

# Guidance on application of EC JRC Certified Reference Material for somatic cell counting in milk



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Harrie van den Bijgaart, Qlip B.V., The Netherlands, [bijgaart@qlip.nl](mailto:bijgaart@qlip.nl)

Silvia Orlandini, International Committee on Animal Recording, The Netherlands,  
[silvia@icar.org](mailto:silvia@icar.org)

Werner Luginbühl, ChemStat, Switzerland, [info@chemstat.ch](mailto:info@chemstat.ch)

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On behalf of IDF and ICAR, the authors Harrie van den Bijgaart, Silvia Orlandini and Werner Luginbühl, as well as all contributors, are warmly thanked for a key step in reaching equivalence in somatic cell counting. The organisations also wish to thank the European Commission Joint Research Centre, and more specifically Dr Reinhard Zeleny, for making this step a reality.

## **Guidance on application of EC JRC Certified Reference Material for somatic cell counting in milk**

### **ABSTRACT**

Milk somatic cell count is a widely used indicator for monitoring the udder health of several mammalian species and is relevant in food hygiene regulations, milk payment testing, farm management and breeding programmes. In 2020, the EC Joint Research Centre launched new certified reference materials for somatic cell counting in milk.

This paper provides relevant guidance on how to apply this material for method performance verification of both the microscopic reference method and routine methods, for verification and possibly required adjustment of calibration settings of routine methods, for assigning reference values to secondary reference materials and for use in proficiency testing.

Application of this new certified reference material for calibration of routine methods might come with a significant change in calibration settings. In such cases, it is advised that laboratories consult the relevant regulatory and supervising bodies and other local stakeholders in order to arrange for an optimal transition.

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## JOINT IDF/ICAR FOREWORD

Milk somatic cell count is a widely used indicator for monitoring the udder health of several mammalian species and is relevant in food hygiene regulations, milk payment testing, farm management and breeding programmes. Since 2008, IDF and the International Committee for Animal Recording (ICAR) have closely collaborated in a joint project to develop solutions and tools to promote a better global equivalence in somatic cell counting in milk. As part of this project, IDF and ICAR approached and involved the European Commission Joint Research Centre (JRC) to develop a certified reference material for somatic cell counting in milk, which was launched in February 2020.

This IDF Bulletin provides relevant guidance on how to apply this material for method performance verification of both the microscopic reference method and routine methods, for verification and possibly required adjustment of calibration settings of routine methods, for assigning reference values to secondary reference materials, and for use in proficiency testing.

We hope this Bulletin will encourage all stakeholders to use this reference material, and in so doing, promote a better equivalence of somatic cell count results.

Caroline Emond, IDF Director General and Martin Burke, ICAR CEO

## BACKGROUND

Milk somatic cell count (SCC) is a widely used indicator for monitoring the udder health of several mammalian species and is relevant in food hygiene regulations, milk payment testing, farm management and breeding programmes [3]. In February 2020, the European Commission Joint Research Centre (EC JRC) launched a new certified reference material (CRM ERM<sup>®</sup>-BD001) for somatic cell counting in milk. The launch is one of the tangible outcomes of a close cooperation between the International Dairy Federation (IDF), the International Committee for Animal Recording (ICAR) and EC JRC in developing solutions and tools to promote a better global equivalence in somatic cell counting in milk.

## DESCRIPTION OF THE EC JRC CERTIFIED REFERENCE MATERIAL

Sets of CRM ERM<sup>®</sup>-BD001 are available from EC JRC and its authorised distributors and they are delivered in sachets, see <https://crm.jrc.ec.europa.eu/p/ERM-BD001>. Each sachet contains two bottles with 14g spray-dried cow milk each in an inert gas atmosphere (argon), ERM<sup>®</sup>-BD001a with a low SCC and ERM<sup>®</sup>-BD001b with a high SCC. Following accompanying instruction protocols for reconstitution in double-distilled or ultrapure/type 1 quality water of 40 °C, the resulting samples will contain about 60 000 and 1 200 000 cells/mL respectively. The stated certified reference values for the resulting two liquid samples are based on direct microscopic counting according to ISO 13366-1|IDF 148-1 [6] or Chapter 10 in the Standard Methods for the Examination of Dairy Products [1] and fluoro-opto-electronic counting according to ISO 13366-2|IDF 148-2 [7] or Chapter 11.032 in the Standard Methods for the Examination of Dairy Products [1]. For the first batch, a total of thirty two laboratories around the globe participated in the characterisation exercise. The stated certified reference values are to be considered as robust estimates of the true SCC values of these materials.

Upon reconstitution, the samples can be used as such, but the two reconstituted samples can also be mixed in different ratios to arrive at samples with values that lie between the SCC values stated for the two reconstituted samples. More background on the materials and their characterisation can be found in the certification report [2], which is available through the website mentioned above.

# APPLICATION OF THE EC JRC CERTIFIED REFERENCE MATERIAL

CRM ERM®-BD001 can be used for multiple purposes:

## 1. Method performance verification

The CRM ERM®-BD001 materials and their certified reference values, with stated uncertainty information, can be used by both reference method users and routine method users to verify whether their method operates correctly. With method performance verification of the reference method, the stated certified reference values and related uncertainty information based on the accepted reference method data sets are to be used. These stated values are based solely on the accepted results from ISO 13366-1|IDF 148-1 [6] compliant measurements. With method performance verification of a routine method, the recommendation is to work with the values based on the 50/50 merged data pool stemming from the reference method data sets and the randomly selected routine method data sets. These assigned values come with a lower uncertainty.

Before verifying the performance of a method with CRM ERM®-BD001 materials, it should be assessed that the method is properly functioning. For the reference method, this means fulfilling the requirements with regard to the correct dimensions of the microscope field and the repeatability. For the common applied routine method with fluoro-opto-electronic counting, this means fulfilling requirements with regard to blank checks, carry-over, linearity effect, other method-specific critical aspects and repeatability. For guidance, see ISO 13366-2|IDF 148-2 [7].

For verifying method performance, the procedure according to the certification report [2] can be applied:

- Measure each sample in at least duplicate and calculate the mean measured value,  $\bar{y}_i$ .
- Calculate for each sample the absolute difference,  $\Delta_{i,meas}$  between  $\bar{y}_i$  and the certified value,  $x_{i,CRM}$ :

$$\Delta_{i,meas} = |\bar{y}_i - x_{i,CRM}| \quad (1)$$

- Combine the measurement uncertainty,  $u_{i,meas}$  of  $\bar{y}_i$  with the uncertainty of the certified value,  $u_{i,CRM}$  on the certificate:

$$u_{i,\Delta} = \sqrt{u_{i,meas}^2 + u_{i,CRM}^2} \quad (2)$$

- Calculate the expanded uncertainty,  $U_{i,\Delta}$  from the combined uncertainty,  $u_{i,\Delta}$  using an appropriate coverage factor, corresponding to a level of confidence of approximately 95%:

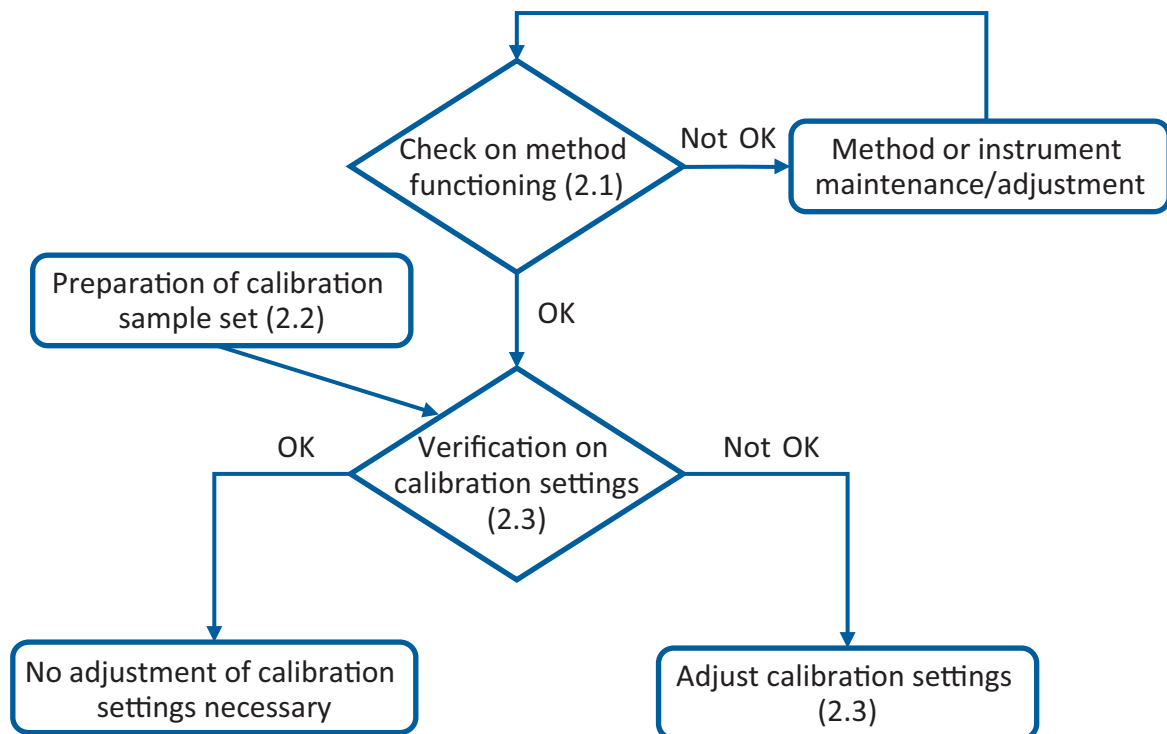
$$U_{i,\Delta} = 2 \cdot u_{i,\Delta} \quad (3)$$

- e. If  $\Delta_{i,meas} \leq U_{i,\Delta}$  then no significant difference exists between the measurement result and the certified value at a confidence level of approximately 95%.

## 2. Verification/adjustment of calibration settings with routine methods

Large scale somatic cell counting relies on the application of routine methods, such as fluoro-opto-electronic counting. Results with these methods are subject to variation between different methods, between different laboratories, between different instruments, and in time. Therefore, it is relevant to have routine methods properly calibrated against a reference and to frequently verify whether the calibration settings are still correct. Where results of the direct microscopic reference method for somatic cell counting come with limited precision, a calibration sample set prepared from CRM ERM<sup>®</sup>-DB001 materials with stated reference values may serve as a stable and robust alternative.

The advice is to follow a multi-step procedure according to Figure 1.



**Figure 1:** Flow chart showing verification of calibration settings of routine methods.

### 2.1. Check on proper functioning of the routine method

Before verifying the calibration settings of a routine method, it should be assessed that the routine method is properly functioning and fulfilling the requirements with regard to blank checks, carry-over effect, other method-specific critical aspects and repeatability. For further guidance on these checks with fluoro-opto-electronic counters, see ISO 13366-2|IDF 148-2 [7].

## 2.2. Preparation of a sample set for verification/adjustment of the calibration settings

A sample set for the verification/adjustment of the calibration settings of a routine method can be prepared by mixing reconstituted sample material from ERM<sup>®</sup>-BD001a and ERM<sup>®</sup>-BD001b in different ratios according to the instruction protocols provided with the materials. This will result in a sample set with at least five cell count levels with an equidistant distribution from the certified reference value of ERM<sup>®</sup>-BD001a up to the certified reference value of ERM<sup>®</sup>-BD001b. The recommendation to use the stated reference values based on the 50/50 merged data pool stemming from the reference method data sets and the randomly selected routine method data sets [2] is in accordance with the guidance in ISO 13366-2|IDF 148-2 [7] on the use of suitable calibration materials. The reference value for each sample can be calculated from the relative amount of ERM<sup>®</sup>-BD001a and ERM<sup>®</sup>-BD001b in each sample. The resulting sample set allows the verification and, if necessary, the adjustment of the calibration settings in accordance with the recommendations of ISO 13366-2|IDF 148-2 [7] and ISO 8196-2|IDF 128-2 [5], see next paragraph.

## 2.3. Verification/adjustment of calibration settings

### NOTE 1

The approach presented in this paragraph is based on an ordinary least square regression model with the underlying prerequisite of an approximate constancy in the distribution of the residuals throughout the calibration range. Otherwise, transform the data so that the residual variance throughout the range is equalized. From experience, a square root transformation could be a good option for this.

### NOTE 2

When working with certified reference materials, it is assumed that the error in the calculated reference values for the samples in the calibration sample set is negligible. Therefore, the calculated reference values are to be plotted on the x-axis and the mean of the routine method values on the y-axis. It is noted that this is contrary to the situation in routine testing, where the true value is estimated from a routine method measurement through applying slope and intercept settings:

$$x_{est} = slope * y + intercept \quad (4)$$

with

$x_{est}$  = estimate of the true value

$y$  = instrument read out

- For the verification of slope and intercept settings of a routine method, the calibration sample set is to be measured with the current settings of slope and intercept.
- Measure each sample of the calibration sample set with the routine method in at least duplicate. Calculate the mean value,  $\bar{y}_i$ , for each sample.

- c. Plot the calculated reference values,  $x_i$ , and the individual mean values obtained with the routine method,  $\bar{y}_i$ , of the  $q$  samples in an XY-axis diagram. Check the distribution of the data points, which should appear linear, regular and homogeneous. If one or more data points deviate considerably from the linear tendency, check the preparation of the sample set, the calculation of the reference values and the functioning of the routine method. If necessary, repeat the measurements.
- d. For the collected values with the calibration sample set, calculate the regression equation,  $\bar{y}_i = b \cdot x_i + a$ , using the ordinary least squares method.

- e. Calculate the residual standard deviation from the regression,  $s_{yx}$ :

$$s_{yx} = \sqrt{\frac{\sum_{i=1}^q (\bar{y}_i - a - b \cdot x_i)^2}{q-2}} \quad (5)$$

- f. Collect or calculate the values for  $b_c$  and  $a_c$ , corresponding with the current settings for slope and intercept:

$$b_c = 1/\text{slope}_c \quad (6)$$

and

$$a_c = -\text{intercept}_c/\text{slope}_c \quad (7)$$

with

$\text{slope}_c$  = current setting of the slope

$\text{intercept}_c$  = current setting of the intercept

- g. Test whether the calculated value for  $b$  differs statistically significant from the value of  $b_c$ .

For that, calculate the standard deviation of  $b$  :

$$s_b = \left( \frac{s_{yx}^2}{SS_x} \right)^{1/2} \quad (8)$$

with

$$SS_x = \sum_{i=1}^q (x_i - \bar{x})^2 \quad (9)$$

Applying a 95% confidence interval, the current slope is still correct if:

$$b - t_{0,975} \cdot s_b \leq b_c \leq b + t_{0,975} \cdot s_b \quad (10)$$

where  $t_{0,975}$  is the 0,975 quantile of the Student distribution with  $q - 2$  degrees of freedom.

- h. Test whether the mean bias  $|\bar{x} - \bar{y}(\bar{x})|$  differs statistically significant from the value of  $a$  with the current calibration,  $a_c$ .

For that, calculate the standard error of the regression equation:

$$s_{\bar{y}\bar{x}} = \frac{s_{yx}}{\sqrt{q}} \quad (11)$$

where  $q$  is the number of calibration samples.

The mean bias is still correct if in agreement with:

$$|\bar{x} - \bar{y}(\bar{x})| - t_{0,975} \cdot s_{\bar{y}\bar{x}} \leq a_c \leq |\bar{x} - \bar{y}(\bar{x})| + t_{0,975} \cdot s_{\bar{y}\bar{x}} \quad (12)$$

where  $t_{0,975}$  is the 0,975 quantile of the Student distribution with  $q - 2$  degrees of freedom and  $\bar{y}(\bar{x})$  being the predicted value from  $\bar{x}$  by  $\bar{y}(\bar{x}) = b \cdot \bar{x} + a$ .

If both the test for slope and mean bias are negative,  $a$  does not statistically differ from  $a_c$  if

$$a - t_{0,975} \cdot s_a \leq a_c \leq a + t_{0,975} \cdot s_a \quad (13)$$

with

$$s_a = s_{yx} \left( \frac{1}{q} + \frac{\bar{x}^2}{SS_x} \right)^{1/2} \quad (14)$$

For more background information on this verification procedure, see ISO 8196-2|IDF 128-2 [5].

- i Adjustment of the calibration is necessary if one of the tests under g. or h. is negative, that is, if one of the requirements is not fulfilled. In such a case, the resulting values for the new slope,  $slope_n$ , and new intercept,  $intercept_n$  are:

$$slope_n = 1/b \quad (15)$$

and

$$intercept_n = -a/b \quad (16)$$

Record the calculated values for  $a$ ,  $b$ ,  $slope_n$  and  $intercept_n$  for future verification.

For commonly applied fluoro-opto-electronic methods according to ISO 13366-2|IDF 148-2 [7], expect slope values of  $1.00 \pm 0.10$  and intercept values of  $0 \pm 50\,000$  cells/mL in case of ordinary least square regression with untransformed data.

### 3. Assigning reference values to Secondary Reference Material (SRM)

In many situations, so-called SRMs could provide more practical and/or more economical means for a traceable anchoring of routine test results. SRMs can be used for calibration purposes or as pilot samples between measurements. Proper reference values can be assigned to each SRM by applying a comparative approach in SRM characterization [8].

For this, a similar SRM with about an equal SCC is to be analysed simultaneously with a CRM using a well-functioning routine method under constant conditions. The SCC of the CRM material can be tuned by mixing ERM®-BD001a and ERM®-BD001b in an appropriate ratio and calculating the corresponding reference value, see paragraph 2.2.

The uncertainty of the mixed CRM material,  $u_{mixed\ CRM}$ , can be calculated from:

$$u_{mixed\ CRM} = \sqrt{u_{CRM\ BD001a}^2 * f_{CRM\ BD001a}^2 + u_{CRM\ BD001b}^2 * f_{CRM\ BD001b}^2} \quad (17)$$

with

$u_{CRM\ BD001a}$  = uncertainty with the certified value for CRM ERM®-BD001a

$u_{CRM\ BD001b}$  = uncertainty with the certified value for CRM ERM®-BD001b

$f_{CRM\ BD001a}$  = volume fraction of CRM ERM®-BD001a in the mixed sample

$f_{CRM\ BD001b}$  = volume fraction of CRM ERM®-BD001b in the mixed sample

a. Prepare an amount of (mixed) CRM and SRM that allows for at least 15 ( $= n$ ) replicate pairwise measurements with the same instrument in the same laboratory, directly after one another, providing paired values of  $C_{i,CRM}$  and  $C_{i,SRM}$  coming from two subsequent measurements.

b. Calculate for each pairwise measurement the difference  $E_i$ , between the two results:

$$E_i = C_{i,SRM} - C_{i,CRM} \quad (18)$$

c. Assuming additivity of bias for about equal SCC values, the reference value for the SRM,  $x_{SRM}$ , can now be calculated:

$$x_{SRM} = C_{CRM} + E_{avg} \quad (19)$$

with

$$C_{CRM} \text{ is the reference value of the (mixed) CRM and } E_{avg} = \sum_{i=1}^n E_i / n. \quad (20)$$

d. The standard uncertainty with the reference value for the SRM,  $u_{SRM}$ , is:

$$u_{SRM} = \sqrt{u_{CRM}^2 + u_{E_{avg}}^2} \quad (21)$$

with  $u_{CRM}$  being the standard uncertainty of the value carried by the CRM and

$$u_{E_{avg}}^2 = \sum_{i=1}^n (E_i - E_{avg})^2 / ((n - 1) \cdot n) \quad (22)$$

e. The expanded uncertainty,  $U_{SRM}$ , amounts to:

$$U_{SRM} = 2u_{SRM} \quad (23)$$

#### 4. Use in proficiency testing

CRM ERM<sup>®</sup>-BD001 is stable and comes with certified reference values. It can therefore serve as a robust anchor sample when included in sample sets for proficiency testing. As an alternative, one or more SRMs with assigned reference values according to the earlier described procedure according to Kuselman et al. (2002) [8] can be included for this purpose.

##### NOTE 3

An Excel calculation file that easily performs the above described calculations for the verification of method performance, the verification of the calibrations settings of a routine method and the assignment of reference values to SRMs can be downloaded at <https://www.icar.org/Excel-templates-with-guidance-on-use-ECJRCCRMSCC.xlsx>.

This Excel calculation file thereby provides the means to combine the verification of method performance, the linearity and the calibration settings in one procedure. The only extra prerequisite is that each sample of the sample set for verifying the calibrations settings is measured in at least 15 replicates.

## TRANSITION WITH CALIBRATION SETTINGS OF ROUTINE METHODS

Applying CRM ERM®-BD001 for calibration of routine methods means a change in the anchoring of somatic cell counting in milk. This might come with a significant change in calibration settings with routine methods and, as a consequence, in routine measurement results. The extent of this will differ between laboratories and geographies, depending on anchoring systems that have been applied thus far. It is to be noted that in case of an expected bigger shift, regulatory limits, limit values in milk payment systems and/or in udder health monitoring programmes might also need reassessment. It is therefore advised that laboratories, which notice a large shift in counting level when switching to the use of CRM ERM®-BD001, contact the relevant regulatory and supervising bodies and other stakeholders in order to arrange for an optimal transition.

Aspects for consideration amongst others might be:

- Extent of the shift in the counting level when applying CRM ERM®-BD001 materials with their stated certified reference values.
- Involvement of somatic cell counting entities (laboratories, veterinarians, farmers) in the concerned geography and need for alignment in transition.
- Involvement of regulatory and supervising bodies. Need and possibilities for accompanying reassessment of regulatory limits, limit values in milk payment and/or udder health programmes.
- Consequences for the performance of laboratories in proficiency testing. What anchoring system do other participating laboratories apply? Are changes in proficiency testing and the assessment of performance therein necessary?
- Consequences for the laboratory protocols.
- Timing of the transition. When is the transition best made? Will it be done in one step or in multiple steps?
- Communication to relevant stakeholders. What? To whom?

As situations will considerably differ, tailored approaches are to be developed locally.

If the choice is made for a transition in multiple steps, the local counting level will be gradually adapted to a counting level that fully coincides with the stated reference values of CRM ERM®-BD001. It is expected that, when applicable, a transition in two or three steps will suffice.

A pragmatic procedure in making these steps is under closely controlled and properly safeguarded conditions to measure the reconstituted CRM ERM®-BD001 materials under the current calibration settings in plural and, after removal of possible outlying

results, to calculate the mean value for both samples ERM<sup>®</sup>-BD001a and ERM<sup>®</sup>-BD001b, respectively  $\bar{x}_{a,local}$  and  $\bar{x}_{b,local}$ . From these values and the stated reference values with CRM ERM<sup>®</sup>-BD001,  $x_{a,CRM}$  and  $x_{b,CRM}$ , temporary local reference values,  $x_{a,CRM\ intermediate}$  and  $x_{b,CRM\ intermediate}$  can be calculated and assigned to the two reconstituted reference materials.

Example: In case of a two-step transition, intermediate local reference values can be set at:

$$x_{a,CRM\ intermediate} = (\bar{x}_{a,local} + x_{a,CRM})/2 \text{ and } x_{b,CRM\ intermediate} = (\bar{x}_{b,local} + x_{b,CRM})/2 \quad (24)$$

Such materials with intermediate locally assigned reference values can during a transition phase be used according to referenced international standards and the guidance in this document, thereby noting the intermediate status of these assigned reference values and avoiding any suggestion that resulting counting levels in routine will be traceable to CRM ERM<sup>®</sup>-BD001.

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INTERNATIONAL COMMITTEE FOR ANIMAL RECORDING

Arthur van Schendelstraat 650, 3511 MJ Utrecht, The Netherlands - <https://www.icar.org>

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