IDF/ICAR Project on Reference System for Somatic Cell Counting in Milk

Newsletter 9 – January 2023

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The project's aim is to create better equivalence with somatic cell counting in milk worldwide and all our work is naturally centred around that. We are continuously supporting the introduction and usage of the certified reference material (CRM) for somatic cell counting (SCC). This newsletter includes a study that clearly demonstrates the positive effect of the usage of the material in terms of harmonisation of SCC levels around the world. We further explored how providers of secondary reference material are using the CRM. It becomes clear that, despite enormous progress made, there is still work left to further improve equivalence of SCC levels around the world. At the same time, new knowledge in the field of improving the reference method for SCC has been developed and will be shared briefly here. In terms of project leadership, we need to inform you that Vesela Tzeneva had stopped as a co-project leader end of 2021 due to a job change and Adelheid Joris took over until August 2022, when she also changed jobs. However, Pierre Broutin was elected as co-project leader in November 2022. Thank you to Vesela and Adelheid for their support of the project.
Last, we will share our short- and long-term ambitions for the project further below.

We wish you a good read of the 9th edition of the newsletter!
Pierre Broutin and Daniel Schwarz

About Pierre Broutin

I graduated in 1992 from the Polytech'Lille (France) Graduate School of Engineering with a Master in Science and New Technologies. I have been involved in dairy herd management and milk quality/safety testing since 1994, with the development of new FTIR and Flow Cytometry-based analytical methods for the determination of milk fine chemical composition and hygienic quality. In 1997, I joined the AOAC as an Associate Referee in the area of somatic cells counting and the IDF. Subsequently, I have been actively involved in several IDF Action Teams within the Standing Committee on Statistics and Automation. The same year, I joined Bentley Instruments in the United States as Senior Scientist and set up, in 2001, our Western Europe subsidiary in France where I am currently acting as General & Innovation manager. For further details: LinkedIn profile
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About Daniel Schwarz
I grew up on a dairy farm in Germany and have had an interest in dairy cows and milk production since my early childhood. After graduation as an Animal Scientist and completion of a PhD in the field of bovine mastitis at the University of Goettingen, Germany, I started working at FOSS in 2013. In my role as Senior Scientist I am focused on developing tools that allow to improve dairy herd management and milk quality through milk analysis. My involvement with IDF started in 2014 and I have been part of several Action Teams since. For further details: LinkedIn profile
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Harrie van den Bijgaart receives ICAR Outstanding Contribution award

By Daniel Schwarz (FOSS, DK)

Harrie van den Bijgaart, who was heading the IDF AT S09 from 2010 to 2020, has been awarded with the ICAR Outstanding Contribution award. ICAR recognises Harrie's outstanding work in the field of milk testing with this award. More specifically, his input to ISO and IDF standards for milk analysis and, not the least, significant share of work leading to the launch of certified reference material (CRM) for somatic cell counting contributed evidently to the high accuracy and precision of milk analysis around the world. Through excellent networking work between ISO, IDF, ICAR and many other national and international organisations and institutions Harrie helped to spread important knowledge and implement standards in daily routine of milk testing laboratories.

Christian Baumgartner *(left)* received the award on behalf of Harrie van den Bijgaart during the ICAR 2022 conference in Montreal from ICAR president Daniel Lefebvre *(right)*

Success story – Implementation of certified reference material for somatic cell counting

By Silvia Orlandini (ICAR, IT) and Daniel Schwarz (FOSS, DK)

The certified SCC reference material[1] is available since spring 2020 and can be ordered here. A joint IDF/ICAR webinar entitled "Development and application of a certified reference material for somatic cell counting in milk" was conducted in December 2020 to introduce the material and its application (full webinar and presentations are available here). In addition, a guideline ("Bulletin of the IDF N° 508/ 2021") on the application of the reference material was developed by the IDF Action Team S09 and is available for free here.

The status quo on the implementation of the certified SCC reference material was described in detail in Newsletter 8 (available here) of the IDF/ICAR Project on Reference System for Somatic Cell Counting in Milk. While there was no need for adjustment of SCC levels in several countries, others faced a situation where they had to adjust.

ICAR is conducting two proficiency tests per year with participation of 30+ milk testing laboratories from around the globe. Somatic cell count is one of the parameters being checked in this context. Hence, results from the ICAR proficiency tests are suitable to investigate the effect of the implementation of the CRM.

We compared results from the ICAR proficiency test conducted in September 2018 (*full report*) with results from September 2022 (*full report*) (Figure 1). Large difference of SCC levels were observed in September 2018 and only 47% of laboratories revealed results within the target (i.e. +/- 10% of mean and SD <10). However, a clearly improved situation was found in September 2022 where 67% of the laboratories reported SCC results within target. While 4 laboratories participating in 2018 applied ISO 13366-1|IDF 148-1, it was 5 laboratories in 2022 using that method. All other laboratories were reporting results generated by ISO 13366-2|IDF 148-2. Nevertheless, the anticipated effect of working with certified reference material (i.e., harmonisation of SCC levels) becomes apparent based on the development of results reported in the frame of the ICAR proficiency test.

It is common practise in milk testing laboratories to work with secondary (e.g., national) reference material and thus cannot be expected that all laboratories work with certified material. Hence, suppliers of secondary reference material should align their material against CRM. A survey was performed to understand to what extent this has already be done (see below).

[1]Official name: EC JRC CRM® ERM-BD001

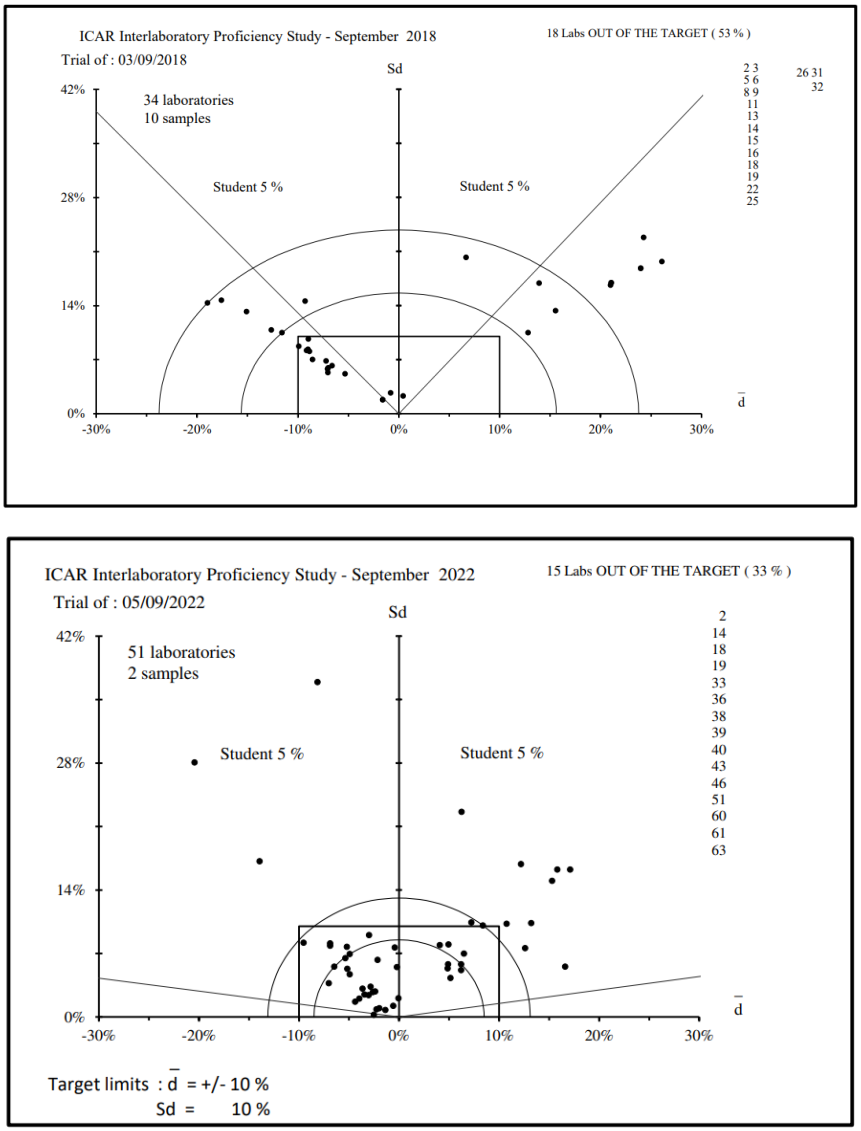

Figure 1. ICAR proficiency test results on somatic cell count from September 2018 *(top, full report)* and September 2022 *(bottom, full report)*. Mean of differences is shown on the x-axes and standard deviation (Sd) of difference shown on y-axis.

Survey on secondary SCC reference material (SRM)

By Silvia Orlandini (ICAR, IT) and Daniel Schwarz (FOSS, DK)

As mentioned above, secondary SCC reference material (SRM) are broadly used among milk testing laboratories. The purpose of this survey was to obtain an overview on secondary SCC reference material and its traceability to CRM. Beyond that, suppliers of secondary reference material were asked about the use and application of the CRM.

A total of 17 suppliers of SRM were contacted from 10 countries and 2 continents were contacted and 11 filled in questionnaires were received and anonymously evaluated.

Table 1. Overview on methods used to determine SCC in secondary SCC reference material (SRM) for 11 suppliers (A-K).

Lab code	ISO 13366-1| IDF 148-1	ISO 13366-2| IDF 148-2	Traceable to CRM	Following IDF Bulletin N° 508/ 2021
A			Yes[1]	
B	Yes[3]			
C	Yes[1]	Yes[1]	Yes[1]	Yes
D			Yes[3]	
E	Yes[1,2,3]	Yes[1,2]	Yes[1,2]	Yes
F		Yes[2,3]		
G	Yes[1]			
H		Yes[2]		
I		Yes[2]		
J	Yes[1]	Yes[2]		
K	Yes[1]	Yes[2]	Yes[1]	

[1]applied in own lab only
[2]applied through a group of labs
[3]applied through a group of trained labs

The results of the survey are summarised in Table 1. In total, six suppliers use ISO 13366-1|IDF 148-1 to determine SCC. Five of them only use the method in their own laboratory, whereas 2 are considering results from other laboratories applying this method as well. Four suppliers work with ISO 13366-1|IDF 148-2 in addition to ISO 13366-1|IDF 148-1. Three other suppliers only use ISO 13366-1|IDF 148-2 to determine SCC in their SRM. Last, five suppliers indicated that their SRM would be traceable to the CRM. Two of these suppliers did not apply the above-mentioned ISO|IDF methods. Two suppliers further indicated to follow the IDF Bulletin N° 508/ 2021.

Comparing the SRM certificates provided by the providers, we found that the declared uncertainty of only one SRM was consistent with the uncertainty of the CRM.

In conclusion, this survey revealed that there is still room for improvement on the SRM. More specifically, the alignment between CRM and SRM could be even higher and uncertainties on SRM consistent with the CRM (see Table 2). The SRM uncertainty cannot be smaller than the CRM uncertainty at the same level of cell concentration. The AT S09 will offer the SRM providers to work with them on further improving the situation.

Table 2. Overview on cell concentration and uncertainty of the certified reference material

Cell concentration		
	Certified value[3] (cells/mL)	Uncertainty[4] (cells/mL)
Somatic cell count (SCC) [1]	64,000	8,000
Somatic cell count (SCC) [2]	62,000	6,000

1) As defined in ISO 13366-1|IDF 148-1. The certified value is the mean value of 14 accepted data sets obtained from ISO 13366-1|IDF 148-1 compliant measurements.
2) As defined in ISO 13366-1|IDF 148-1 and ISO 13366-2|IDF 148-2. The certified value is the mean value of 14 accepted data sets obtained from ISO 13366-1|IDF 148-1 compliant measurements and 14 data sets randomly selected out of 32 accepted data sets obtained from ISO 13366-2|IDF 148-2 compliant measurements.
3) Certified values are values that fulfil the highest standards of accuracy and represent the unweighted mean value of the means of accepted sets of data, each set being obtained in a different laboratory and with methods of determination referred to in footnotes 1 and 2. The certified value and its uncertainty are traceable to the International System of units (SI).
4) The uncertainty of the certified value is the expanded uncertainty with a coverage factor k = 2 corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

ISO 13366-1|IDF 148-1 and ISO 13366-2|148-2 are under revision, brief description of status

By Silvia Orlandini (ICAR, IT) and Philippe Trossat (Actalia, FR)

The main reasons to revise these two ISO|IDF standards were to include the latest developments regarding quality assurance tools, to improve the text and to create Annexes that describe the latest methods for microscopic and flow cytometric analysis of cells in milk. These new sections can promote the application of the standards in routine laboratories and the possibility to organise interlaboratory studies in the nearby future that will allow to calculate the methods' precision.

ISO 13366-1|IDF 148-1 **Error! Reference source not found.**was published in 2008 and the IDF Standing Committee on Statistics and Automation (SC SA) decided to propose its revision for the following reasons:

- some editorial revisions are necessary to include the connected corrigenda and to better explain the procedure to prepare the samples using dry smears for methylene blue dye solution,
- to add an annex to describe an optimised procedure to analyse liquid milk. This procedure has been improved using Ethidium bromide and Yo-Pro 1 as dye solutions within the MIAMi project. The results obtained during the project will be reported as well.

ISO 13366-2|IDF 148-2 was published in 2006 and the IDF Standing Committee on Statistics and Automation (SC SA) decided to:

- add a text where the secondary reference materials for SCC (SRMs for SCC) have to be traceable to the EU JRC CRM
- To update the precision figures based on the results obtained in the proficiency testing scheme (i.e. ICAR PT, more information available here).
- To add an annex where the procedure developed within the Antoinette project will be reported.

An IDF New Work Item proposal is underway for the revision of ISO 13366-1|IDF 148-1. ISO New work proposals are expected for the revision of both parts in the course of 2023.

Publication of the "Antoinette" method

By Thomas Berger, Lotti Egger, Jan-Erik Ingenhoff (Agroscope, CH)

Link
The "Antoinette" project (Antoinette = DNA and Antibody Stained Total and Differential Somatic Cell counting in Milk using Flow Cytometry) was successfully terminated by publishing the method in the *Journal of Dairy Science: Jérôme Widmer, Laurence Descloux, Cédric Brügger, Marielle-Louise Jäger, Thomas Berger, and Lotti Egger. 2022. 105, 11. Direct labelling of milk cells without centrifugation for counting total and differential somatic cells using flow cytometry. Full article freely available here*

Abstract
Somatic cell count (SCC) in milk is an essential indicator for defining and managing udder health. However, analysing differential SCC (dSCC) can be helpful in determining the type or evolution stage of mastitis. A high abundance of polymorphonuclear cells (PMNC) is associated with acute mastitis; however, the status of a chronic disease is less well characterized. A method capable of analysing SCC and dSCC can prove to be a helpful tool for monitoring the status of evolution of mastitis disease in a better way. Therefore, a new direct-flow cytometry method was developed to count and differentiate somatic cells in milk without the steps of centrifugation or washing, avoiding variabilities that occur due to enrichment or loss of specific cell types. In this new method, SCC is analysed using the method of DNA staining with Hoechst stain, whereas dSCC are analysed using specific antibodies targeting 2 main cell types associated with mastitis: PMN cells and antigen-presenting cells, which are associated with innate and adaptive immunity. Equivalent SCC values were obtained between the new method and the routine ISO 13366-2|IDF 148-2 method in a comparison of 240 raw milk samples. Furthermore, dSCC results were confirmed by microscopy after May-Gründwald-Giemsa staining in 165 quarter milk samples from healthy and diseased cows. The method was verified with fluorescence microscopy on the 2 targeted cell types and in raw milk samples. The newly developed method can be performed on any flow cytometer that is capable of measuring three different channels (2-3 lasers) and can be further designed to differentiate other cell types and animal species by selecting appropriate antibodies.

Key words: total somatic cells in milk, mastitis, differential somatic cells in milk, flow cytometry

Future activities
The new method will be used in research projects and, beyond that, Agroscope aims to expand the the existing Agroscope-Suisselab-SCC-Standard with a dSCC standard.

MIAMi (Microscopic Image Analysis in Milk) project – Applying modern microscopic techniques for somatic cell counting in milk

By Silvia Orlandini (IT)

The MIAMi project was concluded in July 2022. The project resulted in an optimized procedure to dye the liquid milk sample and to acquire digital images. The developed procedure describes the use of two possible dye solutions: Ethidium Bromide and Yo-PRO 1.

As described in the previous newsletter (available here) the repeatability of the MIAMi method is better than the dry smear and direct reading on the microscope field (i.e. ISO 13366-1|IDF 148-1). The possibility to analyse milk cells on a computer screen, to identify them one by one, and to indicate as counted clearly improve the outcome of the work done by the technician.

The final protocol has been tested on 43 samples analysed in two replicates using Yo-Pro1 and Ethidium Bromide each. The results showed that the relative repeatability of the MIAMI methods was even below the limits of ISO 13366-2|IDF 148-2 despite the fact that they are based on microscopy. The relative mean bias (relative difference from the CRM) was lower than relative reproducibility (absolute difference between two results of the same method tested under reproducibility conditions). The relative reproducibility was 7% and 6%, the relative mean bias was 6% and 5%, respectively for Yo-Pro1 and EtBr dye solution (Figure 2).

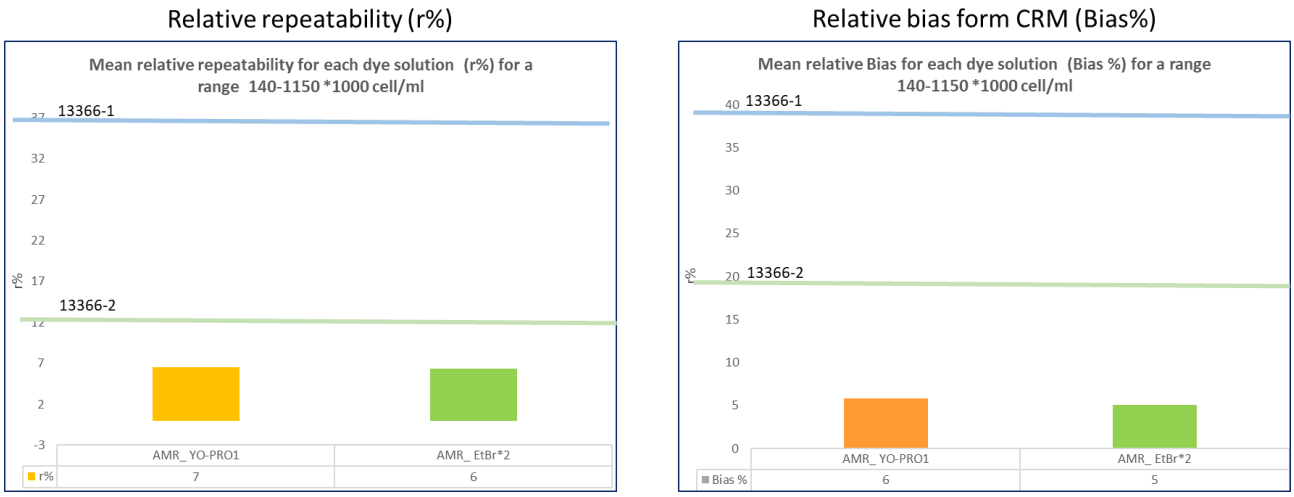

Figure 2. Relative repeatability (r%) and relative bias from certified reference material (CRM) (Bias %) testing 43 samples with the newly developed protocols from the MIAMi project where Ethidium Bromide (EtBr) and Yo-Pro 1 are used as dyes

Official Supporters of MIAMi:

Outlook on future work of the joint IDF/ICAR Action Team S09

By Pierre Broutin (Bentley, FR) and Daniel Schwarz (FOSS, DK)

The AT S09 project's aim is to create and safeguard a worldwide equivalence of the somatic cell counting in raw milk to have the most standardised, traceable, and reliable results for the benefits of the entire dairy industry value chain, from the cow to the consumer. The use of the same anchor method is paramount to benefit from a global knowledge and implement best practises.

Significative improvements have been made since the setup of the Project Group in 2011 but we are only at the beginning of this global standardisation process. A strong foundation has been built with the launch of the CRM in 2020 and significant progress has been made over the last 2 years with over 67% of the laboratories in target in the latest 2022 ICAR PT report, and over 45% of the SRM providers now being aligned with the CRM. We need to keep the momentum to reach our goal of achieving a still broader implementation, with ideally 80-100% of the laboratories within target, by identifying with the stakeholders the remaining technical, regulatory, economic, logistic and communication stumbling blocks. We will also need to involve more countries, especially in the Southern hemisphere to reach that goal.

Most of the payment and dairy herd improvement somatic cell analyses are performed on alternative instrumental methods (i.e. ISO 13366-2|IDF 148-2) calibrated against different SRM materials, over 50% not being traceable yet with the CRM. It seems that the most efficient way to accelerate a global SCC equivalence would be through the greater involvement of the SRM and instruments manufacturers as well as the central milk testing (CMT) laboratories. The AT S09 needs to keep working with the different stakeholders for further acceptance and proper implementation of the CRM worldwide.

In that frame, the requirement of the use of the CRM in the revision of the ISO 13366-2|IDF 148-2standard is an important step as well as the improvement of the reference method with the introduction of the MIAMi and ANTOINETTE projects in the ISO 13366-1|IDF 148-1standards. These revisions will help us reaching our goal faster. In the short run, AT S09 will also continue to be a platform for communication of the achievements obtained within the ANTOINETTE and MIAMi projects.

The global SCC equivalence is now within reach with the help of all the stakeholders. Looking forward to the 2023 ICAR PT results which will hopefully bring us even closer to our goal. Thank you to all the Project Group members for their work and support.

Members of the joint IDF/ICAR Action Team S09:

Börkur Arnvidarson (DK), Berte Asmussen (DK), Dave Barbano (US), Ben Bartlett (UK), Christian Baumgartner (DE), Thomas Berger (CH), Pierre Broutin (FR, co-project leader), Hendrik de Vries (NL), Charlotte Egger (CH), Marina Gips (IL), Steve Holmes (US), Jan-Erik Ingenhoff (CH), Paul Jamieson (NZ), Adelheid Joris (BE), Véronique Ninane (BE), Silvia Orlandini (IT), Jacqueline Page (US), Anne Pécou (FR), Looknauth Ramsahoi (CA), Dalia Riaukiene (LT), Paul Sauvé (CA), Daniel Schwarz (DK, co-project leader), Stewart Stockdale (UK), Wendy Warren (US), Philippe Trossat (FR) , Harrie van den Bijgaart (NL).

IDF (International Dairy Federation)

Helping nourish the world with safe and sustainable dairy

The IDF is the leading source of scientific and technical expertise for all stakeholders of the dairy chain. Since 1903, IDF has provided a mechanism for the dairy sector to reach global consensus on how to help feed the world with safe and sustainable dairy products. A recognized international authority in the development of science-based standards for the dairy sector, IDF has an important role to play in ensuring the right policies, standards, practices and regulations are in place to ensure the world's dairy products are safe and sustainable.

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ICAR (International Committee for Animal Recording)

ICAR is the recognised global standard for livestock recording. Since its inception in 1951 ICAR has promoted the development and improvement of animal identification, performance recording and evaluation in farm animal production. This is achieved through the establishment of guidelines and standards, specific for the purpose of identifying animals, the registration of their parentage, recording their performance and evaluating their genetics, (including their bearing on animal health, care, productivity, food safety and the environment). Through its global network of some 170 professionals in ICAR's 14 sub committees and working groups, these guidelines are published and maintained for all on www.icar.org.

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