THE GLOBAL STANDARD
FOR LIVESTOCK DATA

ICAR CERTIFICATION OF MILK ANALYSER

Company name: Foss Analytical A/S
Instrument name: Fossomatic™ 7 DC
Milk species: Cow milk

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Preface

Fossomatic™ 7 DC (FM 7 DC) was introduced onto the market in June 2017. It is an automatic, dedicated fluoro-optoelectronic instrument, based on flow cytometry, used for the rapid determination of somatic cell count in raw milk.

Fossomatic™ 7 DC can measure the total somatic cell count in fresh or preserved raw milk and can also perform differential somatic cell counting of lymphocytes and polymorphonuclear neutrophils (PMN). The instrument is applied in central milk testing and dairy laboratories for payment and Dairy Herd Improvement (DHI) analyses. In this certification for ICAR DHI purposes, its performance has been evaluated for total somatic cell counting in two phases: phase I, considering the results from single laboratory testing obtained during the Microval certification process and phase II, an interlaboratory study for total somatic cell count in cow milk and a robustness test.

The performances will be evaluated against criteria listed in:

ICAR protocol “Procedure 1 of Section 12 of ICAR Guidelines – Protocols for Evaluation of Milk Analyses for ICAR Approval”, which, in turn, is aligned with the

- 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 and
- ISO 13366-2|IDF 148-2 Milk — Enumeration of somatic cells — Part 2: Guidance on the operation of fluoro-opto-electronic counters
- ISO 8196-3|IDF 128-3:2009 is currently under revision and the next edition will report limits to validate a new instrument against the previous generation. These limits will be considered as informative in this report.

Summary

1.1 Principle

Fossomatic™ 7 DC is an automatic, dedicated fluoro-optoelectronic instrument, based on flow cytometry, used for a rapid determination of somatic cell count in raw milk.

1.2 Scope

The scope of the validation is total somatic cell counting in raw cow milk.

1.3 Data evaluated

Microval validation report executed at Qlip (NL) (Phase I)

Milchprüfring (mpr) Bayern (DE) data 2019 (Phase II)

Interlaboratory study (ILS) and statistical elaboration by Qlip (NL) in 2020

Table. Performance summary FM 7 DC

			*10^{3}cells/ml	r%		Limit r %
		Low	153	11		17
		Medium	516	6		11
		High	1.516	3		9
				Single cow milk sample	Bulk milk sample	
Repeatability Phase I	r		*10^{3}cells/ml	r%	r%	Limit r %
		Level 1	130	12	12	17
		Level 2	265	8	7	14
		Level 3	485	7	4	11
		Level 4	750	5	4	8
		Level 5	1.350	4	3	8
			Overall	7,2	6,0	11
			*10^{3}cells/ml	$CO_{H/L}$ %	$CO_{L/H}$ %	
Carry over Phase I	$CO_{H/L}$	Low	500	1,49	0	Limit $CO_{H/L}$ ≤ 2%
		Medium	1.000	0,05	0,12	
		High	3.000	1,44	0,64	
			*10^{3}cells/ml	R_{intra}%		Limit ICAR - ISO 13366 -2/IDF 148-2
						%
Reproducibility Intralaboratory Phase I	R_{intra}%	Low	153	22		20
		Medium	516	12		14
		High	1.516	6		11
		Overall		14		15
			*10^{3}cells/ml	%		
Linearity Phase I	r linearity		0-10.000	1,7		Limit r linearity ≤ 2%
Accuracy	S_{yx} comparison FM 7 DC vs FM FC Qlip Phase I		*10^{3}cells/ml Range 50-1900 n=144	0,06 ln *10^3 6%		Limit S_{yx} ≤ 8%
	S_{yx} comparison FM 7 DC vs FM FC mpr Phase II		*10^{3}cells/ml Range 50-1900 n=674	% 8		Limit S_{yx} ≤ 8%

		Level of concentration *10³cells/ml	r *10³cells/ml	r %	ICAR %	Limit ISO 13366-2/ IDF 148-2 cells/ml	%
Repeatability (r) Interlaboratory Study (ILS) Phase II	Sample 1	133	21	11,9		22	17
	Sample 2	119	18	14		21	17
	Sample 3	265	19	6,3		38	14
	Sample 4	359	45	11,9		45	13
	Sample 5	528	30	5,9		57	11
	Sample 6	1.019	61	6,0		87	8
	Sample 7	1.070	**115**	9,1		91	8
	Sample 8	1.153	47	3,5		98	8
	Sample 9	192	40	18,1		30	16
	Sample 10	198	18	6,9		29	15
	Overall			9,3	11		

	R_{intra}%	n		sR_{intra}	R_{intra}	R_{intra}	Limit ICAR and ISO 13366-2/ IDF 148-2
			*10³cells/ml	*10³cells/ml	*10³cells/ml	%	%
Reproducibility intralaboratory R_{intra} (mpr) Phase II		91	214	8,6	24,1	11	18

		Level of concentration *10³cells/ml	R *10³cells/ml	R %	Limit ICAR and ISO 13366-2/ IDF 148-2 *10³cells/ml	%
Reproducibility Interlaboratory Study (ILS) Phase II	Sample 1	133	23	13,0	33	25
	Sample 2	119	18	14	31	26
	Sample 3	265	31	10,5	60	23
	Sample 4	359	59	15,5	76	21
	Sample 5	528	61	12,0	102	19
	Sample 6	1.019	107	10,7	173	17
	Sample 7	1.070	**190**	15,0	182	17
	Sample 8	1.153	81	6,0	196	17
	Sample 9	192	30	13,7	46	24
	Sample 10	198	25	9,6	46	23

Note: the values in red are going to be approved in the next version of ICAR protocol and ISO 8196-3/IDF128-3

1.4 Robustness

The robustness test on FM 7 DC was conducted at Milchprüfring Bayern from March 2018 to November 2019. The performance was within the limits indicated in the ICAR protocol, in ISO 8196-3, and in ISO 13366-2|IDF 148-2. During daily routine analyses of about 4000-5000 DHI samples, the instruments showed initially some critical functions with rinse procedures and delayed-carry over, with consequent interruption of the work flow and repeated analysis of samples. The manufacture provided a new software version, which solved these challenges.

The intra laboratory reproducibility was calculated based on pilot samples analysed at Milchprüfring Bayern over 3 working days. This resulted in an intralaboratory reproducibility of 11%, which complies with the limit of 18% as stated in the ICAR protocol and in ISO 13366-2 /IDF 148-2 for a concentration level of 200.000 cells/ml.

Final Conclusion

The outcome from the Microval validation, data from Milchprüfring Bayern (DE) on the instrument robustness and results of an interlaboratory study (ILS) organised by Qlip described the instrument performance well. The adequate instrument performance in phase I was confirmed in phase II. The instrument complies with all criteria defined in the ICAR "Procedure 1 of Section 12 of ICAR Guidelines – Protocols for Evaluation of Milk Analyses for ICAR Approval", which, in turn, is aligned with the ISO 8196-3:2009 – 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 for all the criteria tested and EN ISO 13366-2|IDF 148-2:2006 Milk - Enumeration of somatic cells - Part 2: Guidance on the operation of fluoro-optoelectronic counters.

Based on the results of our investigations, as described in this report, the Fossomatic™ 7 DC can be granted the ICAR certificate for milk analysers.

2 Introduction

ICAR certification for milk analysers is intended to serve as a standardised process for certification of milk analysers and to describe instrument performance according to ICAR international guidelines for the DHI analyses of cow milk.

Fossomatic™ 7 DC is a new generation of Fossomatic instruments for somatic cell counting in raw milk.

FOSS launched Fossomatic™ 7 DC in June 2017. Fossomatic™ 7 DC allows for the simultaneous determination of total somatic cell count (SCC) and differential somatic cell count (DSCC in raw milk). The differentiation between the different cells is intended to classify the mastitis status, and can provide relevant information for farm management purposes.

The purpose of this ICAR certification process was solely to evaluate the total somatic cell counting in cow milk.The method, to determine differential somatic cell count in milk, it is not yet described in an international standard and, for this reason, ICAR cannot evaluate this parameter. Nowadays, there are some publications and research studies under development.

Fossomatic™ FC was granted the ICAR grandfather exception in February 2020. Fossomatic™ 7 DC accuracy was compared with performance of Fossomatic™ FC during the Microval validation study as conducted by Qlip in 2017/2018.

For phase I of the certification process, ICAR made use of the information and data provided with an earlier conducted Microval certification, see https://microval.org/en/issued-certificates/. Milchprüfring Bayern (DE) and Qlip were subcontracted as experts and accreditated laboratories. Milchprüfring Bayern (DE) provided data on the instrument robustness. Qlip (NL) prepared an Interlaboratory study (ILS) and performed statistical analysis.

The tests performed in phase I, phase II and in the ILS study are robust and independent tools to calculate the performance of Fossomatic™ 7 DC.

3 Company name and instrument under evaluation

Manufacturer: FOSS Analytical A/S
Nils Foss Allé 1, 3400 Hillerød, Denmark

Instrument: Fossomatic™ 7 DC

4 Data evaluated by ICAR

Microval validation report (Phase I)

Milchprüfring Bayern data 2019 (Phase II)

Interlaboratory study (ILS) and statistical elaboration by from Qlip (NL) in 2020

5 Instrument principle

Fossomatic™ 7 DC 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 - acridine orange - 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, identified by an algorithm, 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. Furthermore, the determination of SCC is done using an algorithm based on dot-plots on Fossomatic™ 7 DC instead of pulse height amplitude (PHA) diagrams. The instrument is designed to count total cells and to differentiate the different cells populations. Twelve incubation chambers work in parallel and acridine orange is used as fluorescent dye.

The instruments performance characteristics have been tested during phase I and have been confirmed in phase II. Fossomatic™ 7 DC accuracy for total somatic cell count is demonstrated by comparison with results obtained with the Fossomatic™ FC.

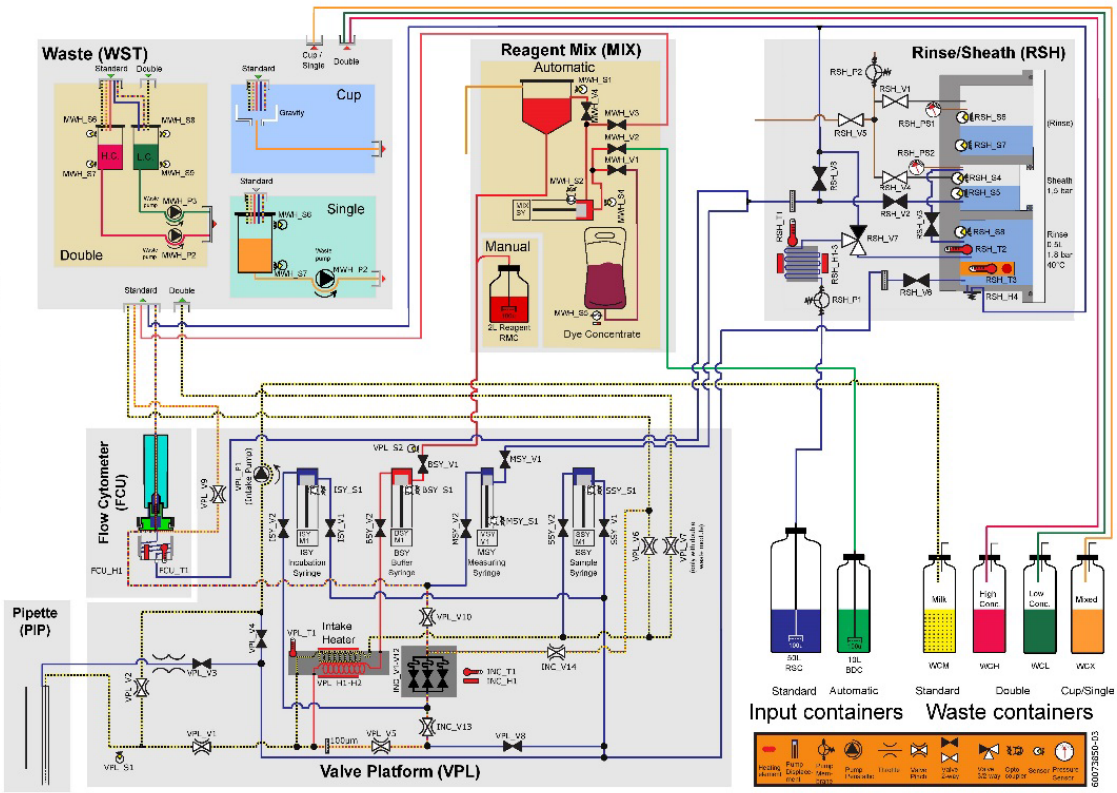

Figure 1. Scheme for measurement principle Fossomatic™ 7 DC (Source: Fossomatic™ 7 / 7 DC User Manual 6007 1937 / Rev. 4)

Figure 2. Fossomatic™ 7 DC.

6 ICAR evaluation criteria

The Fossomatic™ 7 DC was evaluated against the limits in ICAR protocol "Procedure 1 of Section 12 of ICAR Guidelines – Protocols for Evaluation of Milk Analyses for ICAR Approval". Fossomatic™ 7 DC accuracy was obtained through result comparison with Fossomatic FC, which already obtained the ICAR Grandfather exception. Fossomatic FC accuracy was evaluated comparing the instrument and the microscopic reference method ISO 13366-1/IDF 148-1 and the instrument was found to be at least equivalent to the reference method; in the Microval validation Fossomatic FC was used as anchor method to evaluate FM 7 DC.

Limits for this comparison were not yet approved in the current ICAR protocol, but will be approved in the next revision of the ICAR protocol as soon the ISO IDF 8196-3 will be published. The values that are going to be approved are indicated in red in Table 1.

Table 1. ICAR evaluation criteria limits.

Measurand		Criteria	Criteria limits
(units)	SCC		SCC
	*10^3 cells/ml	Intra laboratory reproducibility ($s_{R\ intra}$)	%
Range, DLrange - Overall	0 to 2 000	Relative standard deviation of reproducibility intra, sRintra % - Overall	5
- Low (L)	0 to 100	- Low (L)	7
- Medium (M)	100 to 1 000	- Medium (M)	5
- High (H)	>1 000	- High (H)	4
Carry-over ratio limit, LC		Accuracy (s_{yx}) (1)	
	2%	Comparison of alternative against reference method	
Sequence number, NC	20	Individual animal milk samples	
Minimum range, DLtest	500	Relative standard deviation of s_{yx} %	10 %
Linearity: ratio limit, De/DL		Number of individual animal milk samples, Na	100
	2%	Herd bulk milk samples'	
Replicate number for linearity, NL	8	Relative standard deviation of s_{yx} %	10 %
Maximun range, DLtest	2 000	Number of herds, Nh1	5
Repeatability (s_r)		Number of herd bulk milk samples'	60
Relative standard deviation of repeatability , sr% Overall	4 %	Accuracy (s_{yx}) (2)	
- Low (L)	6%	Comparison between two different instrument model	
- Medium (M)	4 %	Individual animal milk samples	
- High (H)	3 %	Relative standard deviation of s_{yx} %	8 %
Calibration		Number of individual animal milk samples, Na	100
Mean bias		Herd bulk milk samples'	
Relative mean bias	± 5%	Relative standard deviation of s_{yx} %	8 %
Slope,b	1 ± 0,05	Number of herds, Nh1	5
		Number of herd bulk milk samples'	60

7 Phase I - Repeatability

The repeatability test is one of the basic and most important tests to perform during the validation study. The repeatability describes the minimum instrument variance results and it is part of intra- and interlaboratory reproducibility.

The data obtained during the stability tests performed in Phase I on three pilot samples and one instrument have been used to calculate the instrument's repeatability. The pilot samples with low, medium and high concentration of cells were prepared using a blank milk sample (semi skimmed UHT milk with 1ml/L polypropylene glycol 2000 and 0,04% Bronopol) and spiked with milk leucocyte suspension. Samples from each cell count level were measured in triplicate (n=3) with the Fossomatic™ 7 DC in random order each 20 minutes during a working day with 20 checks in total. Routine individual cow milk samples were run in between.

The standard deviation of repeatability (sr), was calculated according to the ICAR protocol and ISO 8196-3:2009.

The results reported in Table 2 are r_{low} =12%, r_{medium} =6%, r_{high} =3%, and and $r_{overall}$ = 7%.

Table 2. Repeatability (r) on three pilot samples.

				sr	r	sr%	r%	Unit	Limit r %
Repeatability Phase I	r	low	153	6,6	18,68	4%	12	103 cell/ml	17
		Medium	516	11,6	32,83	2%	6	103 cell/ml	11
		High	1.516	15,9	45,00	1%	3	103 cell/ml	8
		Overall					7		11

7.1 Conclusion

The instrument's repeatability evaluated in phase I fits amply with the ICAR limits in the entire range tested and at the single level of concentrations. The repeatability will be further evaluated in phase II.

8 Phase I - Intralaboratory reproducibility

Intralaboratory reproducibility (Rintra) was checked by analysing three pilot samples in random order each 20 minutes during a working day with 20 checks in total. Routine individual cow milk samples were run in between.

The standard deviation of repeatability (sr), the standard deviation of means (sx), the standard deviation between checks (sc) and the standard deviation of daily reproducibility (sRdaily) have been calculated and reported in Table 3. ICAR compared the data of standard deviation of daily reproducibility with the limit of standard deviation of intralaboratory reproducibility (sRintra). The reason for this is that the pilot samples have been analysed during the working day (different times on the same instrument) so the operative condition were intermediate between the repeatability and reproducibility. This situation is described by ISO 13366-2 IDF 148-2 as intra-laboratory reproducibility.

Table 3. Instrument Intralaboratory reproducibility (phase I).

			s_r	s_x	s_c	s_{Rintra}	R_{intra}	s_{Rintra} %	R_{intra} %	Unit	Limit R_{intra} %
	Low	153	6,6	10,8	10,1	12,1	33,88	8%	22	10^3 cells/ml	20
R_{intra}	Medium	516	11,6	20,9	19,8	23,0	64,40	4%	12	10^3 cells/ml	14
	High	1.516	15,9	31,8	30,4	34,3	96,04	2%	6	10^3 cells/ml	11
	Overall								14		15

8.1 Conclusion

The instrument daily reproducibility (R intra) in phase I complies with the ICAR limit with the exception at lower concentration that slightly exceeded the limit of 20%. However, this SCC level will be analysed in more detail during phase II (12.1).

The overall intralaboratory reproducibility of phase I complies with the ICAR limit of 15%.

9 Phase I - Carry over

ICAR protocol, ISO 8196-3|IDF 128-3 and ISO 13366-2|IDF 148-2 define the carry-over as the residual volume of the previous sample as a percentage of the total volume of the instrument flow cell after a single pumping sequence of a sample through the instrument cell.

Internal factors/issues affecting carry over include pump settings, flow system deficiencies and compensation factors. External factors affecting carry-over include transfer from the stirrer and pipette.

After each measurement, the analytical circuit of FM 7 DC is cleaned to minimize the transfer of a portion of a milk sample to the successive sample.

The carry-over tests have been executed during Microval validation. One preserved blank milk and spiked milk have been analysed according to the following sequence:

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;

* repeated twenty times for total somatic cell count.

The efficiency of this is measured by the ratio:

CO= [(∑ (BLANK 1) - ∑ (BLANK 2)) / (∑ (MILK 2) - ∑ (BLANK 2))] x 100

This test has been executed for three different level of spiked milk samples

The obtained value has to be lower than 2% according to the ICAR protocol.

For each somatic cell level the ratio CH/L and CL/H have been calculated (see Table 4).

Table 4. Carry-over results.

	Carry Over ratio H/L	Carry Over ratio L/H
High 1 (ca. 500*10^3 cells/ml)	1,49	0,00
High 2 (ca. 1000*10^3 cells/ml)	0,05	0,12
High 3 (ca. 3000*10^3 cells/ml)	1,44	0,64
Limit CO		2%

9.1 Conclusion

The outcome of the CO results complied with the ICAR limit of 2% for all the concentrations tested.

10 Phase I - Linearity

Linearity expresses the constancy of the ratio between the increase in the concentration of an analyte and the corresponding result of an alternative method. The linearity test can be helpful to check the calibration adjustment, when 2 certified reference materials are used to prepare the intermediate concentrations

The ratio De=range of residuals and Dc= range of concentration were calculated according to the formula:

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

emax is the numerical value of the maximum residual from the regression;

emin is the numerical value of the minimum residual from the regression;

Mmax is the numerical value of the upper measured value for the concerned set of samples;

Mmin is the numerical value of the lower measured value for the concerned set of samples

During the Microval validation Qlip prepared two sets of samples with a concentration ranges from 0 to 10.000.000 cells/ml. The blank milk was spiked with milk leucocytes suspension for a total of 22 samples each set. The first set was analysed in increasing concentration of cells with four replicates of each sample and the second set was analysed in decreasing concentration of cells with four replicates of each sample for a total of eight results for each sample of linearity test.

The linearity resulted ratio was r range 0-10.000*103 = 1,70% r range 100*103 -1.500*103 = 1,08%.

10.1 Conclusion

Linearity of Fossomatic™ 7 DC shows linear behavior in the routine working range (0-1.500.000 cells/ml) and also in the wider range of 0-10.000.000 cells/ml. The instrument complies with the requirements of equal or less than 2% in the ICAR protocol, ISO 8196-3|IDF 128-3 and ISO 13366-2|IDF 148-2.

11 Phase I – Accuracy

The accuracy of Fossomatic™ 7 DC was evaluated with method Fossomatic™ FC as anchor method. The accuracy test was performed as follows:

a. Phase I: Fossomatic™ 7 DC vs Fossomatic™ FC (11.1)

b. Milchprüfring Bayern: Fossomatic™ 7 DC vs Fossomatic™ FC (11.2)

c. Calibration data (12.8)

d. Comparison of the instrument measurement results with calibration sample values (12.10)

11.1 Accuracy test Fossomatic™ 7 DC vs Fossomatic™ FC (Qlip)

Qlip selected 144 individual cow milk samples preserved with 0,05% bronopol and 0,005% kathon in a range from 50.000-2.000.000 cells/ml. These samples were analysed in parallel with the two instruments. The standard deviation of accuracy (syx) calculation was performed by ICAR using the raw data.

The standard deviation of accuracy (s_{yx}) was found to be 6% (Table 5).

11.2 Accuracy test Fossomatic™ 7 DC vs Fossomatic ™ FC (Milchprüfring-Bayern)

Milchprüfring Bayern (mpr) selected 657 single cow milk samples in a range form 50.000-2.000.000 cells/ml. The samples were analysed in single replicate, with the two instruments. This experimental plan is slightly different from the ICAR protocol, which prescribes to analyse the sample in 2 or 3 replicates and to consider the mean instrument value for the comparison with the anchor (reference) value. However, in this extensive accuracy test the experimental condition are actually more restrictive due to the single analysis executed on each instrument.

The results obtained is standard deviation of accuracy (s_{yx}) = 8% (Table 5).

Table 5. Accuracy FC7 DC versus FC.

	Individual cow milk samples (Qlip)	Individual cow milk samples (mpr)
	144	647
n	cell/ml	
Range	50000-2000000	50000-1900000
Slope	1,00	0,99
Bias	-2587	-2324
s_{yx}	31437	19934
s_{yx} %	6	8

11.3 Conclusion

The accuracy test executed comparing Fossomatic™ 7 DC with previous generation of instrument being FM FC resulted in a standard deviation of accuracy syx below the proposed limit of 8% for the tests executed at Qlip and Milchprüfring Bayern.

For this first part of Fossomatic™ 7 DC accuracy evaluation (i.e. 10.1 and 10.2), the results are positive. The overall accuracy evaluation is reported in section 11.

12 Phase II - Interlaboratory study

An interlaboratory study (ILS) was organised in order to validate some of the criteria obtained in phase I in one single laboratory and to calculate the Fossomatic™ 7 DC reproducibility.

Qlip organised the ILS according to ISO 5725-1, ISO 5725-2 and IDF Bulletin 453/2012. It shall be noted that Qlip is accredited with ISO 17043 and thus the ILS statistical treatment is within the scope of this accreditation.

12.1 Participant laboratories

Six laboratories from three different countries participated in the validation study with a total number of 9 Fossomatic™ 7 DC. The names of the laboratories are presented in alphabetic order in Table 6.

Table 6. Participant laboratories in the interlaboratory study.-

Laboratory Name	Country
Associazione Regionale Allevatori Lombardia	Italy
Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta S.S. Centro Latte	Italy
LIGAL	Spain
Milchprüfring Bayern	Germany
Qnetics	Germany
Salchim	Italy

12.2 Interlaboratory Study (ILS) design

The participant laboratories have been provided with the following material (per instrument):

- 1 vial of 60 ml containing water for temperature control upon arrival (code: water)
- 2 vials of 60 ml containing Fossomatic™ rinse sheath solution, (code: blank)
- 2 vials of 60 ml containing UHT semi skimmed milk, (code: blank milk)
- 1 vial Fossomatic™ Adjustment Sample DC (code:FMA DC)
- Eight single cow milk samples, 2 bulk milk samples and 2 UHT semi skimmed milk samples were split in double blind each. In this way a set of, in total, twenty-four vials was created (24 vials of 60 ml each containing preserved raw milk for measurements with Fossomatic™ 7 DC (code: FM 1 to FM 24, see Table 7)).
- Note: samples were preserved with 0,04% bronopol (end concentration in the milk).
- 5 vials with QSE calibration samples, lyophilized, (code SCC 1 to SCC 5).QSE calibration material is accredited by DAkkS number D-RM-20961-01-00 for DIN EN ISO 17034:2017. The material has been characterized considering data obtained with both the microscope method ISO 13366-1 | IDF 148-1 and the fluoroptoelectronic method ISO 13366-2 | IDF 148-2.

Table 7. Sample identification and type of milk.

Milk		Sample ID	
Individual cow Milk	1	FM 4	FM 10
	2	FM 16	FM 22
	3	FM 6	FM 19
	4	FM 1	FM 7
	5	FM 9	FM 21
	6	FM 12	FM 18
	7	FM 3	FM 5
	8	FM 15	FM 17
Herd mIlk	9	FM 11	FM 13
	10	FM 23	FM 24
UHT Semi Skimmed Milk	11	FM 2	FM 8
	12	FM 14	FM 20

12.3 Transport condition and delivery time

All samples were delivered in the laboratories on 27 of November The sample temperature at arrival was between 1°C and 4°C. The sample quality reported by all the laboratories was good and all the samples delivered have been analysed.

12.4 ILS samples homogeneity

Each of the 12 FM samples was tested in two replicates. The sample homogeneity was checked through the fat concentration. According to ISO 13528 the variance between vials (i.e., se) should not be bigger than $0,3*s_R$ of the method used to check the homogeneity. Table 8 lists the results obtained and the calculated limit for each level of concentration. All the samples tested showed a standard deviation between vials (se) that was lower than the calculated limit.

Table 8. Sample homogeneity between vials.

	*10^3 cells/ml									
sample	1	2	3	4	5	6	7	8	9	10
mean value	172	131	294	372	515	1023	1263	1352	228	273
s_r	3,742	5,79	5,48	16,34	4,60	17,79	4,135	14,54	7,25	9,35
r	10,77	10,47	15,34	45,75	12,88	49,81	4,37	40,71	20,30	26,18
s_r%	2,2	2,9	1,9	4,4	0,9	1,7	0,1	1,1	3,2	3,4
s_R ISO 13366-2	15,2	12,0	23,6	28,0	34,9	57,7	67,2	70,3	19,3	22,3
se	2,57	2,35	3,69	0,00	6,37	10,24	20,01	0,59	0,00	1,27
se%	1,5	1,8	1,3	0,0	1,2	1,0	1,6	0,0	0,0	0,5
Limit se	4,57	3,59	7,09	8,39	10,48	17,30	20,15	21,08	5,80	6,70
Limit se%	2,7	2,7	2,4	2,3	2,0	1,7	1,6	1,6	2,5	2,5

12.5 ILS execution

All the participant laboratories received detailed instructions on how to treat the samples and how to prepare the instrument according to specific manufactures instructions in order to obtain the best

standardised conditions. All the tests have been executed on 29 November 2019 with the exception of two sets of samples analysed on 28 November. It was requested by ICAR to set slope=1 and bias=0 on all the instruments before analysing the ILS samples.

As initial step, it was requested to analyse the blank and the blank milk samples in twelve replicates each. The mean results should not exceed 3000 cells/ml and all the individual results should be below 8000 cells/ml - if not the procedure had to be repeated. Thereafter FMA DC were analysed in three replicates. Besides, the milk standardization procedure was conducted according to the manufacturer's instructions, FOSS technical note 2214, some time prior to the ILS study. The ILS samples (FM1-FM24) were analysed in two replicates. Successively, the laboratories reconstituted the lyophilized milk provided (QSE calibration material) and analysed each calibration material in five replicates.

12.6 Laboratory results and statistical treatment

The results of total somatic cells have been statistically treated as follows:

a. Evaluation of instrument checks as blank, blank milk, FMA DC and standardization (FL 1)

b. Calibration data (12.8)

c. Comparison of the instrument measurement results with calibration sample values (12.10)

d. Precision calculation (12.12)

12.7 Evaluation of the instrument checks

The results are reported in Table 9.

Table 9. Results of instrument check.

	*10^3 cells/ml			
Laboratory	Blank Mean	Blank milk Mean	FMADC	FL1
2	1	0	168	
3	0	0	155	
4	0	0	165	
8	0	1	156	1,519
9	0	0	159	
11	0	0	167	
12	0	0	169	
14	0	0	109	
18	0	1	168	

All the results fit the indicated limit.

With the exception of one laboratory that reported the correct milk standard factor value for criteria FL1, all others reported the instrument milk cell target value probably for a misinterpretation of the instructions provided.

12.8 Calibration data

During the interlaboratory study, the ILS samples were analysed using nine different instruments with the basic manufacture calibration of slope=1 and bias=0. The laboratories analysed five calibration samples in five replicates, from which the mean bias and slope were calculated considering the calibration material value (y-axis) and the instrument results (x-axis). Furthermore, the confidence interval for slope was calculated for each instrument.

Laboratory 4 did not reported results for calibration material 2 and 3.

Table 10. Mean bias of the instrument measurement results with calibration sample values.

	SCC 1	SCC 2	SCC 3	SCC 4	SCC 5	
Laboratory			% Bias			Mean Bias %
2FM 7DC	31	4	3	9	1	10
3FM 7DC	5	2	2	3	3	3
4FM 7DC	4			3	5	4
8FM 7DC	2	7	5	3	3	4
9FM 7DC	1	1	5	1	3	2
11FM 7DC	18	17	16	16	17	17
12FM 7DC	26	5	27	7	22	17
14FM 7DC	7	3	28	1	0	8
18FM 7DC	3	3	0	10	1	4

Table 11. Instrument slope value with the calibration material.

	*10^3 cells/ml							
Laboratory	SCC 1	SCC 2	SCC 3	SCC 4	SCC 5	Slope	Lowest 95%	Highest 95%
2FM 7DC	181	257	397	673	1042	0,93	0,78	1,09
3FM 7DC	131	259	403	721	1031	0,97	0,96	0,99
4FM 7DC	133			723	1003			
8FM 7DC	135	247	391	720	1027	0,98	0,95	1,00
9FM 7DC	136	263	391	737	1024	0,97	0,93	1,01
11FM 7DC	113	221	344	624	880	0,84	0,82	0,85
12FM 7DC	173	254	300	690	822	0,76	0,50	1,01
14FM 7DC	129	258	294	735	1054	1,03	0,79	1,26
18FM 7DC	133	257	408	673	1047	0,97	0,84	1,10

12.9 Conclusion

All the calculated slopes are within the confidence interval. The confidence interval of instrument 3FM 7 DC and 11 FM 7 DC showed a slope statistically different from 1. Because value 1 is not included in the interval. As the instrument accuracy was already evaluated in phase I in comparison with Fossomatic FC and the results have not been recalculated on the slope and bias obtained using the calibration material, these results are reported only as informative for the laboratories that participated in the ILS study.

12.10 Comparison of the instrument measurement results with calibration sample values

The difference between the calibration material and measured value with its uncertainty were calculated and reported as informative.

The comparison of $\Delta m \leq U\Delta$ where:

Δm = the absolute difference between the mean measured value and calibration sample value

$U\Delta$ = is the expanded uncertainty of Δm

$U\Delta = 2\ u\Delta$ with K=2 with a confidence level of 95%

$u\Delta$ = combined uncertainty of Δm

$u\Delta = \sqrt{u2m + u2SRM}$

Defining:

$U\Delta$ is the expanded uncertainty of $u\Delta$

$U\Delta = u\Delta * 2$

$u2\Delta = u2m + u2SRM$

um = combined uncertainty of measurement result

uSRM =combined uncertainty of the calibration material

um was considered the standard deviation of reproducibility (sR) of ISO 13366-2 interpolated for each level of concentration of the calibration material

Table 12. Comparison between instruments results and calibration sample value ($\Delta m \leq U\Delta$).

	*10^3 cells/ml									
						Absolute difference Inst.- SRM				
Laboratory	SCC 1	SCC 2	SCC 3	SCC 4	SCC 5	SCC 1	SCC 2	SCC 3	SCC 4	SCC 5
2FM 7DC	181	257	397	673	1042	43	9	13	71	15
3FM 7DC	131	259	403	721	1031	7	7	7	23	27
4FM 7DC	133			723	1003	5			21	54
8FM 7DC	135	247	391	720	1027	3	20	19	24	30
9FM 7DC	136	263	391	737	1024	2	3	19	7	34
11FM 7DC	113	221	344	624	880	25	45	66	120	177
12FM 7DC	173	254	300	690	822	35	12	110	54	235
14FM 7DC	129	258	294	735	1054	9	8	116	9	4
18FM 7DC	133	257	408	673	1047	5	9	2	71	11
SRM Value	138	266	410	744	1057					
U_{SRM}	7,3	8,2	14,8	18	23,8					
u_{SRM}	3,65	4,1	7,4	9	11,9					
u_{SRM} /SqRout(5)	1,63	1,83	3,31	4,02	5,32					
s_R ISO 13366-2 (interpolated)	12,4	22,0	30,0	45,0	59,0					
Bias Limit						25,1	44,2	60,4	90,4	118,5

SRM = Calibration material

12.11 Conclusion

The comparison of the instrument measurement results with calibration sample values indicate that instrument 11 and 12 with 4 or 3 samples are out of the range and should be calibrated.

Because the reproducibility evaluation was done on the raw data obtained with slope =1 and bias= 0 these results are reported only as informative.

12.12 Precision calculation

The precision calculation, specifically repeatability and interlaboratory reproducibility, was done according to ISO 5725-2.

In Table 13 the results of the first replicate of each vial and the first replicate of its double blind sample obtained with slope=1 and bias=0 are reported for each laboratory.

Table 13. ILS participant laboratory results.

	*10^3 cells/ml									
	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
Laboratory	FM4	FM10	FM16	FM22	FM6	FM19	FM1	FM7	FM9	FM21
2FM 7DC	180	178	137	130	320	312	411	409	550	534
3FM 7DC	174	176	120	126	301	305	361	367	540	513
4FM 7DC	178	168	126	132	292	295	374	391	488	503
8FM 7DC	180	168	138	129	304	298	386	385	499	519
9FM 7DC	175	179	140	129	290	276	399	370	502	514
11FM 7DC	180	165	146	127	304	284	369	338	473	480
12FM 7DC	188	174	129	129	299	292	386	386	526	512
14FM 7DC	177	161	135	127	307	302	396	377	495	498
18FM 7DC	158	162	137	138	284	287	334	381	484	489
	Sample 6		Sample 7		Sample 8		Sample 9		Sample 10	
Laboratory	FM12	FM18	FM3	FM5	FM15	FM17	FM11	FM13	FM23	FM24
2FM 7DC	1076	1085	1368	1361	1371	1422	232	225	270	281
3FM 7DC	1009	999	1280	1295	1344	1373	216	217	262	266
4FM 7DC	1011	1001	1259	1325	1378	1343	225	225	269	256
8FM 7DC	998	1027	1335	1247	1319	1328	215	233	259	268
9FM 7DC	988	983	1281	1221	1317	1321	201	239	244	261
11FM 7DC	915	993	1199	1167	1350	1345	205	230	249	257
12FM 7DC	1004	1037	1249	1314	1382	1384	217	227	259	257
14FM 7DC	1017	1027	1256	1250	1373	1370	204	236	267	265
18FM 7DC	976	983	1209	1113	1343	1327	215	226	253	251

The statistical analyses did not identify any outliers using the Cochran and Grubbs test. The precision results have been compared with the interpolated values reported in the ISO 13366-2|IDF 148-2. In this standard some indicative levels are reported. Considering the ILS samples somatic cell concentration, the precision values limit were interpolated and recalculated. Table 14 the calculated precision values are reported.

Table 14. Repeatability (r) and Reproducibility (R).

		ILS result				Interpolated limits ISO 13366-2/IDF 148-2			
	Mean Instruments result	r		R		r		R	
Sample	*10^3 cells/ml	*10^3 cells/ml	%	*10^3 cells/ml	%	*10^3 cells/ml	%	*10^3 cells/ml	%
Sample 2	132	18	14	18	14	21	17	31	26
Sample 1	173	21	12	23	13	22	17	33	25
Sample 9	222	40	18	40	18	30	16	46	24
Sample 10	261	18	7	25	10	29	15	46	23
Sample 3	297	19	6	31	11	38	14	60	23
Sample 4	379	45	12	59	16	45	13	76	21
Sample 5	502	30	6	61	12	57	11	102	19
Sample 6	1014	61	6	107	11	87	8	173	17
Sample 7	1263	115	9	190	15	91	8	182	17
Sample 8	1355	47	3	81	6	98	8	196	17
Overall			9		12		13		21
ICAR Limit							11		

12.13 Conclusion repeatability

Sample 9 and sample 7 resulted with a repeatability slightly larger than the limit but the repeatability with samples with the same level of concentration and the overall repeatability was favourable to interpolated values from ISO 13366-2|IDF 148-2 limits. Furthermore, the overall repeatability $r_{overall}$ = 9% is smaller than indicated ICAR limit r = 11 %.

The repeatability value means that the absolute difference between two independent single test results (r) 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, should not be more than 5 % of cases greater than 9% for the instrument FM 7DC.

12.14 Conclusion reproducibility

The results show that the overall instrument reproducibility values are lower than interpolated ISO 13366-2|IDF 148-2 limits. Furthermore, the overall reproducibility equal to Roverall = 12% is smaller than indicated ISO/IDF limit of RLimit =21 %.

The reproducibility value means that the absolute difference between two independent single tests results (R) obtained using the same method on identical test material in different laboratories by different operators using the same equipment within a short interval of time, should not be more than 5 % of cases greater than 12% for FM 7 DC

13 Phase II- Robustness

The aim of the robustness test is to challenge the instrument to highlight possible technical aspects that could be improved. This is also an important moment in the validation process for the manufacturer given that feedback on the instrument performance from operative situations is highly valuable and allows to optimise the instrument further. FM 7 DC has been tested in terms of robustness at Milchprüfring Bayern from 2016 to 2019 in close collaboration with FOSS. The data provided for ICAR robustness evaluation cover the period March 2018 November 2019.

13.1 Phase II- Intralaboratory reproducibility

At Milchprüfring Bayern each day a new pilot sample is prepared based on bulk tank milk. The assigned SCC target value is determined after all the instruments are checked with calibration samples. When the Dairy Herd Improvement (DHI) single cow milk samples are analysed, two consecutive pilot samples are analysed each 45 DHI samples in single replicate. We have extracted,

randomly, the results from 3 working days for a total of 91 pilot samples (one instrument) analysed. The standard deviation of intralaboratory reproducibility (sRintra) and reproducibility R= sRintra*2,8 was calculated considering the pilot repeatability (sr) and the pilot standard deviation (sL) of three working routine days.

Table 15. Intralaboratory reproducibility.

Reproducibility Intralaboratory Phase II 3 days (mpr)		n	Concentration *10^{3}cell/ml	sR_{intra}	R_{intra}	R_{intra} %	R_{intra} Limit ICAR - ISO 13366-2/IDF 148-2 %
	R_{intra}%	91	214	8,6	24,1	11	18

Relative intralaboratory reproducibility was below the ICAR and ISO/IDF 13366-2/IDF 148-2 limit and positively confirms the results obtained in phase I for FM 7 DC.

The intralaboratory reproducibility value means that the absolute difference between two independent single test results (Rintra) obtained using the same method on identical test material in the same laboratory by different operators and or different equipment in different time, should not be more than 5 % of cases greater than 14% in a range of concentration 150-1.500 *103 cells/ml. Reproducibility intralaboratory value of 14% was obtained considering the test executed in phase I and phase II.

13.2 Robustness - technical aspects

A part of the robustness test have been conducted from March 2018 to November 2019. Three instruments were monitored.

During the testing period, it was noted that blank values at the beginning of the working session were within the limit and during the day they increased over the limit of 8000 cells/ml. After the execution of extra cleaning processes correct values were again obtained. Several times, at the beginning of the working day, the values for FL1 and FL2 for FMA DC were too high. After measuring some milk samples, correct values were again obtained. Frequently, the rinse sheath unit (RSH) caused challenges in terms of pressure and sensor functioning.

In October 2019, it was noticed that if a sample with a very high somatic cell concentration (e.g. >5.000.000 cell/ml) is analysed a “delayed carry-over” can occur. Delayed carry over means that a milk sample can be contaminated by another one which is first analysed 1 minute afterwards. The reason for this is that the instrument has an incubation unit consisting of 12 individual chambers. The carry over may appear delayed because the sample sitting in chamber 1 goes through an area (specifically, the area upstream of VPL_V10 and INC_V14 in Figure 1) of the flow system on its way to the flow cell where a small fraction of milk from sample 11 (just sitting in the incubation unit) passes through as a results of flushing the system after sample intake. So, sample 11 might contaminate sample 1 but this can first be noticed with a delay of 1 minute because each sample incubates for 1 minute in the incubation unit.

FOSS was informed of the problem and after some internal tests solved the problem by releasing a software update (i.e., extended cleaning and flushing between samples). Furthermore, an additional column named “Delayed CO” is provided in the software Foss Integrator. Customers were informed about this in November 2019 through a customer letter.

13.3 Conclusion

The tests performed at Milchprüfring Bayern confirm the results of phase I with a favourable intralaboratory reproducibility as compared to the limits in the ICAR protocol and those in ISO 13366-2|IDF 148-2. The instrument manufacturer followed constantly the robustness tests conducted at Milchprüfring-Bayern and provided solutions to solve challenges observed in this phase. All instruments were updated/upgraded and users were informed. The overall conclusion is that the FM 7 DC is an instrument that requires somewhat more attention and is somewhat more sensitive to the quality of milk samples compared to Fossomatic FC or 7. This is clearly explainable by the fact that new and different technology and dye solution are used compared to Fossomatic FC and 7. The sample quality is critical for obtaining reliable results.

14 Conclusion

The data obtained during phase I, phase II, and interlaboratory study (ILS) provided robust evidence of an adequate instrument' performance. In phase II the result of intralaboratory reproducibility for a low level of concentration was better than phase I. The overall FM 7 DC precision obtained in phase I and phase II considering the range tested 130.000-1.500.000 cells/ml is:

Repeatability (r)=9%

Reproducibility intralaboratory (Rintra)=10%.

Reproducibility (R) =12%

The repeatability and reproducibility values are reported in detail in Table 14 for the different levels of SCC.

The instrument complies with all limits defined in the ICAR "Procedure 1 of Section 12 of ICAR Guidelines – Protocols for Evaluation of Milk Analyses for ICAR Approval" aligned with the ISO 8196-3:2009 – 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 for all the criteria tested and ISO 13366-2|IDF 148-2.

ICAR certifies the performance of Fossomatic™ 7 DC based on above described studies and results thereof for total somatic cell counting in milk. All studies were executed by independent and accredited laboratories. In addition an extensive international accredited validation study was performed.

15 Acknowledgment

ICAR thanks the laboratories Milchprüfring Bayern (DE),QLIP (NL), all the laboratories that participated in the interlaboratory study for the qualified work done and and last but not least the ICAR members of Milk Analysis Sub Committee for the report revision and approval.

16 Reference documents

- Protocol for the Evaluation of Milk Analysers for ICAR Approval: 2019. https://www.icar.org/index.php/icar-recording-guidelines/
- ISO 8196 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
- EN ISO 13366-2|IDF 148-2:2006 Milk - Enumeration of somatic cells - Part 2: Guidance on the operation of fluoro-optoelectronic counters.

- T.P.J. Linsinger, ERM Application note 1: Comparison of a measurement result with the certified value, https://crm.jrc.ec.europa.eu/e/132/User-support-Application-Notes (last accessed on 16 January 2020)