_C$ ≤ 2 %)*
- Lower limit of quantification ($L_Q$): 17.10$^3$ cells/mL
- Upper limit of quantification: 10 000.10$^3$ cells/mL
- High fat (up to 8 %) and protein (up to 5,5 %) content of the milk do not relevantly influence the somatic cell count results with the Fossomatic™ 7.

### Conclusions of the comparison of Fossomatic™ 7 and Fossomatic™ FC

The results obtained from the comparison of Fossomatic™ 7 and Fossomatic™ FC are:

- Intra-laboratory reproducibility ($R_{intra\text{-}lab}$) per cell count level in raw herd bulk cow's milk:
  - Low (50-200.10$^3$ cells/mL) 11 % *(ISO 13366-2: ≤ 19 %)*
  - Medium (201-400.10$^3$ cells/mL) 9 % *(ISO 13366-2: ≤ 19 %)*
  - High medium (401-650.10$^3$ cells/mL) 9 % *(ISO 13366-2: ≤ 14 %)*
  - Low high (651-1 000.10$^3$ cells/mL) 7 % *(ISO 13366-2: ≤ 14 %)*
  - High (1 000-1 500.10$^3$ cells/mL) 10 % *(ISO 13366-2: ≤ 11 %)*

- Intra-laboratory reproducibility ($R_{intra\text{-}lab}$) per cell count level in individual raw cow's milk:
  - Low (50-200.10$^3$ cells/mL) 13 % *(ISO 13366-2: ≤ 19 %)*
  - Medium (201-400.10$^3$ cells/mL) 4 % *(ISO 13366-2: ≤ 19 %)*
  - High medium (401-650.10$^3$ cells/mL) 12 % *(ISO 13366-2: ≤ 14 %)*

- Low high (651-1 000.$10^3$ cells/mL) 11 % *(ISO 13366-2: ≤ 14 %)*
- High (1 000-1 500.$10^3$ cells/mL) 7 % *(ISO 13366-2: ≤ 11 %)*

- Standard error ($s_{yx}$) of the results is small and demonstrates a close relationship between the results obtained with Fossomatic™ 7 and Fossomatic™ FC. Standard error ($s_{yx}$) is:
  - for individual raw cow's milk $s_{yx}$= 5,8 %
  - for raw herd bulk cow's milk $s_{yx}$= 4,1 %

- The performed statistical tests (t-test and F-test) demonstrated that the results obtained with Fossomatic™ 7 and Fossomatic™ FC are not significantly different.

- It is concluded that the results obtained with Fossomatic™ 7 and Fossomatic™ FC are equivalent for all cell count levels.

## 4. Final conclusion methods' comparison study

The final conclusion of the validation study is:

- The Method Comparison Study of Fossomatic™ 7 (FOSS Analytical A/S) and the direct comparison with Fossomatic™ FC (MicroVal certificate 2015LR55) show that the results obtained with both instruments are equivalent and comply with the criteria of the EURL MMP document.

## 5. References


1. EURL MMP document - Criteria for the validation of instrumental (epifluorescent) methods for the enumeration of somatic cells in raw cow's milk, version 2, 21/01/2013.
2. ISO 8196-3|IDF 128-3:2009 Milk - Definition and evaluation of the overall accuracy of alternative methods of milk analysis - Part 3: Protocol for the evaluation and validation of alternative quantitative methods of milk analysis.
3. EN ISO 13366-2|IDF 148-1:2006 Milk - Enumeration of somatic cells - Part 2: Guidance on the operation of fluoro-optoelectronic counters.
4. EN ISO 13366-1|IDF 148-1:2008 Milk - Enumeration of somatic cells - Part 1: Microscopic method (Reference method).
5. Di Marzo, L.,Wojciechowski, K. L., and Barbano D. M. (2016) Preparation and stability of milk somatic cell reference materials. Journal of Dairy Science. 99:1-11.