# CERTIFICATION REPORT

# The certification of the concentration of somatic cells (somatic cell count, SCC) in cow's milk: ERM®-BD001

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European Commission
Joint Research Centre
Directorate F – Health, Consumers and Reference Materials

Contact information
Reference materials sales
Address: Retieseweg 111, 2440 Geel, Belgium
E-mail: jrc-rm-distribution@ec.europa.eu
Tel.: +32 (0)14 571 705

JRC Science Hub
*https://ec.europa.eu/jrc*



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Abstract

This report describes the production of ERM-BD001, which is a set of two milk powder materials certified for their concentration of somatic cells. These materials were produced following ISO 17034:2016 and were certified in accordance with ISO Guide 35:2006.
Raw cow's milk was skimmed and cells enriched by means of microfiltration. Thereafter, the milk was converted into milk powders using spray-drying. Two cell count concentration levels were produced. A tailor-made processing procedure was developed to minimise the alteration of the cells during processing. The resulting milk powders were bottled, and packaged in sets (one set consisting of one bottle with low count and one bottle with high count material; ERM-BD001a and ERM-BD001b, respectively).
Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006.
The material was characterised by an inter-laboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025:2017. Technically invalid results were removed but no outlier was eliminated on statistical grounds only.
Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties related to possible inhomogeneity, instability and characterisation.
The materials are intended for the calibration of methods for enumeration of somatic cells using fluoro-opto-electronic cell counters according to ISO 13366-2:2006 and for the assessment of the method performance of somatic cell count (SCC) reference methods based on microscopy according to ISO 13366-1:2008 and routine methods operating fluoro-opto-electronic cell counters according to ISO 13366-2:2006. As with any reference material, the materials can be used for establishing control charts or validation studies. The CRMs are available in 14 g milk powder portions in glass bottles that were filled and sealed under an atmosphere of argon. The minimum amount of sample to be used is 3 g.

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*R. Zeleny, K. Busschots, J. Charoud-Got, H. Schimmel*

European Commission, Joint Research Centre
Directorate F – Health, Consumers and Reference Materials
Geel, Belgium

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Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

# Summary

This report describes the production of ERM-BD001, which is a set of two milk powder materials certified for their concentration of somatic cells. These materials were produced following ISO 17034:2016 [1] and were certified in accordance with ISO Guide 35:2006 [2].

Raw cow's milk was skimmed and cells enriched by means of microfiltration. Thereafter, the milk was converted into milk powders using spray-drying. Two cell count concentration levels were produced. A tailor-made processing procedure was developed to minimise the alteration of the cells during processing. The resulting milk powders were bottled, and packaged in sets (one set consisting of one bottle with low count and one bottle with high count material; ERM-BD001a and ERM-BD001b, respectively).

Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 [2].

The material was characterised by an inter-laboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025:2017 [3]. Technically invalid results were removed but no outlier was eliminated on statistical grounds only.

Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties related to possible inhomogeneity, instability and characterisation.

The materials are intended for the calibration of methods for enumeration of somatic cells using fluoro-opto-electronic cell counters according to ISO 13366-2:2006 [5] and for the assessment of the method performance of somatic cell count (SCC) reference methods based on microscopy according to ISO 13366-1:2008 [6] and routine methods operating fluoro-opto-electronic cell counters according to ISO 13366-2:2006 [5]. As with any reference material, the materials can be used for establishing control charts or validation studies. The CRMs are available in 14 g milk powder portions in glass bottles that were filled and sealed under an atmosphere of argon. The minimum amount of sample to be used is 3 g.

The following values were assigned for ERM-BD001a (low SCC):

| Cell concentration | | |
|---|---|---|
| | Certified value [3] [cells/mL] | Uncertainty [4] [cells/mL] |
| Somatic cell count (SCC) [1] | 64000 | 8000 |
| Somatic cell count (SCC) [2] | 62000 | 6000 |

[1] As defined in ISO 13366-1. The certified value is the mean value of 14 accepted data sets obtained from ISO 13366-1-compliant measurements.

[2] As defined in ISO 13366-1 and ISO 13366-2. The certified value is the mean value of 14 accepted data sets obtained from ISO 13366-1-compliant measurements and 14 randomly selected data sets out of 32 accepted data sets obtained from ISO 13366-2-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.

The following values were assigned for ERM-BD001b (high SCC):

| Cell concentration | | |
|---|---|---|
| | Certified value [3] [cells/mL] | Uncertainty [4] [cells/mL] |
| Somatic cell count (SCC) [1] | 1202000 | 121000 |
| Somatic cell count (SCC) [2] | 1166000 | 79000 |

[1] As defined in ISO 13366-1. The certified value is the mean value of 13 accepted data sets obtained from ISO 13366-1-compliant measurements.

[2] As defined in ISO 13366-1 and ISO 13366-2. The certified value is the mean value of 13 accepted data sets obtained from ISO 13366-1-compliant measurements and 13 randomly selected data sets out of 32 accepted data sets obtained from ISO 13366-2-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.

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# Glossary

| | |
|---|---|
| ANOVA | Analysis of variance |
| CRM | Certified reference material |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DMSCC | Direct microscopic somatic cell count |
| DNA | Deoxyribonucleic acid |
| EC | European Commission |
| EN | European norm (standard) |
| ERM® | Trademark of European Reference Materials |
| EURL | European Union Reference Laboratory |
| EtBr | Ethidium bromide |
| GUM | Guide to the Expression of Uncertainty in Measurement [ISO/IEC Guide 98-3:2008] |
| ICAR | International Committee for Animal Recording |
| IDF | International Dairy Federation |
| IEC | International Electrotechnical Commission |
| ISO | International Organization for Standardization |
| JRC | Joint Research Centre of the European Commission |
| $k$ | Coverage factor |
| $MS_{between}$ | Mean of squares between-unit from an ANOVA |
| $MS_{within}$ | Mean of squares within-unit from an ANOVA |
| $n$ | Number of replicates per unit |
| $N$ | Number of samples (units) analysed |
| PT | Proficiency testing |
| QC | Quality control |
| QCM | Quality control material |
| RM | Reference material |
| $s$ | Standard deviation |
| $s_{bb}$ | Between-unit standard deviation; an additional index "rel" is added when appropriate |
| $s_{between}$ | Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate |
| SCC | Somatic cell count |
| SI | International System of Units |
| $s_{within}$ | Standard deviation within groups as obtained from ANOVA; an additional index "rel" is added as appropriate |
| $s_{wb}$ | Within-unit standard deviation |

| | |
|---|---|
| T | Temperature |
| $t$ | Time |
| $t_i$ | Time point for each replicate |
| $t_{sl}$ | Proposed shelf life |
| $t_{tt}$ | Proposed transport time |
| $u$ | standard uncertainty |
| $U$ | expanded uncertainty |
| $u^*_{bb}$ | Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by method repeatability; an additional index "rel" is added as appropriate |
| $u_{bb}$ | Standard uncertainty related to a possible between-unit inhomogeneity; an additional index "rel" is added as appropriate |
| $u_{char}$ | Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate |
| $u_{CRM}$ | Combined standard uncertainty of the certified value; an additional index "rel" is added as appropriate |
| $U_{CRM}$ | Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate |
| $u_{\Delta}$ | Combined standard uncertainty of measurement result and certified value |
| $U_{\Delta}$ | Combined expanded uncertainty of measurement result and certified value |
| UHT | Ultra-high temperature processing |
| $u_{lts}$ | Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate |
| $u_{sts}$ | Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate |
| V-KFT | Volumetric Karl Fischer titration |
| $\alpha$ | significance level |
| $\Delta_{meas}$ | Absolute difference between mean measured value and the certified value |
| $\nu_{MSwithin}$ | Degrees of freedom of $MS_{within}$ |

# 1 Introduction

## 1.1 Background

Somatic cells are white blood cells naturally occurring in raw cow's milk and are defined as all cells with nuclei, i.e. all leucocytes (lymphocytes, macrophages, and polymorphonuclear neutrophils) and epithelial cells [7,8].

Somatic cell count (SCC) is an important parameter for hygienic quality of milk and for animal health. An elevated SCC level in milk can serve as an indicator for udder infection (mastitis) of lactating cows and thus points at insufficient hygiene practices on farms. SCC is used for various purposes such as milk payment, checking compliance with regulations, and milk recording for genetic evaluation and farm management. A SCC < 400 000 cells/mL for raw cow's milk has been defined as a quality criterion in Council Directive 92/46/EEC [9] laying down the health rules for production and placing on the market of raw milk, heated milk and milk-based products, and in Regulation (EC) 853/2004 of the European Parliament and of the Council [10], laying down specific hygiene rules for food of animal origin.

Decisions and respective measures are relying on accurate measurements. At present, a reference method based on microscopy [ISO 13366-1] and a routine method operating fluoro-optic electronic counters (in most cases flow cytometers) [ISO 13366-2] are prescribed. Moreover, laboratories operating those methods participate in proficiency testing (PT) studies in order to establish equivalence of analytical results and employ in-house and other available reference materials for calibration of their methods and/or for method performance verification. However, a certified reference material is lacking, which would also be an integral part of a reference system for SCC in milk. Such a CRM would help to establish analytical equivalence at a global scale by constituting an anchor point to which secondary reference materials could be linked. The outcome of a survey conducted by the former European Union Reference Laboratory (EURL) for Milk and Milk Products (activities discontinued in 2017) and the jointly launched project by the International Dairy Federation (IDF) and the International Committee for Animal Recording (ICAR) on a reference system for somatic cell counting in milk [11] indicated a world-wide interest to have such a CRM available.

## 1.2 Choice of the material

Raw cow's milk in bulk format (milk from different cows and farms) was chosen as raw material to prepare the CRM. The milk was then converted into milk powder by spray-drying. Various in-house reference materials and PT materials exist in the SCC testing community. However, these are mostly milks that are preserved using potassium dichromate, bronopol, or other adequate additives. The shelf life of such milk is limited (typically several weeks). Other formats are heat-treated milks with preservative or micro-filtrated milks with preservative which have a somewhat longer shelf-life (up to a few months). In addition, there is at least one example of a shock-frozen preserved milk with an indicated shelf life of 1 year, and one example of a preserved lyophilised milk RM with an indicated shelf life of 16 months. A suitably stabilised milk (spray-dried or freeze-dried upon process optimisation to minimize cell damage) would largely enhance the shelf life to several years (data obtained during the feasibility study part of the project). It was decided to prepare two materials, one with low SCC, the other one with high SCC. The purpose is that after reconstitution of the powders, calibration samples can be prepared which span the level of interest for measuring SCC in

cow's milk (0 – 1 million cells/mL [12]), thereby complying with the recommendations in ISO 13366-2 (at least 5 calibration levels over the range of interest).

## 1.3 Design of the CRM project

The project was designed, managed and developed at the European Commission, Joint Research Centre (EC-JRC), Directorate F – Health, Consumers and Reference Materials, in collaboration with and on behalf of the IDF/JRCV project group on a reference system for somatic cell counting in milk [11].

First, an initial feasibility study was performed to investigate how to manufacture a homogeneous, stable and commutable reference material in the large scale. A suitable freeze-dried material can be produced in a smaller scale. For a large scale production however and to avoid the production of sub-batches, spray-drying at industrial scale is a better alternative. Therefore, the bulk candidate RMs were processed at a competent collaboration partner, whereas filling and labelling of the containers under optimised conditions was performed at JRC. For assessment of homogeneity and stability, an ISO/IEC 17025 accredited laboratory was tasked.

Characterisation was based on an inter-laboratory comparison involving a large number of expert laboratories in the field of somatic cell counting in milk. Selected laboratories that took part in the material certification campaign were in most cases ISO/IEC 17025 accredited for the particular applications. Two types of methods were used in the characterisation: the microscopy method defined in ISO 13366-1 (reference method), and the routine method, based on flow cytometry and using high-throughput instrumentation compliant with ISO 13366-2. The latter method is used on a routine basis by dairy analysis laboratories performing these analyses on milk samples (an estimated 500 million analyses are performed world-wide per year). The certified values for SCC in the two CRMs are traceable to the SI using both of these methods.

# 2 Participants

## 2.1 Project management and evaluation

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE
(accredited to ISO 17034:2016 for production of certified reference materials, BELAC No. 268-RM)

## 2.2 Processing

NIZO food research BV, Ede, NL
(ISO 9001 certification by RvA; certificate number 187255-2015-AQ-NLD-RvA)

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE
(accredited to ISO 17034:2016 for production of certified reference materials, BELAC No. 268-RM)

## 2.3 Homogeneity study

Melkcontrolecentrum Vlaanderen, Lier, BE
(measurements under the scope of ISO/IEC 17025 accreditation BELAC No. 096-TEST)

## 2.4 Stability study

Melkcontrolecentrum Vlaanderen, Lier, BE
(measurements under the scope of ISO/IEC 17025 accreditation BELAC No. 096-TEST)

## 2.5 Characterisation

Actalia Cecalait, Poligny, FR
(measurements under the scope of ISO/IEC 17025 accreditation by Cofrac; accreditation number 1-5577)

Agroscope, Bern, CH
(measurements under the scope of ISO/IEC 17025 accreditation by SAS; accreditation number STS 077)

Bentley Instruments SARL, Maroeuil, FR

Bulgarian Food Safety Agency, Food Safety and Quality Laboratory, Sofia, BG
(measurements under the scope of ISO/IEC 17025 accreditation by Bulgarian Accreditation Service; accreditation number A 385-2)

Israeli Cattle Breeders' Association, Central milk lab, Caesarea, IL
(measurements under the scope of ISO/IEC 17025 accreditation by ISRAC; accreditation number 164)

Centre Wallon de Recherches Agronomiques, Gembloux, BE
(measurements under the scope of ISO/IEC 17025 accreditation by BELAC; accreditation number 189-TEST)

Delta Instruments B.V., Drachten, NL

Dairy laboratory Ltd., Ulbroka, LV
(measurements under the scope of ISO/IEC 17025 accreditation by LATAK; accreditation number LATAK-T-283-12-2003)

Eastern Laboratory Services (ELS), Medina, US

FOSS Analytical A/S, Hillerød, DK

INTI Lácteos, Rafaela, AR
(measurements under the scope of ISO/IEC 17025 accreditation by OAA; accreditation number LE 075)

Institute of Food Safety, Animal Health and Environment BIOR, Riga, LV
(measurements under the scope of ISO/IEC 17025 accreditation by LATAK; accreditation number LATAK-T-012-29-95)

Istituto Zooprofilattico Sperimentale del Lazio e della Toscana, Roma, IT
(measurements under the scope of ISO/IEC 17025 accreditation by Accredia; accreditation number 0201)

Laboratoire interprofessionnel d'analyses laitieres du massif central, Aurillac, FR
(measurements under the scope of ISO/IEC 17025 accreditation by Cofrac; accreditation number 1-0196)

Laboratorio agroalimentario de Santander, Santander, ES
(measurements under the scope of ISO/IEC 17025 accreditation by ENAC; accreditation number 517/LE1040)

Max Rubner-Institut, Bundesforschungsinstitut für Ernährung und Lebensmittel, Institut für Sicherheit und Qualität bei Milch und Fisch, Kiel, DE
(measurements under the scope of ISO/IEC 17025 accreditation by DAkkS; accreditation number D-PL-18156-02-00)

Melkcontrolecentrum Vlaanderen, Lier, BE
(measurements under the scope of ISO/IEC 17025 accreditation by BELAC; accreditation number 096-TEST)

MilkTestNZ, Hamilton, NZ
(measurements under the scope of ISO/IEC 17025 accreditation by IANZ; accreditation number 1168)

National Milk Laboratories Ltd., Glasgow, GB
(measurements under the scope of ISO/IEC 17025 accreditation by UKAS; accreditation number 2051)

National Milk Laboratories Ltd., Wolverhampton, GB
(measurements under the scope of ISO/IEC 17025 accreditation by UKAS; accreditation number 2700)

National Veterinary Research Institute, Department of Hygiene of Food of Animal Origin, Puławy, PL
(measurements under the scope of ISO/IEC 17025 accreditation by PCA; accreditation number AB 485)

Qlip N.V., Zutphen, NL
(measurements under the scope of ISO/IEC 17025 accreditation by RvA; accreditation number L 099)

QSE GmbH (subsidiary of Milchprüfring Bayern e.V.), Wolnzach, DE

State Enterprise Pieno Tyrimai, Kaunas, LT
(measurements under the scope of ISO/IEC 17025 accreditation by Lithuanian National Accreditation Bureau; accreditation number LA.01.106)

State Veterinary and Food Institute, Bratislava, SK
(measurements under the scope of ISO/IEC 17025 accreditation by Slovak National Accreditation Service; accreditation number S-127)

United States Department of Agriculture, Agricultural Marketing Service (USDA-AMS), Dairy Programme - Southwest Federal Milk Marketing Order 126, Carrollton, US

United States Department of Agriculture, Agricultural Marketing Service (USDA-AMS), Dairy Programme - Central Federal Milk Marketing Order 32, Lenexa, US

United States Department of Agriculture, Agricultural Marketing Service (USDA-AMS), Dairy Programme - Appalachian Federal Milk Marketing Order 5, Louisville, US

University of Guelph, Lab Services Division, Agriculture and Food Laboratory, Guelph, CA
(measurements under the scope of ISO/IEC 17025 accreditation by Standards Council of Canada; accreditation number 100)

University of Zagreb, Faculty of Agriculture, Department of Dairy Science, Zagreb, HR
(measurements under the scope of ISO/IEC 17025 accreditation by Croatian Accreditation Agency; accreditation number 1081)

Valacta, Ste-Anne-de-Bellevue, CA
(measurements under the scope of ISO/IEC 17025 accreditation by Standards Council of Canada; accreditation number 99)

Veterinary Faculty – National Veterinary Institute, Naklo, SI
(measurements under the scope of ISO/IEC 17025 accreditation by Slovenska Akreditacija; accreditation number LP-021)

# 3 Material processing and process control

## 3.1 Origin of the starting material

Raw cow's milk (bulk) was used as raw material to produce the spray-dried milk powders. The raw milk was provided by Friesland Campina, Amersfoort (NL). Ca. 13 000 litres bulk milk for each reference material batch (low and high count material) were shipped to NIZO (Ede, NL) for processing. Friesland Campina confirmed that (i) the provided milks originated from Dutch dairy farms, (ii) were not older than 24 hours when arriving at NIZO, (iii) were kept cold (4 ± 2 ºC) since milking, (iv) fulfilled the hygiene requirements for human consumption according to Regulation (EC) 853/2004 of the Council and the European Parliament [10], and (v) had a SCC between 50 000 and 350 000 cells/mL.

One sub-sample per raw milk batch was analysed for various milk parameters in a laboratory operating its methods under ISO/IEC 17025 accreditation. The results are summarised below in Table 1.

**Table 1:** Analyses results of main milk parameters in the raw materials used to produce ERM-BD001a and ERM-BD001b

| Parameter | Method | Unit | Raw milk used for ERM-BD001a | Raw milk used for ERM-BD001b |
|---|---|---|---|---|
| Fat | ISO 1211[13] | mass % | 4.24 | 4.39 |
| Protein | ISO 8968-1/IDF20-1[14] | mass % | 3.65 | 3.70 |
| Lactose | in-house SOP (HPLC-RI) | mass % | 4.45 | 4.47 |
| Ash | In-house SOP (gravimetric determination) | mass % | 0.77 | 0.76 |
| Dry matter | ISO 6731/IDF 21[15] | mass % | 13.66 | 13.56 |
| Total plate count | ISO 4833-1[16] | cfu/mL | 5200 | 6800 |
| Somatic cell count | ISO 13366-2 [5] | cells/mL | 227 000 | 228 000 |
| Urea | ISO 14637/IDF 195 [17] | mg/100g | 27 | 21 |
| pH | In-house SOP conforming with ISO 10523 [18] | - | 6.77 | 6.77 |
| Antibiotic residue testing[1] | Delvotest® | I.U./mL | n.d.[2] | n.d.[2] |

[1] a broad-spectrum microbial inhibition test (available as kit from company DSM)

[2] not detected

## 3.2 Processing

Processing of raw cow's milk to bulk milk powder was performed at NIZO, Ede, NL. The main steps of processing are outlined below, however, due to confidentiality reasons, more details cannot be revealed in this report. The process was designed to achieve the desired quantity of material (50 kg bulk powder for both SCC levels) while minimising stress to the cells.

Low count milk powder:

The incoming milk was first pasteurised at 72 ºC for 14 s and stored at 5 ºC under stirring in a vessel. Thereafter, the milk was heated to 55 ºC by means of a plate heat exchanger and skimmed using a cream separator with a light phase separation bowl. Three phases were generated in the bowl: cream, skimmed milk and sludge. After removal of the cream, skimmed milk and sludge were combined, mixed and applied to a 5 μm ceramic microfiltration membrane, which separated the sample into retentate (containing the somatic cells) and cell-free permeate. This process was carried out under cooled condition (in-line tubular heat exchanger). Retentate and permeate were separately collected in cooled tanks and stirred. The two products were heated in a plate heat exchanger to 40 ºC and subsequently fed to a two passes falling film evaporator for concentration to about 27 % dry matter. The concentrates were put in a feed vessel of the spray-drying device and heated in plate heat exchanger to 55 ºC prior to be atomised. The obtained powders were collected in food-grade laminated bags and the SCC of both materials was determined. Finally, powders were mixed in a conical screw mixer to obtain a material with a target level of 55 000 cells/g milk. The resulting powder (50 kg) was sieved (0.5 mm) using a vibrating sieving/dosing system and collected in 10 kg quantities in paper-lined plastic bags. Upon finalisation of the process, the bulk material was shipped to JRC for filling into the final storage containers.

High count milk powder:

The incoming milk was first pasteurised at 72 ºC for 14 s and stored at 5 ºC under stirring in a vessel. Thereafter, the milk was heated to 55 ºC by means of a plate heat exchanger and skimmed using a cream separator with a light phase separation bowl. Three phases were generated in the bowl: cream, skimmed milk and sludge. After removal of the cream, skimmed milk and sludge were combined, mixed and applied to a 5 μm ceramic microfiltration membrane, which separated the sample into retentate (containing the somatic cells) and cell-free permeate. The retentate circulated in the system until sufficient enrichment of somatic cells was accomplished. This process was carried out under cooled condition (in-line tubular heat exchanger). Retentate and permeate were separately collected in cooled tanks and stirred. The two products were heated in a plate heat exchanger to 55 and 40 ºC, respectively, and subsequently fed to a four and two passes falling film evaporator to be concentrated to about 24 % and 27 % dry matter, respectively. The concentrates were put in a feed vessel of the spray-drying device and heated in plate heat exchanger to 55 ºC prior to be atomised. The obtained powders were collected in food-grade laminated bags and the SCC of both materials was determined. Finally, powders were mixed in a conical screw mixer as to obtain a material with a target level of 1 100 000 cells/g milk. The resulting powder (50 kg) was sieved (0.5 mm) using a vibrating sieving/dosing system and collected in 10 kg quantities in paper-lined plastic bags. Upon finalisation of the process, the bulk material was shipped to JRC for filling into the final storage containers.

For each bulk material (low and high count), the 10 kg quantities were combined and mixed for 1 hour in a three-dimensional mixer (Dynamix CM-200 WAB, Basel, CH) to ensure homogenization. Fourteen g portions were filled into 50 mL brown glass bottles under inert gas atmosphere in a glove box for optimal product protection during filling. Bottles were closed with a red screw cap with break-ring. One bottle of ERM-BD001a (low SCC) and

ERM-BD001b (high SCC) each were put in an aluminized sachet for double protection. Sachets were stored at -20 ºC.

### 3.3 Process control

The water content in the final materials was measured by volumetric Karl Fischer titration (vKFT) [19]. Ten units of the batch were chosen using a random stratified sample picking scheme and each sample unit was analysed in triplicate. The determined mean water content and its standard deviation was 3.52 ± 0.12 g/100 g for ERM-BD001a and 3.68 ± 0.13 g/100 g for ERM-BD001b.

## 4 Homogeneity

A key requirement for any reference material aliquoted into units is equivalence between those units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty of the certified value, but it is not relevant if this variation between units is significant compared to the analytical variation. Consequently, ISO 17034 [1] requires RM producers to quantify the between unit variation. This aspect is covered in between-unit homogeneity studies.

The within-unit inhomogeneity does not influence the uncertainty of the certified value when the minimum sample intake is respected, but determines the minimum size of an aliquot that is representative for the whole unit.

### 4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified values of the CRMs are valid for all units of the material, within the stated uncertainties.

The number of 30 units selected corresponds to approximately the cube root of the total number of units produced. The 30 units were selected using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. For this, the batch was divided into 30 groups (with a similar number of approximately 100 units) and one unit was selected randomly from each group. Two independent samples were taken from each selected unit, and analysed using the routine method in a laboratory operating this method under ISO/IEC 17025 accreditation. The measurements were performed under repeatability conditions, and in a randomised manner to be able to separate a potential analytical drift from a trend in the filling sequence. Each sample was injected twice, and the mean value of the two results constituted the result for each replicate. The randomized analysis order was prescribed by JRC and strictly followed by the laboratory performing the measurements. The results are shown as graphs in Annex C.

Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. No trends in the filling sequence or the analytical sequence were observed at a 95 % confidence level.

Quantification of between-unit inhomogeneity was undertaken by analysis of variance (ANOVA), which separates the between-unit variation ($s_{bb}$) from the within-unit variation ($s_{wb}$). The latter is equivalent to the method repeatability if the individual samples were representative for the whole unit.

Evaluation by ANOVA requires mean values per unit, which follow at least a unimodal distribution and results for each unit that follow unimodal distributions with approximately the same standard deviations. The distribution of the mean values per unit was visually tested

using histograms and normal probability plots. Minor deviations from unimodality of the individual values do not significantly affect the estimate of between-unit standard deviations. The results of all statistical evaluations are given in Table 2.

**Table 2:** Results of the statistical evaluation of the homogeneity studies

| SCC* | Trends** | | Outliers*** | | Distribution | |
|---|---|---|---|---|---|---|
| | Analytical sequence | Filling sequence | Individual results | Unit means | Individual results | Unit means |
| ERM-BD001a | no | no | none | none | normal | normal |
| ERM-BD001b | no | no | none | none | unimodal | unimodal |

*as defined in ISO 13366-2

** 95 % confidence level

*** 99 % confidence level

It should be noted that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and are therefore subject to random fluctuations. Therefore, the mean square between groups ($MS_{between}$) can be smaller than the mean squares within groups ($MS_{within}$), resulting in negative arguments under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be lower than zero. In this case, $u^*_{bb}$, the maximum inhomogeneity that could be hidden by method repeatability, was calculated as described by Linsinger *et al.* [20]. $u^*_{bb}$ is comparable to the LOD of an analytical method, yielding the maximum inhomogeneity that might be undetected by the given study setup.

Method repeatability ($s_{wb,rel}$), between–unit standard deviation ($s_{bb,rel}$) and $u^*_{bb,rel}$ were calculated as:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}} \qquad \textbf{Equation 1}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}} \qquad \textbf{Equation 2}$$

$$u^*_{bb,rel} = \frac{\sqrt{\frac{MS_{within}}{n}}\sqrt[4]{\frac{2}{v_{MSwithin}}}}{\bar{y}} \qquad \textbf{Equation 3}$$

$MS_{within}$ mean of squares within-unit from an ANOVA

$MS_{between}$ mean of squares between-unit from an ANOVA

$\bar{y}$ mean of all results of the homogeneity study

$n$ mean number of replicates per unit

$v_{MSwithin}$ degrees of freedom of $MS_{within}$

The results of the evaluation of the between-unit variation are summarised in Table 3. The resulting values from the above equations were converted into relative uncertainties.

**Table 3:** Results of the homogeneity studies

| SCC* | $s_{wb}$ | $s_{wb,rel}$ [%] | $s_{bb}$ | $s_{bb,rel}$ [%] | $u^*_{bb}$ | $u^*_{bb,rel}$ [%] | $u_{bb}$ | $u_{bb,rel}$ [%] |
|---|---|---|---|---|---|---|---|---|
| ERM-BD001a | 849.0 | 1.35 | 2081.0 | 3.32 | 2081.0 | 0.49 | 2081.0 | 3.32 |
| ERM-BD001b | 1260.0 | 0.11 | 27275.9 | 2.28 | 1260.0 | 0.04 | 27275.9 | 2.28 |

*as defined in ISO 13366-2

The homogeneity study showed no outlying unit means or trends in the filling sequence. Therefore the between-unit standard deviation can be used as estimate of $u_{bb}$. As $u^*_{bb}$ sets the limits of the study to detect inhomogeneity, the larger value of $s_{bb}$ and $u^*_{bb}$ is adopted as uncertainty contribution to account for potential inhomogeneity.

## 4.2 Within-unit homogeneity and minimum sample intake

The within-unit homogeneity is closely correlated to the minimum sample intake. The minimum sample intake is the minimum amount of sample that is representative for the whole unit and thus should be used in an analysis. Using sample sizes equal or above the minimum sample intake guarantees the certified value within its stated uncertainty.

The prescribed sample intake was 7 g milk powder (to be mixed with 64.6 g water) for the homogeneity study and 5 g milk powder (to be mixed with 46 g water) for the stability studies, respectively. In the characterisation study, 3 g (to be mixed with 27.66 g water) were prescribed as sample intake per replicate analysis. No dedicated study was performed to establish the minimum sample intake, as a larger amount will be reconstituted by the user laboratories in order to have sufficient milk available to prepare the different calibration samples. The minimum sample intake to be used is 3 g.

# 5 Stability

Time, temperature, light, and water content were regarded as the most relevant influences on the stability of the materials. The influence of visible light was minimised by storing the material in containers which reduces light exposure. In addition, materials are stored in the dark and dispatched in boxes, thus removing any possibility of degradation by light. The water content was adjusted to an optimum during processing. Therefore, only the influences of time and temperature needed to be investigated.

Stability testing is necessary to establish the conditions for storage (long-term stability) as well as the conditions for dispatch of the materials to the customers (short-term stability). During transport, especially in summer time, temperatures up to 60 °C can be reached and stability under these conditions must be demonstrated, if the samples are to be transported without any additional cooling.

The stability studies were carried out using an isochronous design [21]. In this approach, samples were stored for a particular length of time at different temperature conditions. Afterwards, the samples were moved to conditions where further degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples were analysed simultaneously under repeatability conditions. Analysis of the material (after various exposure times and temperatures) under repeatability conditions greatly improves the sensitivity of the stability tests.

## 5.1 Short-term stability study

For the short-term stability study, samples were stored at -20 °C, 4°C, 18 °C and 60 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set to -70 °C. Two units per storage time were selected using a random stratified sampling scheme. The milk powders were reconstituted with type 1 ultrapure water as prescribed by JRC. From each unit, three samples were analysed using the routine method in a laboratory operating this method under ISO/IEC 17025 accreditation. Each sample was injected twice, and the mean value of the two results constituted the result for each replicate. The measurements were performed under repeatability conditions, and a randomised sequence was used to differentiate any potential analytical drift from a trend over storage time. The results were reported as number of cells/mL in the reconstituted samples ("milk"), whereby rounding to the nearest thousand cells was applied.

The data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test on a confidence level of 99 %. No outlying individual results were found (Table 4: Results of the short-term stability tests).

In addition, the data were evaluated against storage time, and regression lines of cell concentration versus time were calculated, to test for potential increases/decrease of the measurand due to shipping conditions. The slopes of the regression lines were tested for statistical significance. The detailed results are shown in Annex D. The results of the statistical evaluation of the short-term stability are summarised in Table 4.

**Table 4:** Results of the short-term stability tests

| SCC* | Number of individual outlying results** | | | | Significance of the trend*** | | | |
|---|---|---|---|---|---|---|---|---|
| | -20 ºC | 4 ºC | 18 °C | 60 °C | -20 ºC | 4 ºC | 18°C | 60°C |
| ERM-BD001a | none | none | none | none | no | no | no | no |
| ERM-BD001b | none | none | none | none | yes | yes | no | no |

*as defined in ISO 13366-2

** 99 % confidence level

*** 95 % confidence level

A significant positive trend at -20 °C and 4 °C was found (ERM-BD001b), but the material appeared to be stable at 20 °C and 60 °C. This phenomenon cannot be technically explained; somatic cells cannot grow. Moreover, a possible matrix effect (degradation of the milk powder) can also be excluded (no effect for the two higher temperatures for this material and for all temperatures for the almost identical material ERM-BD001a). In fact, the effects were caused by somewhat higher measurement results for some of the 4 week samples (although those results are not detected as single or double Grubbs outliers). The replicate measurements of the three concerned sample units (488, 638, 2073) were randomly distributed in the analysis order, therefore any such effect stemming from the analysis (e.g. drift) can also be excluded. Consequently, the observed somewhat higher results for those samples and replicates have to be regarded as purely stochastic, but lead to a significant positive trend in two cases, which however can for the reasons explained above be regarded as a statistical artefact.

The material can be dispatched under ambient conditions.

## 5.2 Long-term stability study

For the long-term stability study, samples were stored at 4 °C and -20 °C for 0, 4, 8 and 12 months (at each temperature). The reference temperature was set to -70 °C. Three units per storage time were selected using a random stratified sampling scheme. The milk powders were reconstituted with type 1 ultrapure water as prescribed by JRC. From each unit, three samples were analysed using the routine method in a laboratory operating this method under ISO/IEC 17025 accreditation. Each sample was injected twice, and the mean value of the two results constituted the result for each replicate. The measurements were performed under repeatability conditions, in a random sequence to be able to separate any potential analytical drift from a trend over storage time. The results were reported as number of cells/mL in the reconstituted samples ("milk"), whereby rounding to the nearest thousand cells was applied.

The data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test at a confidence level of 99 %. No outliers were detected for any tested temperatures and materials.

In addition, the data were evaluated against storage time and linear regression lines of SCC versus time were calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to storage). No significant trend was detected for any of the tested temperatures and materials at a 95 % confidence level.

The detailed results of the long-term stability measurements are shown in Annex E. The results of the statistical evaluation of the long-term stability study are summarised in Table 5.

**Table 5:** Results of the long-term stability tests

| SCC* | Number of individual outlying results** | | Significance of the trend*** | |
|---|---|---|---|---|
| | -20 ºC | 4 ºC | -20 ºC | 4 ºC |
| ERM-BD001a | none | none | no | no |
| ERM-BD001b | none | none | no | no |

*as defined in ISO 13366-2

** 99 % confidence level

*** 95 % confidence level

No technically unexplained outliers were observed and none of the trends was statistically significant on a 99 % confidence level for any of the temperatures. The material can therefore be stored at -20 °C.

## 5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results, no study can entirely rule out degradation of materials, even in the absence of statistically significant trends. It is therefore necessary to quantify the potential degradation that could be hidden by the method repeatability, i.e. to estimate the uncertainty of stability. This means that, even under ideal conditions, the outcome of a stability study can only be that there is no detectable degradation within an uncertainty to be estimated.

The uncertainties of stability during dispatch and storage were estimated, as described in [22] for each analyte. In this approach, the uncertainty of the linear regression line with a slope of zero was calculated. The uncertainty contributions $u_{sts}$ and $u_{lts}$ were calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{sts,rel} = \frac{s_{rel}}{\sqrt{\sum\left(t_i - \bar{t}\right)^2}} \cdot t_{tt} \qquad \textbf{Equation 4}$$

$$u_{lts,rel} = \frac{s_{rel}}{\sqrt{\sum\left(t_i - \bar{t}\right)^2}} \cdot t_{sl} \qquad \textbf{Equation 5}$$

$s_{rel}$ relative standard deviation of all results of the stability study

$t_i$ time elapsed at time point *i*

$\bar{t}$ mean of all $t_i$

$t_{tt}$ chosen transport time (1 week at 60 ºC)

$t_{sl}$ chosen shelf life (12 months at -20 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the 60 °C studies. The uncertainty describes the possible change during a dispatch at 60 °C lasting for one week.

- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the -20 °C 1-year isochronous study. The uncertainty contribution describes the possible degradation during 12 months storage at -20 °C.

The results of these evaluations are summarised in Table 6.

**Table 6:** Uncertainties of stability during dispatch and storage. $u_{sts,rel}$ was calculated for a temperature of 60 °C and 1 week; $u_{lts,rel}$ was calculated for a storage temperature of -20 °C and 12 months.

| SCC* | $u_{sts}$ | $u_{sts,rel}$ [%] | $u_{lts}$ | $u_{lts,rel}$ [%] |
|---|---|---|---|---|
| ERM-BD001a | 566.0 | 0.97 | 1494.1 | 2.35 |
| ERM-BD001b | 4521.3 | 0.28 | 7984.4 | 0.65 |

*as defined in ISO 13366-2

No significant degradation during dispatch even at 60 °C was observed. Therefore, the material can be transported at ambient conditions without special precautions. No degradation could be detected when storing the materials for 12 months at 4 ºC and -20 ºC. The latter temperature shall be used as long-term storage temperature of the materials.

After the certification study, the materials will be included in the JRC's regular stability monitoring programme, to control their further stability.

# 6 Characterisation

The material characterisation is the process of determining the property value(s) of a reference material.

This was based on an interlaboratory comparison of expert laboratories, i.e. the SCC was determined in different laboratories that applied one or both of the prescribed measurement procedures to demonstrate the absence of a measurement bias. This approach aims at randomisation of laboratory bias, which reduces the combined uncertainty.

## 6.1 Selection of participants

Thirty-two laboratories were selected based on criteria that comprised both technical competence and quality management aspects. Each participant was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field of somatic cell count measurements in milk using methods compliant with the ISO standards 13366-1 and 13366-2, or, in case of some US laboratories, with similar methods described in the reference "Standard methods for the examination of dairy products" (chapters 10, DMSCC and 11.032, electronic SCC) [23]. Laboratory proficiency was deduced from laboratory results in recent SCC PT studies as well as quality system documentation (e.g. accreditation certificate). Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory. Where measurements are covered by the scope of accreditation, the accreditation number is stated in the list of participants (Section 2.5).

## 6.2 Study setup

Each laboratory using the reference method received 1 set of the CRM (low and high count milk powder), and each laboratory using the routine method received 2 sets of the CRM (low and high count milk powder) The units for material characterisation were selected using a random stratified sampling scheme and covered the whole batch. The sample preparations and measurements had to be spread over two days to ensure intermediate precision conditions.

Reconstitution of the milk powders had to be performed as outlined in a detailed instruction of JRC. Two reconstitution protocols were sent with the sample, and the laboratories could choose between those (annexes A and B).

The study set-up is depicted below (Figures 1 and 2).

Briefly, laboratories operating the reference method were requested to prepare two sample replicates on each analysis day and for each type of sample (ERM-BD001a and ERM-BD001b), and to prepare two smears from each sample replicate. Hence, per material, 4 replicates (reconstituted milks) and 8 smears to count were prepared. The average result of the two smears counted was reported as the result for a given replicate (see Figure 1).The low count milk was measured as such, whereas the high count milk had first to be diluted 1 by 3 (1 part of milk and 2 parts of diluent) in order to obtain a SCC which can be measured with higher precision (concentration closer to the centre of the method working range). Fat-free UHT milk (typically containing SCC below LOD of the method or at least < 10 000 cells/mL) was prescribed as diluent. Labs were requested to first analyse the UHT milk for the absence of somatic cells before using it as diluent. The reason for prescribing UHT milk as diluent was that the matrix is very similar (almost identical) to that of the CRMs after

reconstitution, which is important not to introduce potential dilution effects by using a diluent (e.g. PBS buffer) substantially different from milk.

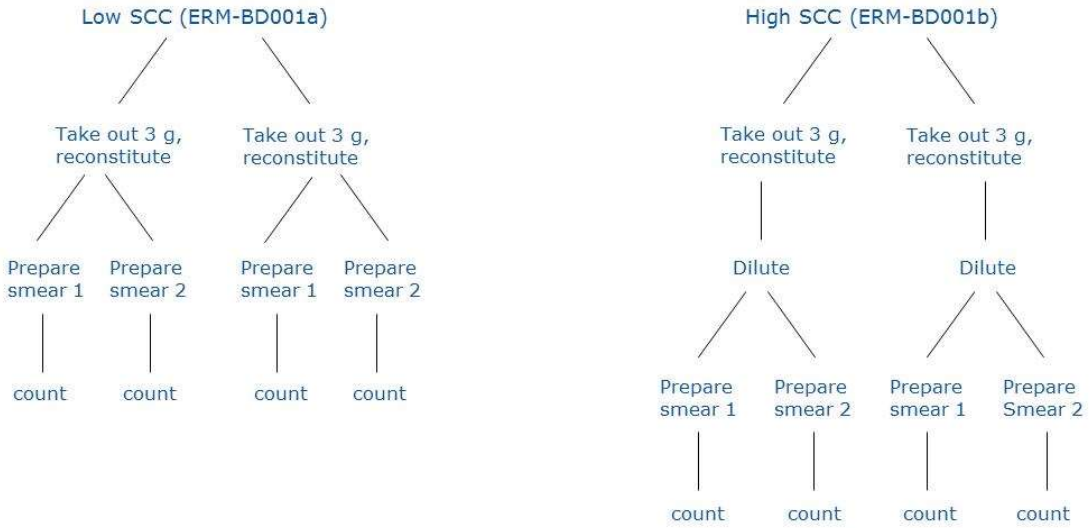


**Figure 1**. Graphical outline of the work to be done in laboratories using the reference method (ISO 13366-1) on one day. On the second day, this work had to be repeated.

Labs which applied the routine method were requested to prepare three sample replicates (reconstituted milks) per day ($n$ = 2) and measure both low and high SCC milks as such (no dilution). Hence, per material, 6 replicates were prepared and counted (Figure 2) in total. Laboratories could use one unit on the first day and the second unit on the second day, which however was not prescribed (suitable material stability confirmed as described above). Again, labs could choose between the two provided reconstitution protocols.

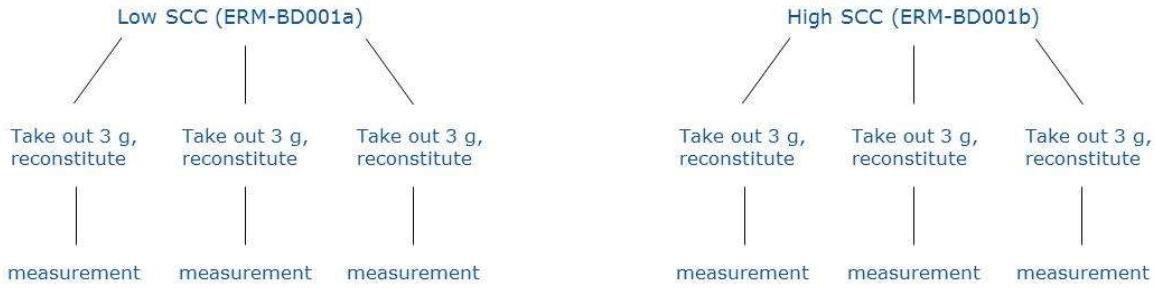


**Figure 2**. Graphical outline of the work to be done in laboratories using the routine method (ISO 13366-2) on one day. On the second day, this work had to be repeated.

Laboratories were also requested to fill out a detailed method questionnaire for a full description of critical method parameters (e.g. type of microscope, magnification factor, staining procedure; instrumentation used). Also, laboratories were requested to describe QC-related measures (e.g. usage of QCM that are co-analysed with unknown samples, adherence of the method's performance with the criteria defined in the two ISO standards).

## 6.3 Methods used

Laboratories used one or both of the following methods: ISO 13366-1 [6] and ISO 13366-2 [5]. In the case of some US laboratories, similar methods listed in the reference "Standard methods for the examination of dairy products" were used. All laboratories were operating those methods under ISO/IEC 17025 accreditation or an equivalent quality system.

All methods used during the characterisation study are summarised in Annex F. The laboratory code (e.g. A1) is a random number and does not correspond to the order of laboratories in Section 2.5. As can be seen in Annex F, several differences among laboratories operating the same method were encountered (e.g. type of microscope, magnification, type of slide, type of QCM, staining reagent, type of flow cytometry equipment, type of QCM, type of RM used for re-calibration in some cases).

## 6.4 Evaluation of results

The characterisation study resulted in the following number of data sets: 19 data sets (ERM-BD001a and ERM-BD001b) obtained from reference method measurements, and 42 data sets obtained from routine method measurements for ERM-BD001a and ERM-BD001b, respectively. All individual results of the participants, grouped per method type are displayed in tabular form in Annex G.

To each laboratory a code letter/dual letter (A, B, ..; AA, AB, …) was assigned. Codes were assigned in the order the results were submitted from a laboratory. If a number follows the lab code (single or double letter), this indicates that this laboratory has reported more than 1 set of results (e.g. one reference and one routine method data set, 2 or more reference method results sets, 2 or more routine method results sets). Laboratories D, G and V used so-called alternative methods (not visual microscopy or flow cytometry), but e.g. image cytometry methods. Consequently, the results were not included in the data evaluation and value assignment.

### 6.4.1 Technical evaluation

The obtained data were first checked for compliance with the requested analysis protocol and for their validity based on technical reasons. The following criteria were considered during the evaluation:

- adherence to the prescribed methods (microscopy-based or flow cytometry-based).

- compliance with the analysis protocol.

- proper documentation for precise method description.

The results were evaluated as such, with the below listed exceptions and reasons (recalculations at JRC):

- If the lab did not round the result to the nearest thousand cells/mL, this was done at JRC.
- For the reference method, the mean of the two smear counts per slide was taken as result and not the individual results per smear as reported in some case. If required, the obtained mean result was rounded to the nearest thousand cells/mL.
- If the exact ratio of water (1 part) to milk powder (9.22 parts) was not respected, results were recalculated accordingly at JRC using a correction factor which compensates for a sample that was either too concentrated (i.e. not enough water added) or too diluted (too much water added).

In a few cases, laboratories deviated from the analysis protocol. However, these data were taken into account for value assignment, as the results agreed with those from other labs and because the deviations were minor (e.g. analysing less samples, analysing reconstituted milk on a second instrument, misunderstandings such as using reconstitution protocol A for ERM-BD001a and reconstitution protocol B for ERM-BD001b).

The following data sets were rejected due to technical shortcomings:

**a.) Reference method data sets removed:**

If there was a large deviation between the counts of the two smears on one slide, this indicated poor accuracy and/or precision of the method applied in the lab (shortcomings related to sample preparation (preparation of smear, staining) and/or counting of cells under the microscope. Such data were therefore disregarded.

Data sets H1 (both materials), AD1 (both materials), and AF1 (both materials): in almost all instances (sample replicates), large smear-to-smear SCC differences (same slide) were encountered. The differences were defined by assuming 100 % for the higher count and calculating the respective % for the lower count smear (e.g. 48000 and 82000 cells/mL, 48000 is 41 % lower than 82000).

The criteria to accept results were set as follows; low count: average of differences among the 4 sample replicates below 20 %, one outlying result allowed with maximum 30 % SCC difference among 2 smears on the same slide. High count: average of differences among the 4 sample replicates below 15 %, one outlying result allowed with maximum 20 % SCC difference among 2 smears on the same slide. The criteria are based on the outcome of this and previous SCC intercomparisons among expert labs. The performances of the 3 labs listed above were as follows (Table 7):

**Table 7:** Differences in SCC among smears for data sets that were rejected

| **ERM-BD001a** | | | | | |
|---|---|---|---|---|---|
| Lab code | Day 1, first replicate, difference among smears [%] | Day 1, second replicate, difference among smears [%] | Day 2, first replicate, difference among smears [%] | Day 2, second replicate, difference among smears [%] | Average difference [%] |
| H1 | 41 | 43 | 41 | 23 | **37.00** |
| AD1 | 44 | 28 | 31 | 18 | **30.25** |
| AF1 | 9 | 42 | 26 | 20 | **24.25** |
| **ERM-BD001b** | | | | | |
| Lab code | Day 1, first replicate, difference among smears [%] | Day 1, second replicate, difference among smears [%] | Day 2, first replicate, difference among smears [%] | Day 2, second replicate, difference among smears [%] | Average difference [%] |
| H1 | 4 | 14 | 23 | 20 | **15.25** |
| AD1 | 21 | 24 | 6 | 3 | **13.50** |
| AF1 | 15 | 17 | 3 | 31 | **16.50** |

Data sets AB1 and AB2 were also excluded for the following reason: the laboratory reported that the Newman-Lampert staining solution used to dye the cells was not filtered before use (although prescribed in ISO 13366-1). Potentially, dye residues can complicate the correct counting of cells, especially in low SCC milks; blue spots of dye residues can be confounded with somatic cells, so false positive results can be obtained. Indeed, data sets AB1 and AB2 showed results for ERM-BD001a that were considerably higher than the rest of the obtained results.

Finally, the data set AH1 was removed (only for ERM-BD001b). An approximately 30 % discrepancy between the results reported by the laboratory in the results sheet (ca. 1.5 x $10^6$ cells/mL when the reconstituted milk was 1:3 diluted in UHT milk as prescribed) and those mentioned in a later written communication (1.2 x $10^6$ cells/mL when the milk was measured

directly without UHT milk dilution). Thus, the dilution with UHT milk (most likely not cell free as required) caused a result bias which led to a rejection of this data set.

**b.) Routine method data sets removed:**

Data set E1 was removed as the laboratory indicated problems with material reconstitution (only ERM-BD001a). The laboratory acknowledged the invalidity of the results and requested more samples to repeat the analysis, reported as data set E2.

Data set J2 was excluded. The reported first set of results including the reported slope gave rise to doubt about the correctness of the results. The lab confirmed the correctness of the reported slope but nevertheless requested to re-analyse new samples. The results for these samples were considerably different: ERM-BD001a, the 2nd result mean value was 23 % higher than 1st result mean value, and ERM-BD001b, the 2nd result mean value 117 % higher than 1st result mean value. The reason for this difference could not be explained. Thus, the confidence in the results was insufficient and the data were not taken into account for value assignment.

**Routine method data sets – significance of the slope**

In addition to this, the slope values reported by the laboratories were checked. The slope is originally established at the instrument manufacturer as follows: a trusted SCC reference material (value assigned with reference method measurements) is taken and measured. If the instrument raw value is not matching the target value, then a slope correction is applied. In case the reference method value is plotted on the x axis and the routine method value on the y axis, the instrumental raw value is divided by the slope; in case the reference method value is plotted on the y axis and the routine method value is plotted on the x axis, the instrumental raw value is multiplied with the slope.

Instrument manufacturers confirmed that they recommend to use the instruments with a slope of 1 or close to 1 (no changes to the original calibration) unless technically required. In the majority of cases, labs in this study reported a slope of 1 or close to 1 (thus no recalibration of the instrument was performed).

In addition to two technical outliers described above for the routine method, routine method data sets with an indicated slope outside the 0.95-1.05 range were not further considered in the statistical data evaluation and subsequent value assignment. The reason for this measure was to establish reference values as independent from the past as possible, i.e. only taking data into account which was not obtained by (repeated) re-calibrations with various types of reference materials, assigned with different strategies.

Two mean values per material were assigned; one based on the 14 (13) reference method data sets, and one based on a 50/50 merged data pool stemming from the 14 (13) reference method data sets and 14 (13) randomly selected from the above-mentioned 32 routine method data sets. The latter is technically valid as the value agrees with the reference method mean value within the respective expanded uncertainties. Moreover, this approach meets the requirements of ISO 13366-2 for the preparation of suitable calibration materials.

### 6.4.2 Statistical evaluation

The datasets accepted based on technical reasons were tested for normality of dataset means using kurtosis/skewness tests and normal probability plots. The data sets were also tested for outlying means using the Grubbs test and the Cochran test for outlying standard deviations, (both at a 99 % confidence level). Standard deviations within ($s_{within}$) and between ($s_{between}$) laboratories were calculated using one-way ANOVA. The results of these evaluations are shown in Tables 8 and 9.

**Table 8:** Statistical evaluation of the technically accepted datasets for ERM-BD001a. *p*: number of technically valid datasets

| | | *p* | Outliers | | Normally distributed | Statistical parameters | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Means | Variances | | Mean [cells/mL] | *s* [cells/mL] | $s_{between}$ [cell/mL] | $s_{within}$ [cells/mL] |
| ERM-BD001a | Reference method | 14 | No | No | Yes | 64482.1 | 9250.5 | 9111.7 | 3193.2 |
| *ERM-BD001a* | *Routine method* | *32* | *No* | *No* | *Yes* | *59255.2** | *6022.1* | *5811.3* | *2915.5* |
| ERM-BD001a | 50%/50% Reference and routine method** | 28 | No | No | Yes | 62068.4 | 7728.6 | 7208.1 | 3056.1 |

* This value is shown for information only. The relevant reference value is listed in the third row.

** 14 routine method data randomly selected from the 32 available.

**Table 9:** Statistical evaluation of the technically accepted datasets for ERM-BD001b. *p*: number of technically valid datasets

| | | *p* | Outliers | | Normally distributed | Statistical parameters | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Means | Variances | | Mean [cells/mL] | *s* [cells/mL] | $s_{between}$ [cells/mL] | $s_{within}$ [cells/mL] |
| ERM-BD001b | Reference method | 13 | No | No | Yes | 1202442.3 | 190011.1 | 189399.9 | 30457.3 |
| *ERM-BD001b* | *Routine method* | *32* | *No* | *No* | *Yes* | *1151968.8** | *70520.6* | *70482.1* | *22309.6* |
| ERM-BD001b | 50%/50% Reference and routine method** | 26 | No | No | Yes*** | 1165618.6 | 139547.0 | 127986.4 | 24383.3 |

* This value is shown for information only. The relevant reference value is listed in the third row.

** 13 routine method data randomly selected from the 32 available

*** Please see remarks in the paragraph below

The laboratory means follow normal distributions. However, in case of ERM-BD001b, combination of 50% reference and 50% routine method data, the situation had to be investigated in more detail: the standard procedure to check for normality did not give a clear picture: the kurtosis test value showed normality at 99 % confidence level, whereas the skewness value was above the respective critical value. The normal probability plot indicated some deviation from a normal distribution. However, a Kolmogoroff-Smirnoff test was applied and indicated a test value below the respective critical value at both 95 and 99 % confidence level. Therefore, a normal distribution can reasonably be assumed.

None of the data contains outlying means and variances. The datasets are therefore consistent and the mean of laboratory means is a good estimate of the true value. Standard deviations between laboratories are considerably larger than the standard deviation within laboratories, showing that confidence intervals of replicate measurements are unsuitable as estimate of measurement uncertainty.

The uncertainty related to the characterisation is estimated as the standard error of the mean of laboratory means. For this, the standard deviation among laboratory means is divided by the square root of the number of laboratories (Tables 10 and 11).

**Table 10:** Uncertainty of characterisation for ERM-BD001a

| ERM-BD001a | *Method* | *p* | Mean [cells/mL] | *s* [cells/mL] | $u_{char}$ [cells/mL] |
|---|---|---|---|---|---|
| SCC | Reference method | 14 | 64482.1 | *9250.5* | *2472.3* |
| SCC | 50/50 Reference method and routine method | 28 | 62068.4 | *7728.6* | *1460.6* |

**Table 11:** Uncertainty of characterisation for ERM-BD001b

| ERM-BD001b | *Method* | *p* | Mean [cells/mL] | *s* [cells/mL] | $u_{char}$ [cells/mL] |
|---|---|---|---|---|---|
| SCC | Reference method | 13 | 1202442.3 | *190011.1* | *52699.6* |
| SCC | 50/50 Reference method and routine method | 26 | 1165618.6 | *139547.0* | *27367.4* |

# 7 Value Assignment

Certified values were assigned.

Certified values are values that fulfil the highest standards of accuracy. Procedures at JRC generally require generally pooling of not less than 6 datasets to assign certified values. Full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [4] were established.

It was decided to assign two certified values per material. The main reason for this is the fact that although the two values agree within their respective uncertainties, the uncertainty of the first value (see below) is somewhat larger than that of the second value (see below), and a lower uncertainty in the second case is essential to obtain a suitable calibration material.

The first value is the mean of the accepted reference method data sets. This value is to be used for method performance verification of reference methods. The second value is the mean of the accepted reference method data set results and the same number ($n$ = 14 for ERM-BD001a, $n$ = 13 for ERM-BD001b) of accepted routine method data sets randomly picked from the $n$ = 32 (both materials) accepted data sets with an indicated slope around 1 (0.95-1.05). The rationale for this approach relates to standard ISO 13366-2 of how a suitable calibration material should be designed.

## 7.1 Certified values and their uncertainties

The unweighted mean of the means of the accepted datasets is shown in Table 12 as certified value for each parameter. Certified values are rounded to the nearest thousand cells/mL. Uncertainties are also rounded, but rounded up to the nearest thousand cells/mL.

The assigned uncertainty consists of uncertainties relating to characterisation, $u_{char}$ (Section 6), potential between-unit inhomogeneity, $u_{bb}$ (Section 4.1), and potential degradation during transport, $u_{sts}$, and long-term storage, $u_{lts}$ (Section 5). These different contributions were combined to estimate the relative expanded uncertainty of the certified value ($U_{CRM,\ rel}$) with a coverage factor $k$ given as:

$$U_{CRM,rel} = k \cdot \sqrt{u^2_{bb,rel} + u^2_{sts,rel} + u^2_{lts,rel} + u^2_{char,rel}} \qquad \textbf{Equation 6}$$

- $u_{char}$ was estimated as described in section 6.4.2
- $u_{bb}$ was estimated as described in section 4.1.
- $u_{sts}$ and $u_{lts}$ were estimated as described in section 5.3

A coverage factor $k$ of 2 was applied, to obtain the expanded uncertainties. The certified values and their uncertainties are summarised in Tables 12 and 13.

**Table 12:** Certified values and their uncertainties for ERM-BD001a

| | Certified value [cells/mL] | $u_{char, rel}$ [%] | $u_{bb, rel}$ [%] | $u_{sts, rel}$ [%] | $u_{lt, rels}$ [%] | $U_{CRM}$ [cells/mL] |
|---|---|---|---|---|---|---|
| SCC, reference method | 64000 | 3.83 | 3.32 | 0.97 | 2.35 | 8000 |
| SCC, 50/50 reference method and routine method | 62000 | 2.35 | 3.32 | 0.98 | 2.35 | 6000 |

**Table 13:** Certified values and their uncertainties for ERM-BD001b

| | Certified value [cells/mL] | $u_{char, rel}$ [%] | $u_{bb, rel}$ [%] | $u_{sts, rel}$ [%] | $u_{lts, rel}$ [%] | $U_{CRM}$ [cells/mL] |
|---|---|---|---|---|---|---|
| SCC, reference method | 1202000 | 4.38 | 2.28 | 0.28 | 0.65 | 121000 |
| SCC, 50/50 reference method and routine method | 1166000 | 2.35 | 2.28 | 0.28 | 0.65 | 79000 |

# 8 Metrological traceability and commutability

## 8.1 Metrological traceability

### Identity

#### a.) Reference method

Somatic cells are white blood cells naturally occurring in raw cow's milk and are defined as all cells with nuclei, i.e. all leucocytes (lymphocytes, macrophages, polymorphonuclear neutrophils) and epithelial cells [7]. ISO 13366-1 gives further guidance of how cells typically look like and defines e.g. a minimum size a cell must have to be taken into account for counting. Somatic cell count is a method-defined measurand and shall be obtained by following the microscopic method laid down in ISO 13366-1.

#### b.) Routine method

ISO 13366-2 defines somatic cells as those cells that show more than a threshold intensity of fluorescence due to the staining of DNA in their nuclei. Somatic cell count is a method-defined measurand and shall be obtained by following the fluoro-opto-electronic counting procedure laid down in ISO 13366-2.

### Quantity value

#### a.) Reference method

Traceability of the obtained results is based on the traceability of all relevant input factors (e.g. calibrated micro-syringe used for sampling a milk drop; slide, pre-marked with an outline shape - circular or rectangular - with a defined area). Consistency in the interlaboratory comparison demonstrates that all relevant input factors were covered. As the assigned values are combinations of agreeing results individually traceable to the SI, the assigned quantity values themselves are traceable to the SI as well.

#### a.) Routine method

Fluoro-opto-electronic counters (flow cytometers in all routine method laboratories in this study) are calibrated by the instrument providers using DMSCC value-assigned calibration standards. Instrument providers also give guidance as to when a re-calibration of the method is necessary. Moreover, traceability of the obtained results is based on the traceability of all relevant input factors. Instruments in individual laboratories were verified and checked for necessary recalibration with tools ensuring traceability to the International System of units (SI). Consistency in the interlaboratory comparison demonstrates that all relevant input factors were covered. As the assigned values are combinations of agreeing results individually traceable to the SI, the assigned quantity values themselves are traceable to the SI as well.

## 8.2 Commutability

Many measurement procedures include one or more steps which select specific (or specific groups of) analytes from the sample for the subsequent whole measurement process. Often the complete identity of these 'intermediate analytes' is not fully known or taken into account. Therefore, it is difficult to mimic all analytically relevant properties of real samples within a CRM. The degree of equivalence in the analytical behaviour of real samples and a CRM with respect to various measurement procedures (methods) is summarised in a concept called 'commutability of a reference material'. There are various definitions that define this concept. For instance, the CLSI Guideline C53-A [24] recommends the use of the following definition for the term *commutability*:

> "The equivalence of the mathematical relationships among the results of different measurement procedures for an RM and for representative samples of the type intended to be measured."

The commutability of a CRM defines its fitness for use and is therefore a crucial characteristic when applying different measurement methods. When the commutability of a CRM is not established, the results from routinely used methods cannot be legitimately compared with the certified value to determine whether a bias does not exist in calibration, nor can the CRM be used as a calibrant.

ERM-BD001 was produced from raw cow's milk, involving the steps described in section 3.2. Care was taken during processing (enrichment of cells, dilution to appropriate levels, conversion of milks to milk powders) to minimize damage to cells as much as possible. Also, in a feasibility study carried out before candidate CRM batch processing, it could be shown that defatting of the milk did not impair the SCC results by laboratories using either microscopic or flow cytometric methods. Defatted milk offers the advantage of somewhat easier reconstitution (keeping the sample homogeneous after reconstitution) and low fat content adds to a longer-life stability of the material (no issues with oxidation/rancidness).

Moreover, the tested techniques in the feasibility study to stabilise the milk preparations (freeze-drying; spray-drying) did not substantially impair the integrity of the cells. This was indirectly confirmed when experienced labs employing both microscopic and flow cytometric methods analysed raw milk samples as well as candidate RM batch samples and found comparable results for a given sample. Moreover, comparable analytical behaviour was observed with real-world samples (raw milk) and stabilised milks (candidate RMs), which also underpins suitable commutability of the established reference materials.

# 9 Instructions for use

## 9.1 Safety information

ERM-BD001 is intended for laboratory use only. The usual laboratory safety measures apply.

## 9.2 Storage conditions

The materials should be stored at (-20 ± 5) °C in the dark. Care should be taken to avoid any change of the moisture content once the units are open, as the material is hygroscopic. The user should close any bottles immediately after taking a sample.

Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially for opened bottles.

## 9.3 Preparation and use of the material/reconstitution

The CRMs are supplied in a set, consisting of 1 bottle of ERM-BD001a and 1 bottle of ERM-BD001b. Each bottle contains 14 g milk powder.

Reconstitution of the materials shall be performed as described in Annex A or Annex B (both protocols yield equivalent results).

## 9.4 Minimum sample intake

The minimum sample intake is 3 g of milk powder (to be reconstituted to milk by adding 27.66 g of water, see Annexes A and B).

## 9.5 Use of the certified value

The reference materials shall be used for two purposes:

a.) Calibration

The certified value given on the certificate obtained from the combination of 14 (ERM-BD001a) or 13 (ERM-BD001b) sets of reference method and routine method data sets represents a robust estimate of the true SCC value in those materials. Materials shall be reconstituted as described in Annex A or B and mixed in the quantities shown in Table 14 below. By this, at least 5 calibration levels shall be prepared (equidistant distribution from about 62000 cells/mL to 1166000 cells/ml) which allow to establish calibration complying with the recommendations of ISO 13366-2.

**Table 14**. Preparation of calibration samples from the reconstituted materials ERM-BD001a and ERM-BD001b. The values in the last two columns assume that 15 mL is a sufficient volume for one calibration level with the appliance used at the customer side. If a larger volume is needed (e.g. 20 mL), 1.33 times higher volumes have to be used in all cases.

| Level | Concentration [cells/mL] | Uncertainty [cells/mL] | mL reconstituted ERM-BD001a | mL reconstituted ERM-BD001b |
|---|---|---|---|---|
| 1 | 1166000 | 79000 | - | 15.00 |
| 2 | 890000 | 59000 | 3.75 | 11.25 |
| 3 | 614000 | 40000 | 7.50 | 7.50 |
| 4 | 338000 | 21000 | 11.25 | 3.75 |
| 5 | 62000 | 6000 | 15.00 | - |

Exemplified case in the table (5 times 15 mL needed): for each CRM, 4.5 g powder need to be reconstituted with 41.5 g water to have sufficient milk for preparation of the calibration samples.

b.) Method performance verification

The other certified value listed on the certificate was established from technically valid reference method data sets. This value is to be used for reference method users who want to verify whether their method operates correctly. Materials shall be reconstituted as described in Annex A or B.

The certified value on the certificate described above under a.) can also be used by routine method users who want to verify that their method operates correctly. It shall however be noted that for a dedicated usage of the material, it can either be used for calibration or for method performance verification, but not for both at the same time.

As any reference materials, they can be used for establishing control charts or validation studies.

The procedure below explains how to assess whether a result obtained in the laboratory complies with the certified value.

<u>Comparing an analytical result with the certified value</u>

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1, https://crm.jrc.ec.europa.eu/e/132/User-support-Application-Notes [25].

When assessing the method performance, the measured values of the CRMs are compared with the certified values. The procedure is summarised here:

- Calculate the absolute difference between mean measured value and the certified value ($\Delta_{meas}$).
- Combine the measurement uncertainty ($u_{meas}$) with the uncertainty of the certified value ($u_{CRM}$): $u_{\Delta} = \sqrt{u_{meas}^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty ($U_{\Delta}$) from the combined uncertainty ($u_{\Delta,}$) using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %
- If $\Delta_{meas} \leq U_{\Delta}$ then no significant difference exists between the measurement result and the certified value, at a confidence level of approximately 95 %.

Use in quality control charts

The materials can be used for quality control charts. Using CRMs for quality control charts has the added value that a trueness assessment is built into the chart.

# 10 Acknowledgments

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# 11 References


1 ISO 17034:2016, General requirements for the competence of reference materials producers, International Organization for Standardization, Geneva, Switzerland

2 ISO Guide 35:2006, Reference materials – General and statistical principles for certification, International Organization for Standardization, Geneva, Switzerland

3 ISO/IEC 17025:2017, General requirements for the competence of testing and calibration laboratories, International Organization for Standardization, Geneva, Switzerland

4 ISO/IEC Guide 98-3:2008, Guide to the Expression of Uncertainty in Measurement (GUM 1995, International Organization for Standardization, Geneva, Switzerland

5 ISO 13366-2:2006/IDF 148-2:2006, Milk – Enumeration of somatic cells – Part 2: Guidance on the operation of fluoro-opto-electronic counters, International Organization of Standardization, Geneva, Switzerland, and International Dairy Federation, Brussels, Belgium

6 ISO 13366-1:2008/IDF 148-1:2008, Milk – Enumeration of somatic cells – Part 1: Microscopic method (Reference method), International Organization of Standardization, Geneva, Switzerland, and International Dairy Federation, Brussels, Belgium

7 I. Zlotnik. Types of cells in cow's milk. J. Comp. Path. 57 (1947) 196-208.

8 H. Sarikaya, C. Prgomet, M.W. Pfaffl, R.M. Bruckmaier. Differentiation of leukocytes in bovine milk. Milchwissenschaft 59 (2004) 586-589.

9 Council Directive 92/46/EEC of 16 June 1992 laying down the health rules for the production and placing on the market of raw milk, heat-treated milk and milk based products. Off. J. Eur. Comm. L 268, 1-32.

10 Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. Off. J. Eur. Union L 139, 55.205.

11 S. Orlandini, H. van den Bijgaart. Reference system for somatic cell counting in milk. Accred. Qual. Assur. 16 (2011) 415-420.

12 O. Leray, S. Orlandini, U. Braun. Requirements for reference materials for the calibration of automated somatic cell counters. Bulletin of the International Dairy Federation 469/2013. International Dairy Federation, Brussels, Belgium

13 ISO 1211:2010/IDF 1:2010, Milk – Determination of fat content – gravimetric method (reference method). International Organization of Standardization, Geneva, Switzerland, and International Dairy Federation, Brussels, Belgium

14 ISO 8968-1:2014/IDF 20-1:2014, Milk and milk products – Determination of nitrogen content – Part 1: Kjeldahl principle and crude protein calculation. International Organization of Standardization, Geneva, Switzerland, and International Dairy Federation, Brussels, Belgium

15 ISO 6731:2010/IDF 21:2010, Milk, cream and evaporated milk – Determination of total solids content (reference method). International Organization of Standardization, Geneva, Switzerland, and International Dairy Federation, Brussels, Belgium

16 ISO 4833-1:2008, Microbiology of the food chain – Horizontal method for the enumeration of microorganisms – Part 1: colony count at 30 degrees C by the pour plate technique

17 ISO 14637:2004/IDF 195:2004, Milk – Determination of urea content – enzymatic method using difference in pH (reference method). International Organization of Standardization, Geneva, Switzerland, and International Dairy Federation, Brussels, Belgium

18 ISO 10523:2008, Water quality – Determination of pH. International Organization of Standardization, Geneva, Switzerland

19 M. Mathlouthi. Water content, water activity, water structure and the stability of foodstuffs. Food Control 106 (2001) 409-417.

20 T.P.J. Linsinger, J. Pauwels, A.M.H. van der Veen, H. Schimmel, A. Lamberty. Homogeneity and stability of reference materials. Accred. Qual. Assur. 6 (2001) 20-25.

21 A. Lamberty, H. Schimmel, J. Pauwels. The study of the stability of reference materials by isochronous measurements. Fres. J. Anal. Chem. 360 (1998) 359-361.

22 T.P.J. Linsinger, J. Pauwels, A. Lamberty, H. Schimmel, A.M.H. van der Veen, L. Siekmann. Estimating the uncertainty of stability for matrix CRMs. Fres. J. Anal. Chem. 370 (2001) 183-188.

23 H.M. Wehr, J.F. Frank (ed.) Standard Methods for the Examination of Dairy Products. ISBN: 978-0-87553-002-4, 2004, American Public Health Association Press, Washington, DC, United States

24 H. Vesper, H. Emons, M. Gnezda, C.P. Jain, W.G. Miller, R. Rej, G. Schumann, J. Tate, L. Thienpont, J.E. Vaks. Characterisation and qualification of commutable reference materials for laboratory medicine, approved guideline, 2010, CLSI document C30-A, Clinical and Laboratory Standards Institute, Wayne, PA, United States

25 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)

# Annexes

Annex A. Reconstitution protocol A

Annex B. Reconstitution protocol B

Annex C. Results of the homogeneity measurements

Annex D. Results of the short-term stability measurements

Annex E. Results of the long-term stability measurements

Annex F. Summary of methods used in the characterisation study

Annex G. Results of the characterisation study

# **Annex A.** Reconstitution protocol A

The following protocol needs to be strictly followed in order to convert the provided milk powders into milks, which are then either mixed in different ratios to prepare calibration samples (see section 9.5 a.)) or used as such for method performance verification purposes (section 9.5 b.)).

On the day of sample preparation and analysis, allow the samples to warm up to ambient temperature before taking out an aliquot for weighing.

The powder to water ratio to be used is 1 to 9.22. Hence, for 3.00 g milk powder (minimal sample intake per sample replicate), 27.66 mL water (double-distilled or ultrapure/type 1 water quality) shall be added.

The water should be pre-warmed to 40 ºC before use.

As container for sample preparation, please use a clean glass bottle (e.g. borosilicate glass) with a tight screw cap. Alternatively, a sterile plastic bottle with a tight cap can be used.

Add the water to the powder. First gently shake by hand till all powder has come into contact with water (wetting), then add a magnetic stirring rod and stir with medium speed (300 rpm) for 30 min on a stirring hot plate set to 40 ºC. Please note that after addition of water and initial gentle shaking by hand, it will take about 10 min until lumps formed will gradually disappear as the powder solubilizes and gets dissolved in the water. Once a homogeneous solution is obtained, take an aliquot and proceed with the analysis without unnecessary delay. Ensure homogenisation and/or mixing before analysis in case the sample/replicate is not analysed right after reconstitution.

# **Annex B.** Reconstitution protocol B

As an alternative to the reconstitution protocol prescribed in Annex A, some laboratories have used a different reconstitution protocol in the laboratory intercomparison. Investigations at JRC as well as study results demonstrated equivalence of the two protocols in terms of completeness of reconstitution.

On the day of sample preparation and analysis, allow the samples to warm up to ambient temperature before taking out an aliquot for weighing.

The powder to water ratio to be used is 1 to 9.22. Hence, for 3.00 g milk powder (minimum sample intake per sample replicate), 27.66 mL water (double-distilled or ultrapure/type 1 water quality) shall be added.

The water should be pre-warmed to 40 ºC before use.

As container for sample preparation, a sterile plastic bottle with a tight cap or a sterile centrifuge tube with a tight cap can be used.

Add the water to the powder. First gently shake by hand till all powder has come into contact with water (wetting). Please note that after addition of water and initial gentle shaking by hand, it will take about 10 min until lumps formed will gradually disappear as the powder solubilizes and gets dissolved in the water. Shake by hand 20 times (inversion mixing), then put bottle for 30 min in a 40 ºC water bath, mix again 20 times by hand (inversion mixing), put sample overnight in a cold room or refrigerator (4 ºC) for protein rehydration, mix again 20 times by hand (inversion mixing), put bottle for 30 min in 40 ºC water bath, final mixing by hand 20 times (inversion mixing), take out aliquot for analysis. Ensure homogenisation and/or mixing before analysis in case the sample/replicate is not analysed right after finalisation of the reconstitution.

# **Annex C.** Homogeneity data

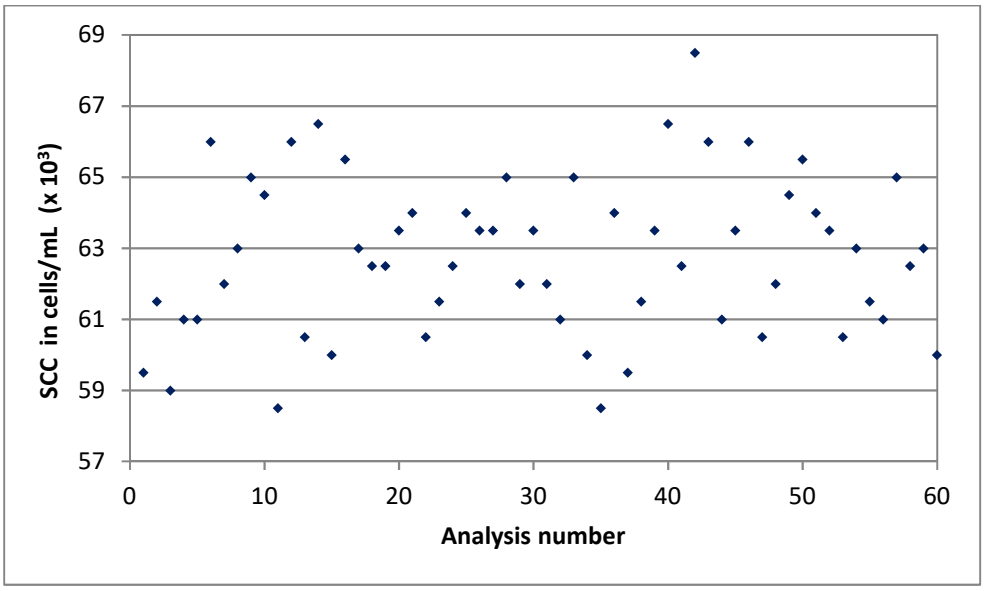


Figure C1. Results of the homogeneity study ERM-BD001a, analytical sequence.

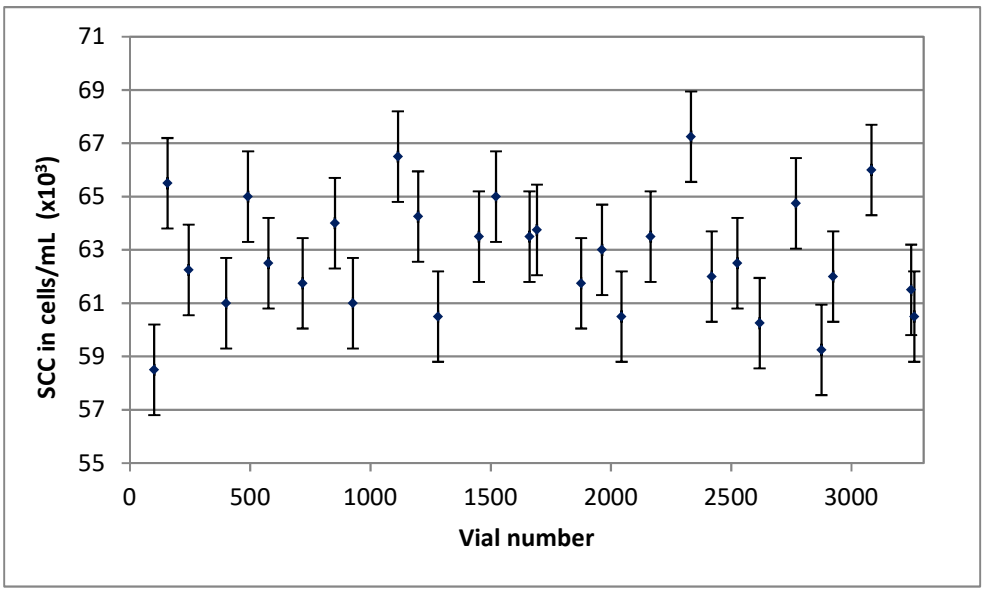


Figure C2. Results of the homogeneity study ERM-BD001a, filling sequence. The error bars are the 95 % confidence interval of the means of each bottle (2 determinations), based on the within-group standard deviation as obtained by a one-way ANOVA.

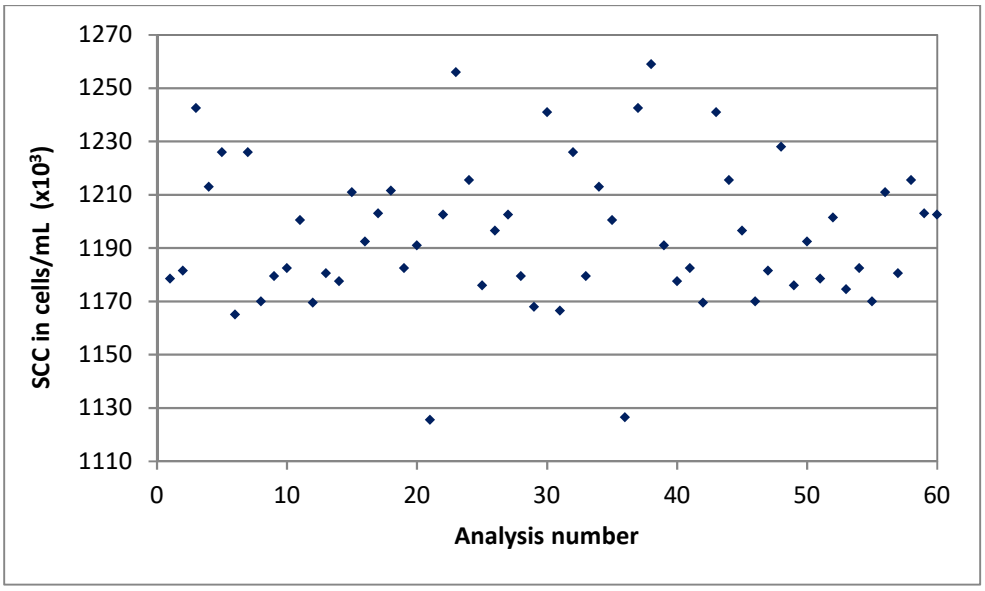


Figure C3. Results of the homogeneity study ERM-BD001b, analytical sequence.

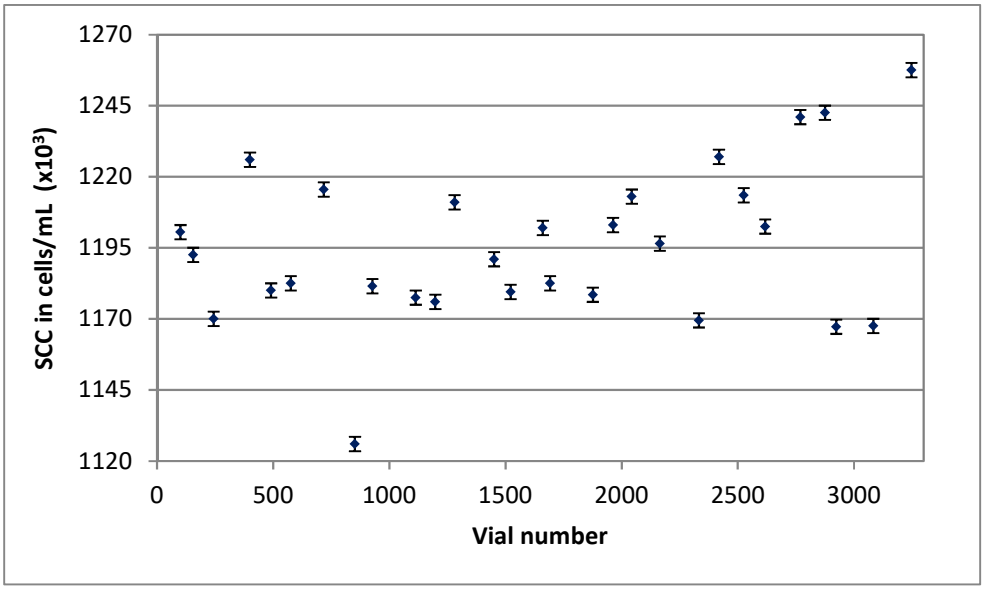


Figure C4. Results of the homogeneity study ERM-BD001b, filling sequence. The error bars are the 95 % confidence interval of the means of each bottle (2 determinations), based on the within-group standard deviation as obtained by a one-way ANOVA.

## **Annex D.** Short-term stability data

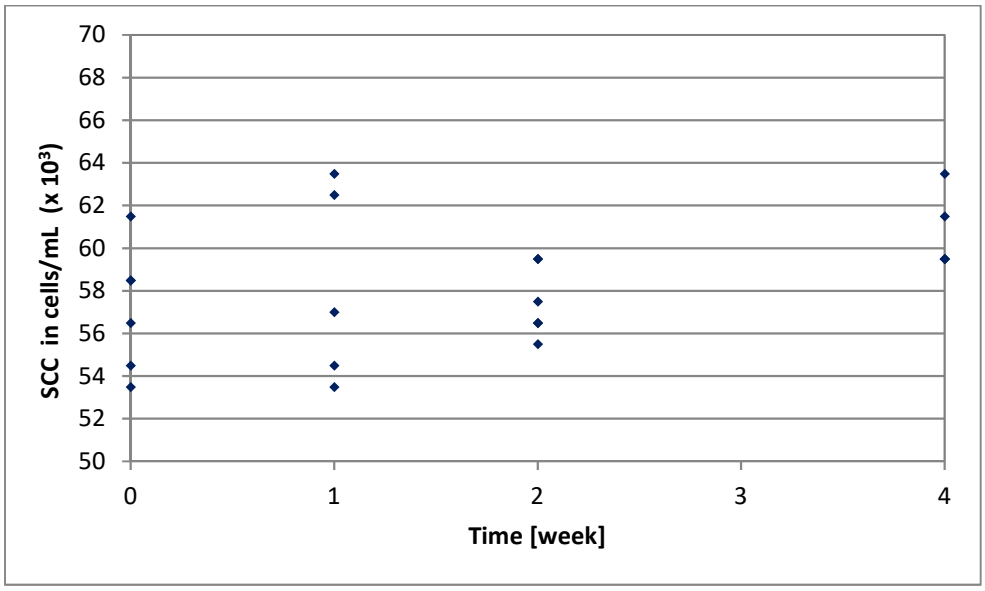


Fig. D1. Results of the short-term stability study ERM-BD001a, storage temperature -20 ºC. The graph provides individual results (6 replicates per time point) to better illustrate the presence, if any, of outliers.

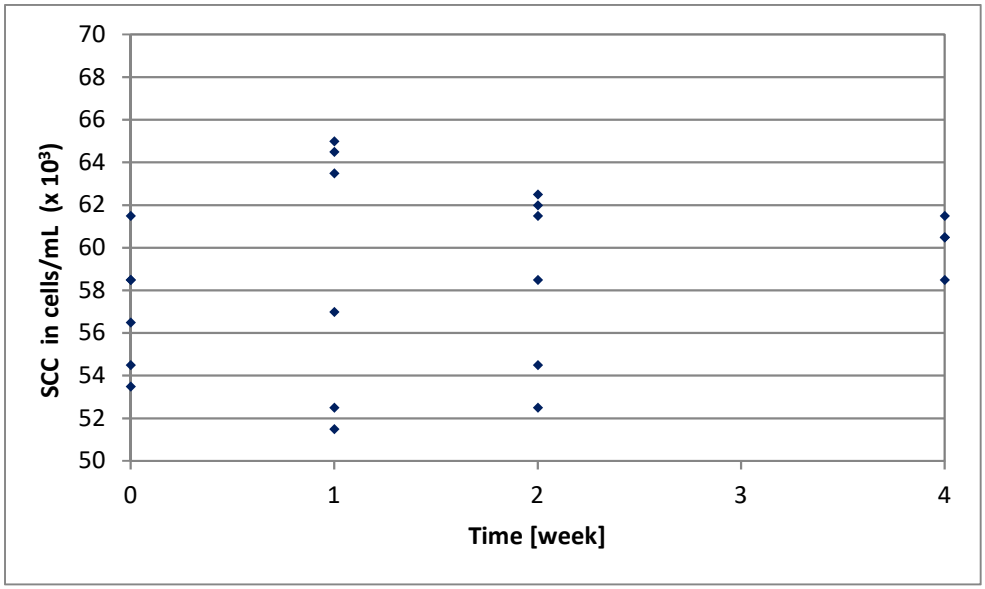


Fig. D2. Results of the short-term stability study ERM-BD001a, storage temperature 4 ºC. The graph provides individual results (6 replicates per time point) to better illustrate the presence, if any, of outliers.

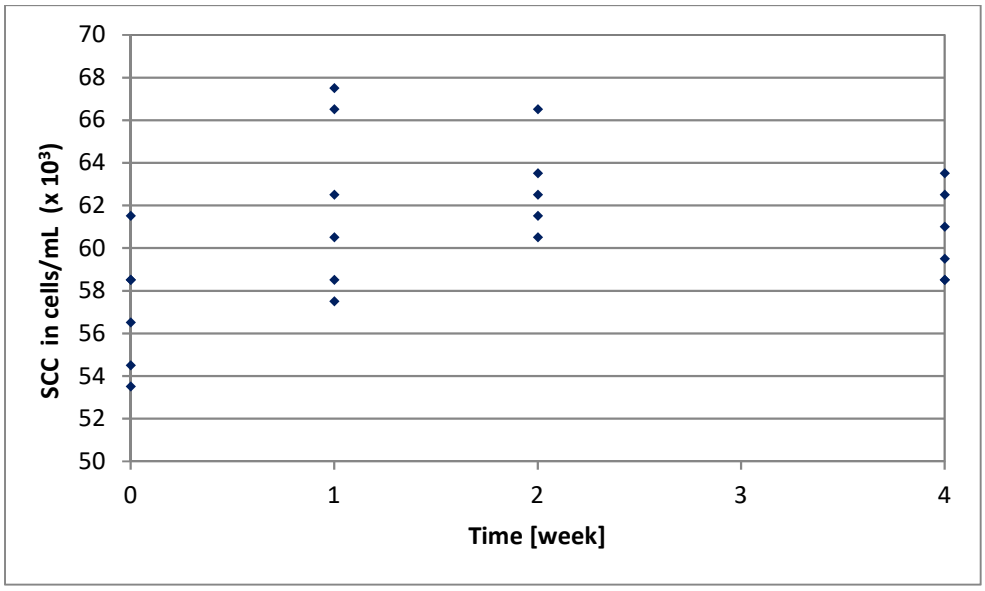


Fig. D3. Results of the short-term stability study ERM-BD001a, storage temperature 18 ºC. The graph provides individual results (6 replicates per time point) to better illustrate the presence, if any, of outliers.

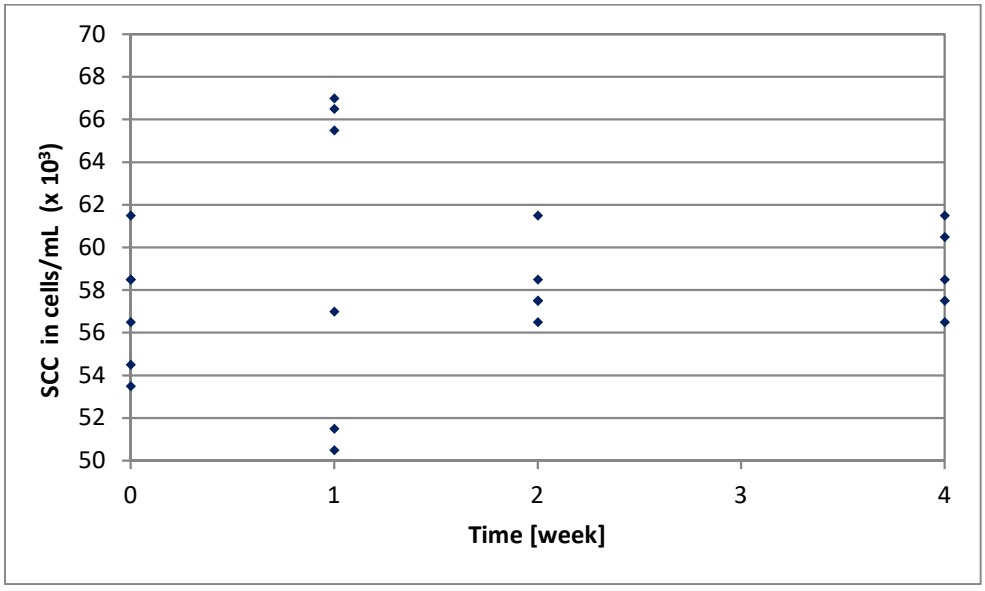


Fig. D4. Results of the short-term stability study ERM-BD001a, storage temperature 60 ºC. The graph provides individual results (6 replicates per time point) to better illustrate the presence, if any, of outliers.

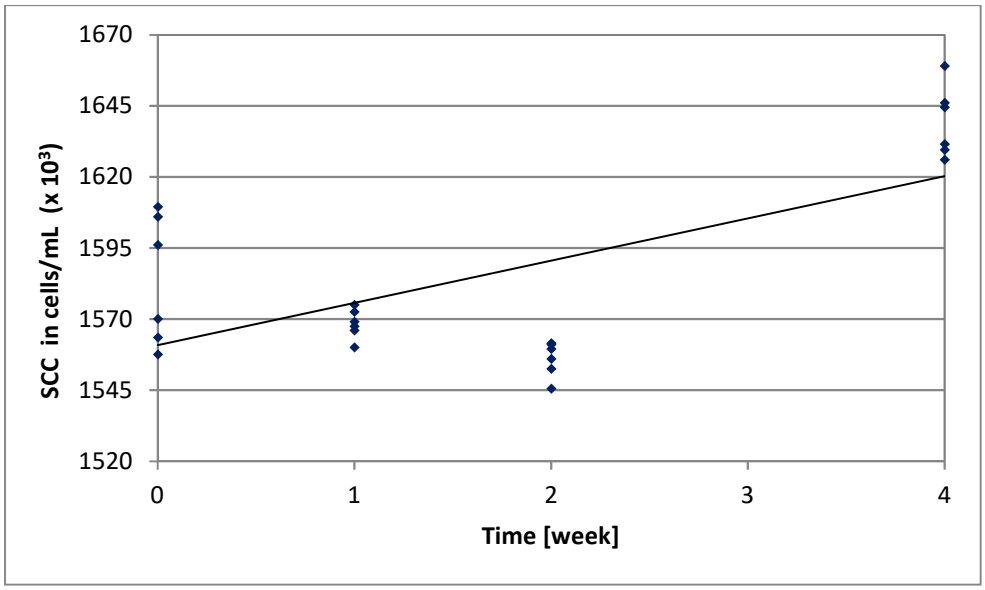


Fig. D5. Results of the short-term stability study ERM-BD001b, storage temperature -20 ºC. The graph provides individual results (6 replicates per time point) to better illustrate the presence, if any, of outliers. A linear regression line displays a statistically significant trend.

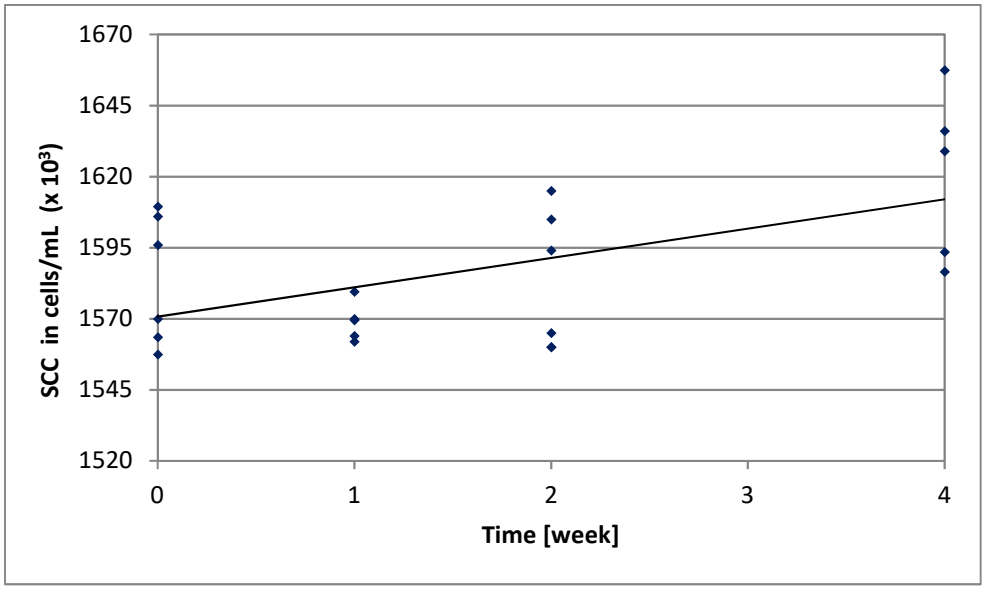


Fig. D6. Results of the short-term stability study ERM-BD001b, storage temperature 4 ºC. The graph provides individual results (6 replicates per time point) to better illustrate the presence, if any, of outliers. A linear regression line displays a statistically significant trend.

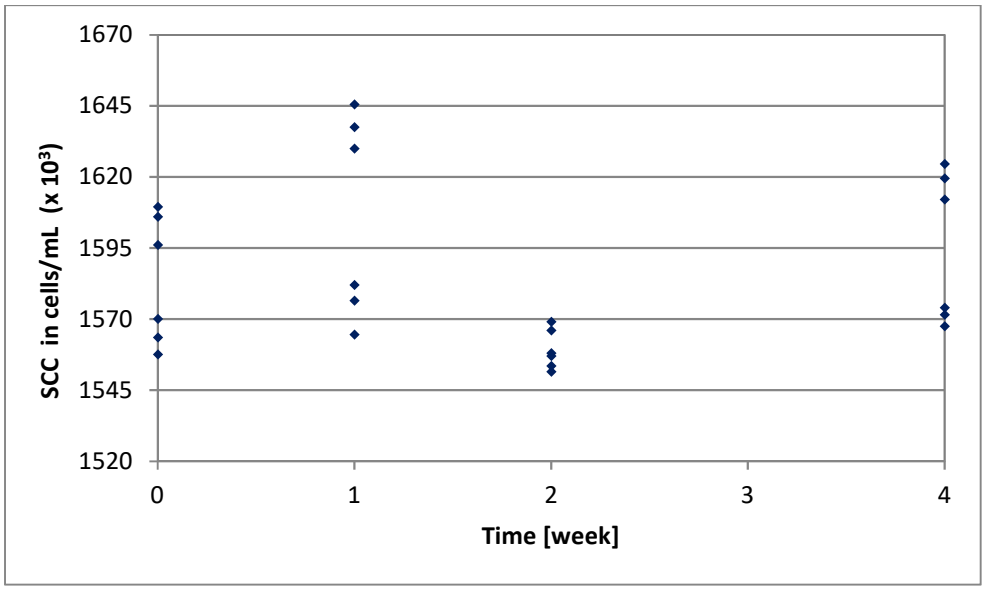


Fig. D7. Results of the short-term stability study ERM-BD001b, storage temperature 18 ºC. The graph provides individual results (6 replicates per time point) to better illustrate the presence, if any, of outliers.

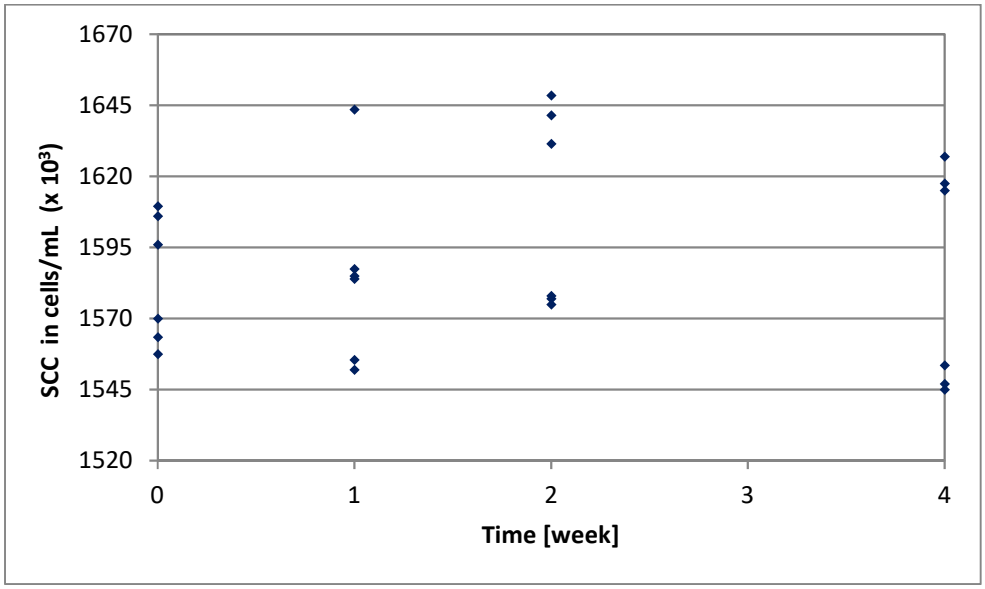


Fig. D8. Results of the short-term stability study ERM-BD001b, storage temperature 60 ºC. The graph provides individual results (6 replicates per time point) to better illustrate the presence, if any, of outliers.

# **Annex E.** Long-term stability data

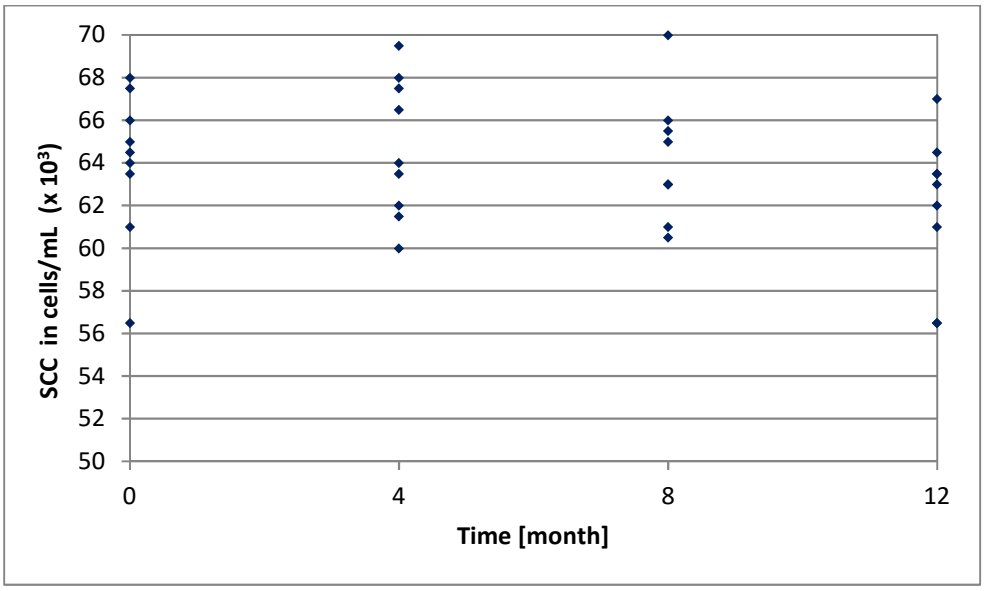


Fig. E1. Results of the long-term stability study ERM-BD001a, storage temperature -20 ºC. The graph provides individual results (9 replicates per time point) to better illustrate the presence, if any, of outliers.

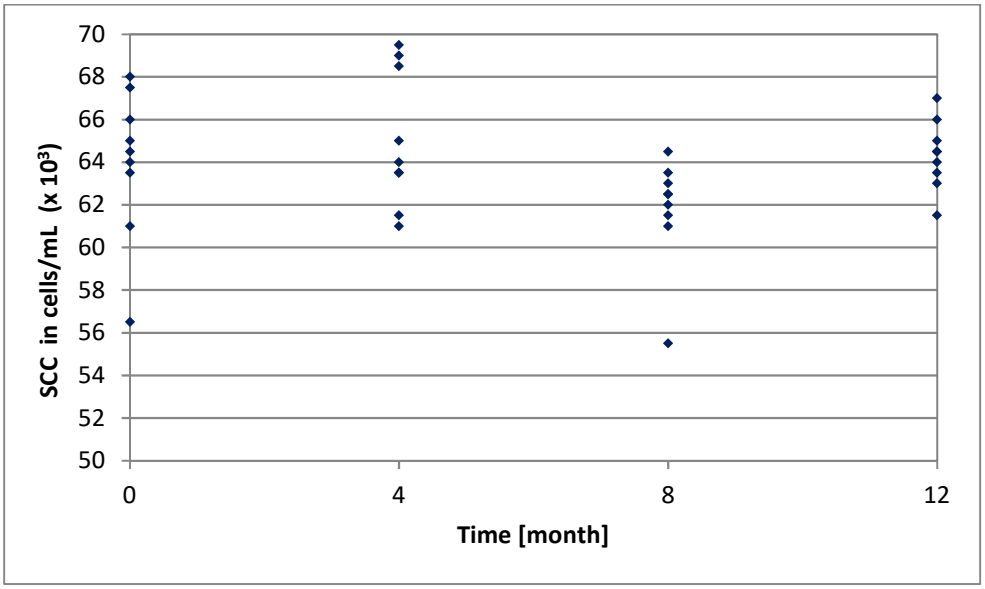


Fig. E2. Results of the long-term stability study ERM-BD001a, storage temperature 4 ºC. The graph provides individual results (9 replicates per time point) to better illustrate the presence, if any, of outliers.

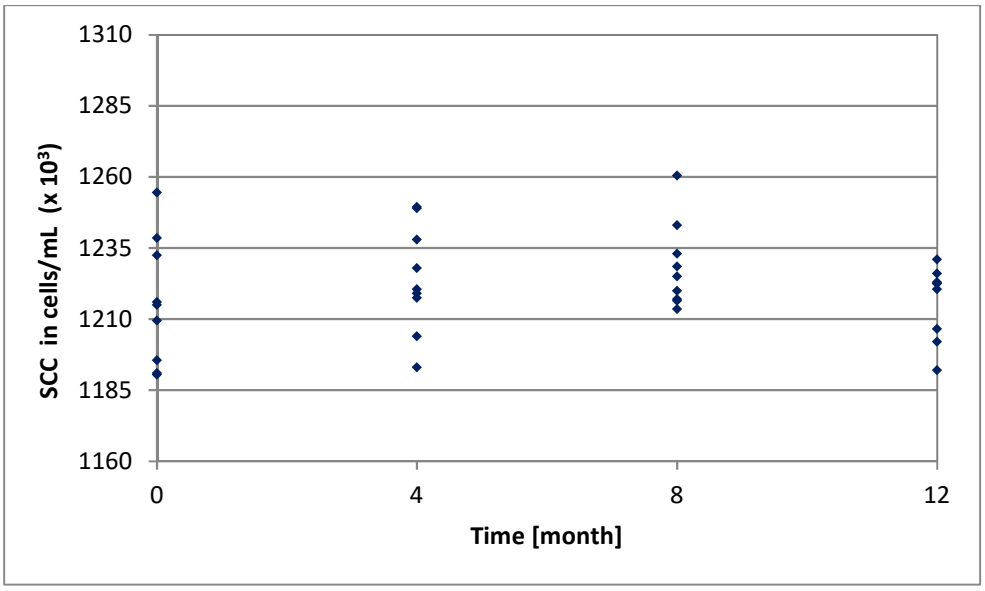


Fig. E3. Results of the long-term stability study ERM-BD001b, storage temperature -20 ºC. The graph provides individual results (9 replicates per time point) to better illustrate the presence, if any, of outliers.

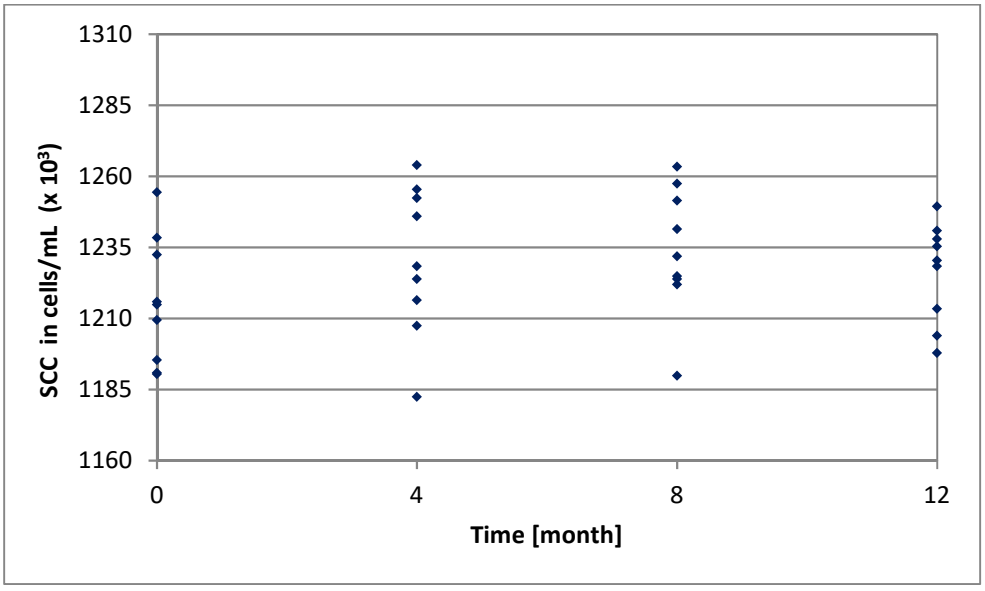


Fig. E4. Results of the long-term stability study ERM-BD001b, storage temperature 4 ºC. The graph provides individual results (9 replicates per time point) to better illustrate the presence, if any, of outliers.

## **Annex F:** Summary of methods used in the characterisation study

**Table F1.** Reference methods used. All laboratories applied methods which are based on microscopy. If a number follows the lab code (single or double letter), this indicates that this laboratory has reported more than 1 set of results (e.g. one set each of reference and routine method, 2 or more reference method results sets, 2 or more routine method results sets). - Laboratories D, G and V are not referenced in this table as those laboratories used so-called alternative methods (not visual microscopy or flow cytometry, but e.g. image cytometry methods).

| Laboratory code | Cell staining reagent | Microscope brand/model | Microscope type | Magnification | Reconstitution protocol used | Slide type (circular/ rectangular) | Counting by bands/fields | Quality control material used | Remarks |
|---|---|---|---|---|---|---|---|---|---|
| B | Modified Newman-Lampert stain solution | Zeiss | optical | 500x | A | circular | fields | preserved liquid sample, 1 level | |
| H1 | Modified Newman-Lampert stain solution | Zeiss Axioskop 2 Plus | optical | 1000x | A | rectangular | bands | preserved liquid sample, 1 level | ERM-BD001b was not diluted with cell-free UHT milk before counting |
| I1 | Modified Newman-Lampert stain solution | Nikon | optical | 500x | A | rectangular | fields | none | |
| J1 | EtBr stain solution | Olympus BX51 | fluorescence | 600x | A and B | rectangular | fields | preserved liquid samples (2 types), several levels each | ERM-BD001a reconstituted with protocol A, BD001b with protocol B |
| K1 | Modified Newman-Lampert stain solution | Zeiss | optical | 500x | A | circular | fields | preserved liquid samples, several levels | |
| O | Modified Newman-Lampert stain solution | Nikon DS-Fi2 | optical | 1000x | A | rectangular | bands | none | |
| P1 | Modified Newman-Lampert stain solution | Motic BA310 | optical | 1000x | A | rectangular | fields | preserved liquid samples, 9 levels | |
| Q1 | EtBr stain solution | Zeiss | fluorescence | 400x | B | rectangular | bands | preserved liquid sample, 1 level | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| U1 | Modified Newman-Lampert stain solution | Delta Optical | optical | 1000x | B | rectangular | bands | preserved liquid sample, 1 level | |
| W1 | Modified Newman-Lampert stain solution | Zeiss | optical | 800x | B | rectangular | bands | preserved liquid samples, 2 levels | |
| X1 | Modified Newman-Lampert stain solution | Leica DM 2000 LED | optical | 1000x | B | circular | fields | in-house control material | Regular PT participation |
| Z1 | Modified Newman-Lampert stain solution | Olympus BX50 | optical | 1000x | B | circular | fields | preserved liquid samples, 2 levels | |
| AB1 | Modified Newman-Lampert stain solution | Nikon Eclipse E200 | optical | 1000x | A | rectangular | fields | none | Staining solution not filtered before use |
| AB2 | Modified Newman-Lampert stain solution | Nikon Eclipse E200 | optical | 1000x | A | rectangular | fields | none | Staining solution not filtered before use. New samples and other operator than AB1. |
| AC | Modified Newman-Lampert stain solution | Leica DMLB | optical | 630x | A | circular | fields | None | Regular PT participation |
| AD1 | Modified Newman-Lampert stain solution | Accu-Scope 3000 LED | optical | 800x | A | circular | fields | raw milk control set, 10 levels | |
| AE1 | Modified Newman-Lampert stain solution | Leica | optical | 1000x | A | circular | fields | raw milk control set, 10 levels | |
| AF1 | Modified Newman-Lampert stain solution | Motic BA 310 | optical | 1000x | A | circular | fields | raw milk control set, 10 levels | |
| AH1 | Modified Newman-Lampert stain solution | Olympus BH2 | optical | 1000x | A | circular | fields | preserved liquid samples, 4 levels | |

**Table F2.** Routine methods used. All laboratories applied methods which are based on flow cytometry. If a number follows the lab code (single or double letter), this indicates that this laboratory has reported more than 1 set of results (e.g. one reference and one routine method data set, 2 or more reference method results sets, 2 or more routine method results sets). – Laboratories D, G and V are not referenced in this table as those laboratories used so-called alternative methods (not visual microscopy or flow cytometry, but e.g. image cytometry methods).

| Laboratory code | Cell staining reagent | Instrument brand | Instrument model | Reconstitution protocol used | QC to check proper calibration | Reference material used for calibration | Remarks |
|---|---|---|---|---|---|---|---|
| A1 | DAPI | Delta | SomaScope Smart | A | preserved liquid milk sample, 1 level | none | |
| A2 | DAPI | Delta | SomaScope Smart | B | preserved liquid milk sample, 1 level | none | |
| A3 | DAPI | Delta | CombiScope 600 FTIR 600 HP | B | preserved liquid milk sample, 1 level | none | only 3 samples prepared and analysed |
| C1 | Acridine Orange | FOSS | Fossomatic 7 | A | preserved liquid milk samples, 3 levels | none | |
| C2 | Acridine Orange | FOSS | Fossomatic 7 | B | preserved liquid milk samples, 3 levels | none | |
| C3 | Acridine Orange | FOSS | Fossomatic 7 DC | A | preserved liquid milk samples, 3 levels | none | C1 samples analysed with second instrument |
| C4 | Acridine orange | FOSS | Fossomatic 7 DC | B | preserved liquid milk samples, 3 levels | none | C2 samples analysed with second instrument |
| E1 | EtBr | FOSS | Fossomatic FC | B | preserved liquid samples, 5 levels | recalibration every 4 weeks with preserved liquid samples, 5 levels | reconstitution problems reported for some low count samples, |
| E2 | EtBr | FOSS | Fossomatic FC | B | preserved liquid samples, 5 levels | recalibration every 4 weeks with preserved liquid samples, 5 levels | |
| F | Acridine orange | FOSS | Fossomatic FC | B | preserved liquid samples, 10 levels | recalibration every 4 weeks with preserved liquid samples, 10 levels | |
| H2 | Propidium iodide | FOSS | Fossomatic Minor | A | preserved liquid sample, 1 level | none | |
| I2 | EtBr | FOSS | Fossomatic FC | A | UHT liquid samples, 2 levels | none | |
| J2 | EtBr | FOSS | Fossomatic 5000 | A and B | preserved liquid samples , 2 levels | recalibration every 12 weeks with preserved liquid samples, 5 levels | ERM-BD001a reconstituted with protocol A, ERM-BD001b with protocol B |
| K2 | EtBr | Bentley | SomaCount SCC150 | A | preserved liquid samples, several levels | recalibration every 4 weeks with preserved liquid samples, 10 levels | |
| L1 | EtBr | FOSS | Fossomatic FC | B | preserved liquid samples (2 types), 1 level each | none | |
| L2 | EtBr | FOSS | Fossomatic FC | B | preserved liquid samples (2 types), 1 level each | none | the reconstituted milks prepared for L1 were re-analysed on a second instrument |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| M | EtBr | FOSS | Fossomatic FC | B | preserved liquid sample, 1 level | recalibration every 4 weeks if needed, with preserved liquid samples, 6 levels | |
| N | EtBr | FOSS | Fossomatic FC | B | preserved liquid samples, 5 levels | none | |
| P2 | DAPI | Delta | SomaScope MK | B | preserved liquid samples, 2 levels | recalibration every 4 weeks with preserved liquid samples, 9 levels | |
| Q2 | EtBr | FOSS | Fossomatic 7 | B | preserved liquid sample, 1 level | recalibration every 2 weeks with preserved liquid samples, 7 levels | |
| R | EtBr | FOSS | Fossomatic 5000 Basic | A | preserved liquid samples, 2 levels | none | |
| S1 | EtBr | FOSS | Fossomatic 6000 | B | preserved liquid samples, 3 levels | none | |
| S2 | EtBr | FOSS | Fossomatic 6000 | B | preserved liquid samples, 3 levels | none | other instrument than S1 |
| S3 | EtBr | FOSS | Fossomatic 6000 | B | preserved liquid samples, 3 levels | none | other instrument than S1 and S2 |
| T1 | EtBr | FOSS | Fossomatic FC | B | adjustment sample (FOSS, DK); preserved liquid samples, 5 levels | none | |
| T2 | EtBr | FOSS | Fossomatic FC | B | adjustment sample (FOSS, DK); preserved liquid samples, 5 levels | none | other instrument than T1 |
| T3 | EtBr | FOSS | Fossomatic 7 | B | adjustment sample (FOSS, DK); preserved liquid samples, 5 levels | none | other instrument than T1 and T2 |
| U2 | EtBr | FOSS | Fossomatic 5300 | B | preserved liquid sample, 1 level | none | |
| W2 | EtBr | FOSS | Fossomatic 5000 | B | preserved liquid samples, 2 levels | recalibration every week if needed, preserved liquid samples, 2 levels | |
| X2 | EtBr | Bentley | SomaCount FC | B | preserved liquid samples (2 types), 2 levels | recalibration every 4 weeks with in-house preserved samples; 5 levels | |
| Y1 | EtBr | FOSS | Fossomatic FC | A | none | recalibration every week when needed with preserved liquid samples, 2 levels, otherwise every 4 weeks with preserved liquid samples (2 types), 2 levels each | |
| Y2 | Acridine Orange | FOSS | Fossomatic 7 DC | A | none | recalibration every week when needed with preserved liquid samples, 2 levels, otherwise every 4 weeks with preserved liquid samples, 5 levels | |
| Z2 | EtBr | FOSS | Fossomatic 5000 | B | preserved liquid samples, 2 levels | none | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| AA | EtBr | FOSS | Fossomatic 7 | B | preserved liquid samples, 2 levels | none | |
| AD2 | EtBr | FOSS | Fossomatic FC | A | Preserved liquid sample, 1 level | recalibration every 2 weeks, with In-house raw milk control set, 10 levels | |
| AE2 | EtBr | FOSS | Fossomatic FC | A | preserved liquid samples (2 types), 10 and 4 levels, respectively | recalibration every 2 weeks, with In-house raw milk control set, 10 levels | |
| AF2 | EtBr | FOSS | Fossomatic FC | A | preserved liquid sample, 1 level | recalibration every 2 weeks, with In-house raw milk control set, 10 levels | |
| AG1 | EtBr | FOSS | Fossomatic FC | B | preserved liquid samples, 3 levels | recalibration every 4 weeks, with preserved liquid samples, 3 levels | only 2 instead of 3 samples prepared per day, each sample replicate analysed three times, mean taken as results |
| AG2 | EtBr | FOSS | Fossomatic FC | B | preserved liquid samples, 3 levels | recalibration every 4 weeks, with preserved liquid samples, 3 levels | as AG1. Sample measured on a different instrument |
| AH2 | EtBr | FOSS | CombiFoss 7 | A | preserved liquid samples, 4 levels | recalibration every 2 weeks, with preserved liquid samples, 12 levels | |
| AI1 | EtBr | Bentley | BactoCount IBC | A and B | lyophilised SCC preparations, 5 levels | none | reconstitution protocol A used on day 1 and B used on day 2 |
| AI1 | EtBr | Bentley | SomaCount FCM | A and B | lyophilised SCC preparations, 5 levels | none | reconstitution protocol A used on day 1 and B used on day 2 |

# **Annex G:** Results of the characterisation measurements

**ERM-BD001a, reference method results.** The individual data shown in the table (given in thousand cells/mL) are those reported by the laboratory with corrections by JRC if necessary (e.g. if the result was not reported as rounded to the nearest thousand cells/mL; in case 8 individual results were reported instead of the 4 replicate results (1 replicate is the mean of the results obtained from two smears on one slide, see section 6.2) and final rounding to the nearest thousand cells if necessary; in case the prescribed 3.00 g powder to be mixed with 27.66 g water was not fully respected)

| Laboratory code | replicate 1 [cells/mL] | replicate 2 [cells/mL] | replicate 3 [cells/mL] | replicate 4 [cells/mL] | mean [cells/mL] | Standard deviation [cells/mL] |
|---|---|---|---|---|---|---|
| B | 65000 | 58000 | 66000 | 58000 | 61750 | 4349 |
| I1 | 55000 | 57000 | 60000 | 55000 | 56750 | 2363 |
| J1 | 69000 | 66000 | 74000 | 75000 | 71000 | 4243 |
| K1 | 79000 | 79000 | 78000 | 79000 | 78750 | 500 |
| O | 79000 | 81000 | 78000 | 81000 | 79750 | 1500 |
| P1 | 73000 | 77000 | 77000 | 77000 | 76000 | 2000 |
| Q1 | 53000 | 54000 | 52000 | 50000 | 52250 | 1708 |
| U1 | 57000 | 57000 | 58000 | 56000 | 57000 | 816 |
| W1 | 59000 | 54000 | 63000 | 60000 | 59000 | 3742 |
| X1 | 63000 | 62000 | 59000 | 53000 | 59500 | 4041 |
| Z1 | 76000 | 70000 | 69000 | 62000 | 69250 | 5737 |
| AC | 54000 | 56000 | 47000 | 54000 | 52750 | 3948 |
| AE1 | 69000 | 65000 | 69000 | 65000 | 67000 | 2309 |
| AH1 | 59000 | 62000 | 65000 | 62000 | 62000 | 2449 |
| *H1* | *65000* | *69000* | *72000* | *73000* | *69750* | *3594* |
| *AB1* | *111000* | *116000* | *93000* | *98000* | *104500* | *10786* |
| *AB2* | *99000* | *100000* | *90000* | *99000* | *97000* | *4690* |
| *AD1* | *67000* | *57000* | *76000* | *48000* | *62000* | *12138* |
| *AF1* | *43000* | *44000* | *59000* | *41000* | *48750* | *8261* |

Please note: 5 data sets (H1, AB1, AB2, AD1, AF1) were rejected for technical reasons and therefore not taken into account for value assignment (see pages 24 and 25 for explanation).

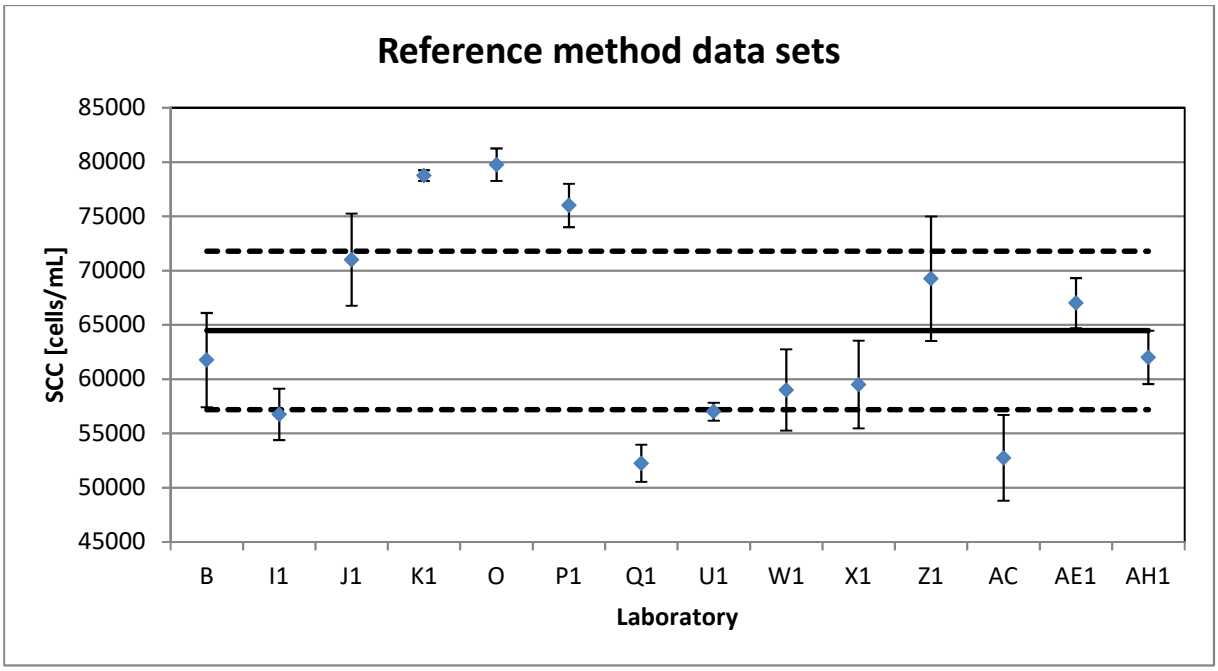


**Figure G1.** ERM-BD001a – accepted reference method data sets. Laboratory means with their standard deviations are represented as error bars. The bold line and the two dashed lines correspond to the mean of laboratory means ± $U_{CRM}$

**ERM-BD001a, routine method results.** The data shown in the table (given is thousand cells/mL) are those reported by the laboratory with corrections by JRC if necessary (e.g. if the result was not reported as rounded to the nearest thousand cells/mL; in case the prescribed 3.00 g powder to be mixed with 27.66 g water was not fully respected)

| Laboratory code | replicate 1 [cells/mL] | replicate 2 [cells/mL] | replicate 3 [cells/mL] | replicate 4 [cells/mL] | replicate 5 [cells/mL] | replicate 6 [cells/mL] | mean [cells/mL] | Standard deviation [cells/mL] | Slope |
|---|---|---|---|---|---|---|---|---|---|
| **A1** | **49000** | **54000** | **52000** | **50000** | **49000** | **50000** | **50667** | **1966** | **1.01** |
| A2 | 52000 | 48000 | 48000 | 47000 | 49000 | 51000 | 48833 | 1472 | 1.01 |
| A3 | 49000 | 50000 | 49000 | | | | 49333 | 577 | 1.01 |
| C1 | 63000 | 58000 | 66000 | 61000 | 62000 | 65000 | 62500 | 2881 | 1 |
| C2 | 66000 | 62000 | 65000 | 67000 | 60000 | 61000 | 63500 | 2881 | 1 |
| C3 | 57000 | 56000 | 53000 | 59000 | 58000 | 55000 | 56333 | 2160 | 1 |
| C4 | 61000 | 59000 | 63000 | 73000 | 66000 | 64000 | 64333 | 4885 | 1 |
| E2 | 80000 | 71000 | 75000 | 77000 | 74000 | 77000 | 75667 | 3077 | 1.3 |
| F | 78000 | 75000 | 72000 | 78000 | 77000 | 81000 | 76833 | 3061 | 1.34 |
| **H2** | **61000** | **58000** | **62000** | **65000** | **60000** | **60000** | **61000** | **2366** | **1** |
| I2 | 67000 | 64000 | 69000 | 64000 | 69000 | 68000 | 66833 | 2317 | 1 |
| K2 | 76000 | 69000 | 70000 | 72000 | 64000 | 68000 | 69833 | 4021 | 0.56 |
| **L1** | **62000** | **63000** | **60000** | **60000** | **58000** | **64000** | **61167** | **2229** | **1** |
| **L2** | **59000** | **67000** | **62000** | **63000** | **63000** | **63000** | **62833** | **2563** | **1** |
| **M** | **57000** | **57000** | **58000** | **66000** | **67000** | **60000** | **60833** | **4535** | **0.99566** |
| N | 60000 | 57000 | 61000 | 59000 | 64000 | 62000 | 60500 | 2429 | 1 |
| P2 | 78000 | 79000 | 79000 | 78000 | 76000 | 72000 | 77000 | 2683 | 1 |
| **Q2** | **52000** | **52000** | **54000** | **57000** | **50000** | **47000** | **52000** | **3406** | **1.0135** |
| **R** | **63000** | **60000** | **61000** | **70000** | **65000** | **64000** | **63833** | **3545** | **1** |
| S1 | 63000 | 67000 | 63000 | 63000 | 66000 | 66000 | 64667 | 1862 | 1.15 |
| S2 | 72000 | 78000 | 75000 | 77000 | 77000 | 88000 | 77833 | 5419 | 1.14 |
| S3 | 58000 | 66000 | 61000 | 63000 | 68000 | 69000 | 64167 | 4262 | 1.10 |
| **T1** | **53000** | **58000** | **58000** | **64000** | **55000** | **60000** | **58000** | **3847** | **1** |
| T2 | 60000 | 51000 | 52000 | 58000 | 58000 | 55000 | 55667 | 3615 | 1 |
| T3 | 61000 | 57000 | 59000 | 57000 | 61000 | 55000 | 58333 | 2422 | 1 |
| U2 | 60000 | 57000 | 57000 | 55000 | 55000 | 53000 | 56167 | 2401 | 1.04 |
| W2 | 58000 | 58000 | 58000 | 56000 | 60000 | 57000 | 57833 | 1329 | 1.011 |
| X2 | 55000 | 54000 | 52000 | 51000 | 55000 | 51000 | 53000 | 1897 | 0.98 |
| **Y1** | **65000** | **66000** | **69000** | **59000** | **62000** | **65000** | **64333** | **3445** | **1** |
| Y2 | 54000 | 51000 | 52000 | 52000 | 49000 | 56000 | 52333 | 2422 | 1 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Z2** | **63000** | **72000** | **73000** | **68000** | **69000** | **64000** | **68167** | **4070** | **1** |
| **AA** | **60000** | **64000** | **59000** | **60000** | **63000** | **61000** | **61167** | **1941** | **1** |
| AD2 | 52000 | 57000 | 66000 | 59000 | 59000 | 67000 | 60000 | 5657 | 0.98256 |
| **AE2** | **63000** | **62000** | **64000** | **61000** | **60000** | **61000** | **61833** | **1472** | **0.97944** |
| **AF2** | **53000** | **59000** | **57000** | **57000** | **60000** | **56000** | **57000** | **2449** | **0.96455** |
| AG1 | 72000 | 67000 | 68000 | 71000 | | | 69500 | 2380 | 1.13 |
| AG2 | 78000 | 70000 | 68000 | 65000 | | | 70250 | 5560 | 1.14 |
| AH2 | 61000 | 67000 | 62000 | 61000 | 65000 | 62000 | 63000 | 2449 | 1.02 |
| **AI1** | **52000** | **54000** | **53000** | **50000** | **54000** | **51000** | **52333** | **1633** | **1** |
| AI2 | 52000 | 56000 | 55000 | 56000 | 57000 | 57000 | 55500 | 1871 | 1 |
| *E1* | *213000* | *183000* | *98000* | *305000* | *142000* | *149000* | *181667* | *71882* | *1.3* |
| *J2** | *86000* | *77000* | *93000* | *86000* | *80000* | *83000* | *85500* | *5831* | *1.4* |

***** 8 values reported, the two not in the table are 85000 and 94000.

Please note: 2 data sets (E1, J2) were rejected for technical reasons. Data sets with a slope outside 0.95-1.05 were not further taken into account for value assignment (see page 25 for explanation). From the 32 data sets with a reported slope within 0.95-1.05, 14 were selected fully randomly (the ones indicated in bold).

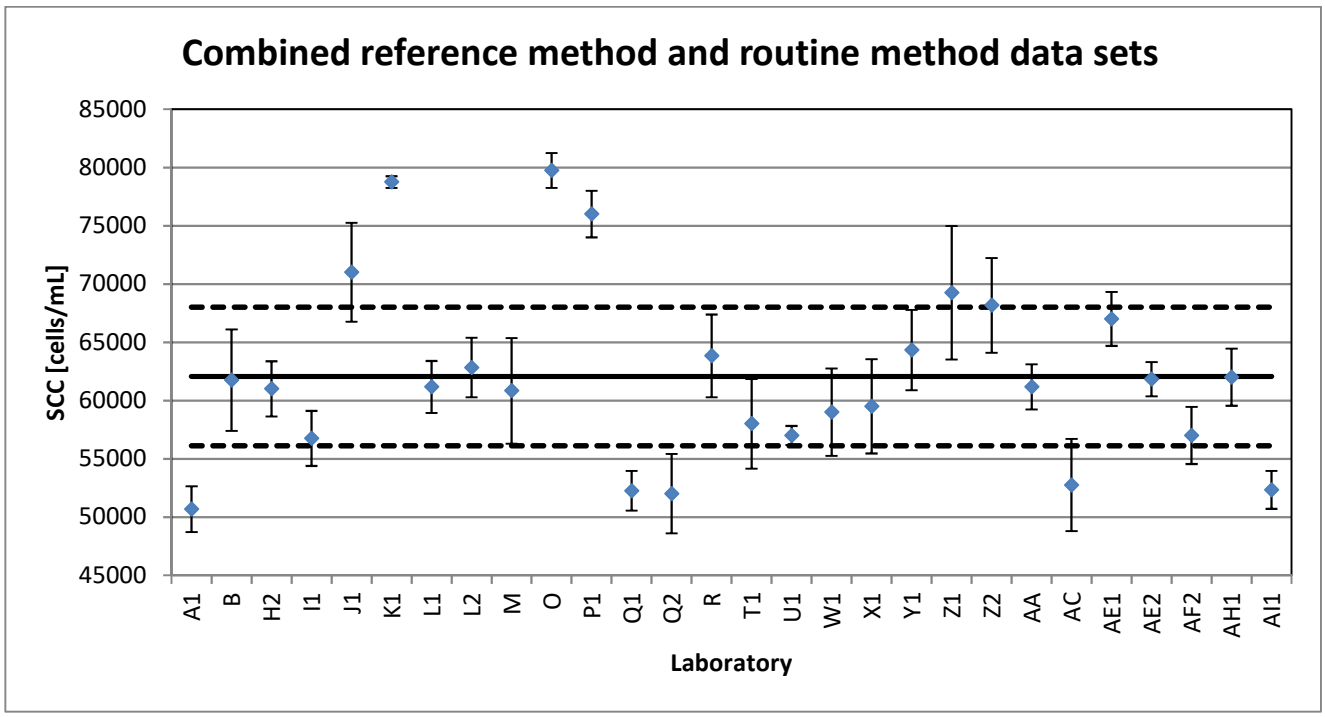


**Figure G2.** ERM-BD001a – accepted reference and routine method data sets. Laboratory means with their standard deviations are represented as error bars. The bold line and the two dashed lines correspond to the mean of laboratory means ± $U_{CRM}$

**ERM-BD001b, reference method results.** The individual data shown in the table (given in thousand cells/mL) are those reported by the laboratory with corrections by JRC if necessary (e.g. if the result was not reported as rounded to the nearest thousand cells/mL; in case 8 individual results were reported instead of the 4 replicate results (1 replicate is the mean of the results obtained from two smears on one slide, see section 6.x) and final rounding to the nearest thousand cells if necessary; in case the prescribed 3.00 g powder to be mixed with 27.66 g water was not fully respected)

| Laboratory code | replicate 1 [cells/mL] | replicate 2 [cells/mL] | replicate 3 [cells/mL] | replicate 4 [cells/mL] | mean [cells/mL] | Standard deviation [cells/mL] |
|---|---|---|---|---|---|---|
| B | 1193000 | 1253000 | 1274000 | 1212000 | 1233000 | 37068 |
| I1 | 1076000 | 1057000 | 1001000 | 1064000 | 1049500 | 33272 |
| J1 | 956000 | 969000 | 995000 | 984000 | 976000 | 17068 |
| K1 | 1545000 | 1582000 | 1523000 | 1508000 | 1539500 | 32151 |
| O | 1540000 | 1540000 | 1545000 | 1554000 | 1544750 | 6602 |
| P1 | 1270000 | 1258000 | 1262000 | 1258000 | 1262000 | 5657 |
| Q1 | 1081000 | 1091000 | 1109000 | 1085000 | 1091500 | 12369 |
| U1 | 1134000 | 1142000 | 1154000 | 1158000 | 1147000 | 11015 |
| W1 | 1186000 | 1114000 | 1213000 | 1251000 | 1191000 | 57845 |
| X1 | 1075000 | 1060000 | 1024000 | 1091000 | 1062500 | 28618 |
| Z1 | 1344000 | 1430000 | 1370000 | 1336000 | 1370000 | 42552 |
| AC | 972000 | 928000 | 994000 | 935000 | 957250 | 31192 |
| AE1 | 1178000 | 1198000 | 1251000 | 1204000 | 1207750 | 30902 |
| *H1* | *1344000* | *1392000* | *1305000* | *1294000* | *1333750* | *44365* |
| *AB1* | *1321000* | *1253000* | *1388000* | *1355000* | *1329250* | *57726* |
| *AB2* | *1082000* | *1078000* | *1338000* | *1113000* | *1152750* | *124487* |
| *AD1* | *1393000* | *1369000* | *1217000* | *1230000* | *1302500* | *91613* |
| *AF1* | *859000* | *798000* | *941000* | *866000* | *866000* | *58589* |
| *AH1* | *1517000* | *1441000* | *1495000* | *1453000* | *1476500* | *35567* |

Please note: 6 data sets (H1, AB1, AB2, AD1, AF1, AH1) were rejected for technical reasons and therefore not taken into account for value assignment (see pages 24-25 for explanation).

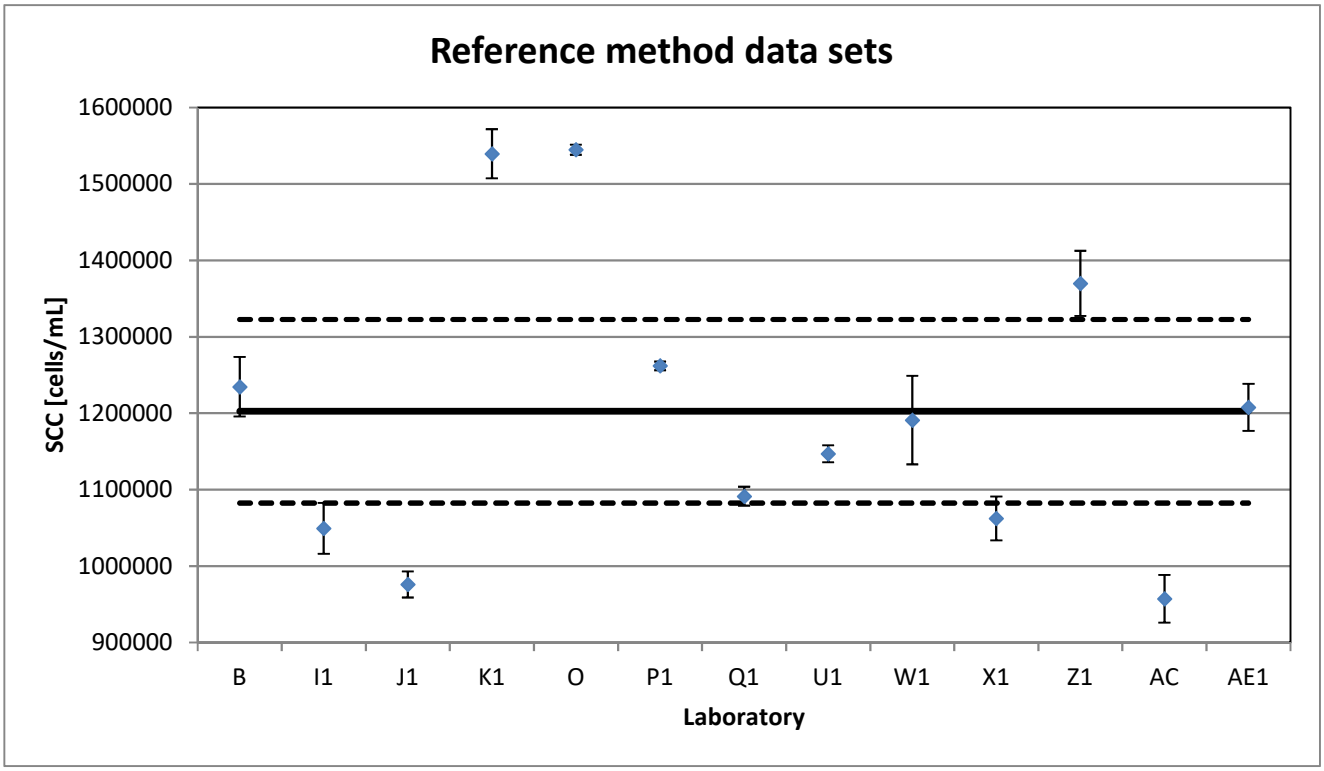


**Figure G3.** ERM-BD001b – accepted reference and routine method data sets. Laboratory means with their standard deviations are represented as error bars. The bold line and the two dashed lines correspond to the mean of laboratory means ± $U_{CRM}$

**ERM-BD001b, routine method results.** The data shown in the table (given is thousand cells/mL) are those reported by the laboratory with corrections by JRC if necessary (e.g. if the result was not reported as rounded to the nearest thousand cells/mL; in case the prescribed 3.00 g powder to be mixed with 27.66 g water was not fully respected)

| Laboratory code | replicate 1 [cells/mL] | replicate 2 [cells/mL] | replicate 3 [cells/mL] | replicate 4 [cells/mL] | replicate 5 [cells/mL] | replicate 6 [cells/mL] | mean [cells/mL] | Standard deviation [cells/mL] | Slope |
|---|---|---|---|---|---|---|---|---|---|
| A1 | 1131000 | 1161000 | 1116000 | 1110000 | 1086000 | 1061000 | 1110833 | 34787 | 1.01 |
| A2 | 1148000 | 1110000 | 1160000 | 1119000 | 1157000 | 1138000 | 1138667 | 20432 | 1.01 |
| **A3** | **1140000** | **1146000** | **1143000** | | | | **1143000** | **3000** | **1.01** |
| C1 | 1186000 | 1167000 | 1163000 | 1213000 | 1202000 | 1169000 | 1183333 | 20598 | 1 |
| C2 | 1175000 | 1175000 | 1199000 | 1121000 | 1119000 | 1141000 | 1153000 | 34176 | 1 |
| C3 | 1123000 | 1142000 | 1131000 | 1140000 | 1119000 | 1141000 | 1132667 | 9933 | 1 |
| C4 | 1169000 | 1192000 | 1170000 | 1122000 | 1118000 | 1133000 | 1150667 | 30395 | 1 |
| E1 | 1500000 | 1524000 | 1525000 | 1526000 | 1514000 | 1510000 | 1516500 | 10387 | 1.3 |
| E2 | 1470000 | 1461000 | 1461000 | 1501000 | 1497000 | 1477000 | 1477833 | 17509 | 1.3 |
| F | 1601000 | 1609000 | 1625000 | 1614000 | 1598000 | 1632000 | 1613167 | 13348 | 1.34 |
| H2 | 1365000 | 1360000 | 1313000 | 1397000 | 1406000 | 1394000 | 1372500 | 34460 | 1 |
| I2 | 1175000 | 1182000 | 1181000 | 1174000 | 1155000 | 1161000 | 1171333 | 10967 | 1 |
| K2 | 1646000 | 1623000 | 1617000 | 1533000 | 1524000 | 1538000 | 1580167 | 54190 | 0.56 |
| **L1** | **1202000** | **1164000** | **1192000** | **1165000** | **1177000** | **1164000** | **1177333** | **16318** | **1** |
| L2 | 1207000 | 1187000 | 1184000 | 1207000 | 1207000 | 1240000 | 1205333 | 20007 | 1 |
| M | 1111000 | 1062000 | 1099000 | 1104000 | 1153000 | 1125000 | 1109000 | 30100 | 0.99566 |
| **N** | **1220000** | **1180000** | **1204000** | **1158000** | **1185000** | **1160000** | **1184500** | **24345** | **1** |
| P2 | 1378000 | 1384000 | 1375000 | 1348000 | 1357000 | 1367000 | 1368167 | 13615 | 1 |
| Q2 | 1029000 | 1028000 | 1011000 | 1071000 | 1046000 | 1051000 | 1039333 | 21078 | 1.0135 |
| **R** | **1025000** | **1100000** | **1045000** | **1037000** | **1057000** | **1036000** | **1050000** | **26698** | **1** |
| S1 | 1299000 | 1374000 | 1360000 | 1332000 | 1355000 | 1327000 | 1341167 | 27155 | 1.15 |
| S2 | 1271000 | 1261000 | 1278000 | 1278000 | 1259000 | 1262000 | 1268167 | 8658 | 1.14 |
| S3 | 1288000 | 1280000 | 1270000 | 1283000 | 1270000 | 1298000 | 1281500 | 10803 | 1.10 |
| T1 | 1077000 | 1084000 | 1112000 | 1114000 | 1133000 | 1176000 | 1116000 | 35928 | 1 |
| **T2** | **1134000** | **1115000** | **1107000** | **1143000** | **1150000** | **1123000** | **1128667** | **16597** | **1** |
| **T3** | **1139000** | **1150000** | **1144000** | **1171000** | **1153000** | **1130000** | **1147833** | **13992** | **1** |
| **U2** | **1111000** | **1099000** | **1114000** | **1114000** | **1105000** | **1119000** | **1110333** | **7202** | **1.04** |
| **W2** | **1189000** | **1192000** | **1183000** | **1110000** | **1171000** | **1170000** | **1169167** | **30367** | **1.011** |
| X2 | 1113000 | 1176000 | 1160000 | 1162000 | 1144000 | 1169000 | 1154000 | 22760 | 0.98 |
| **Y1** | **1151000** | **1135000** | **1113000** | **1120000** | **1122000** | **1117000** | **1126333** | **14194** | **1** |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Y2** | **1099000** | **1116000** | **1075000** | **1109000** | **1145000** | **1111000** | **1109167** | **22825** | **1** |
| Z2 | 1161000 | 1192000 | 1132000 | 1122000 | 1133000 | 1155000 | 1149167 | 25701 | 1 |
| AA | 1205000 | 1203000 | 1192000 | 1203000 | 1231000 | 1217000 | 1208500 | 13590 | 1 |
| **AD2** | **1117000** | **1116000** | **1177000** | **1137000** | **1120000** | **1166000** | **1138833** | **26649** | **0.98256** |
| AE2 | 1114000 | 1085000 | 1119000 | 1068000 | 1109000 | 1092000 | 1097833 | 19610 | 0.97944 |
| **AF2** | **1061000** | **1055000** | **1065000** | **1070000** | **1091000** | **1073000** | **1069167** | **12465** | **0.96455** |
| AG1 | 1423000 | 1438000 | 1303000 | 1349000 | | | 1378250 | 63484 | 1.13 |
| AG2 | 1433000 | 1384000 | 1316000 | 1357000 | | | 1372500 | 49075 | 1.14 |
| AH2 | 1228000 | 1219000 | 1194000 | 1222000 | 1220000 | 1195000 | 1213000 | 14670 | 1.02 |
| AI1 | 1119000 | 1113000 | 1098000 | 1133000 | 1130000 | 1121000 | 1115333 | 10690 | 1 |
| **AI2** | **1125000** | **1113000** | **1098000** | **1133000** | **1130000** | **1121000** | **1120000** | **12869** | **1** |
| *J2** | *765000* | *746000* | *798000* | *773000* | *757000* | *766000* | *773375* | *18776* | *1.4* |

***** 8 values reported, the two not in the table are 798000 and 784000.

Please note: 1 data set (J2) was rejected for technical reasons. Data sets with a slope outside 0.95-1.05 were not further taken into account for value assignment (see page 25 for explanation). From the 32 data sets with a reported slope within 0.95-1.05, 13 were selected fully randomly (the ones indicated in bold).

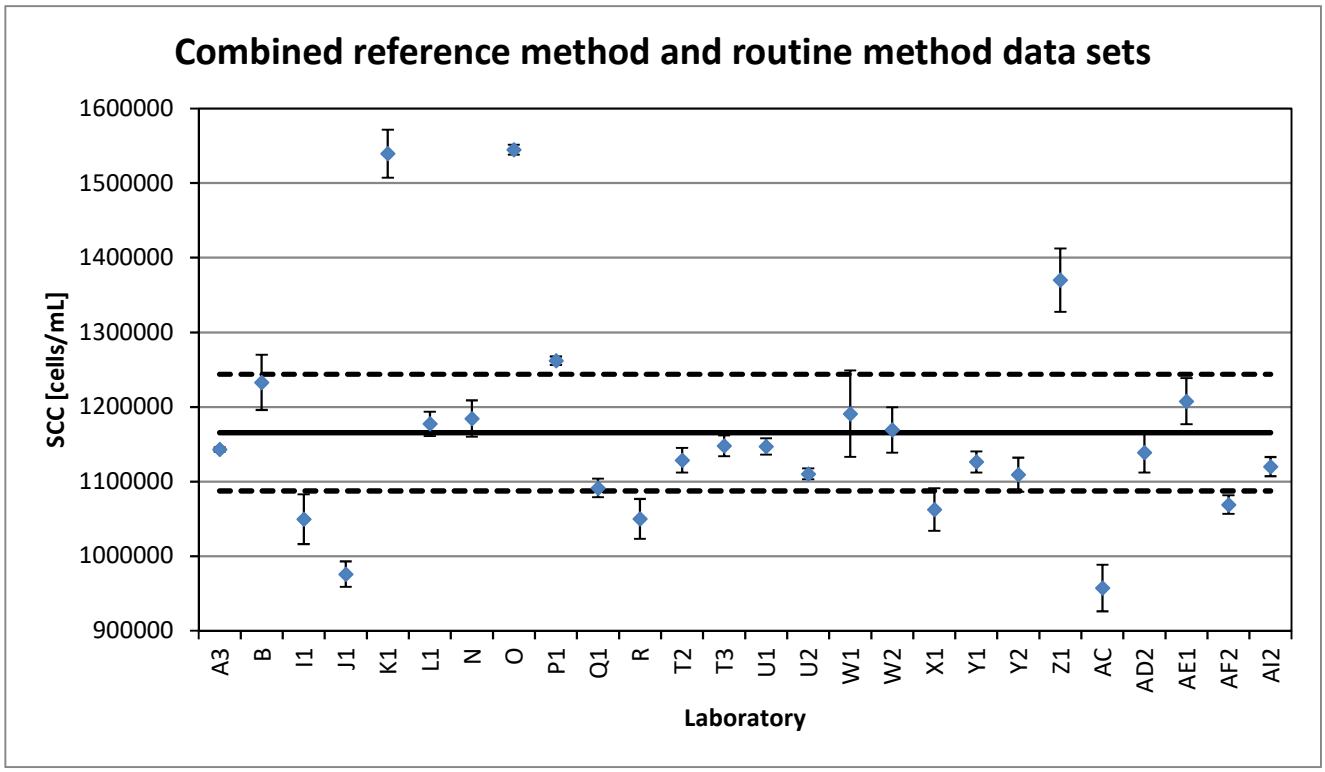


**Figure G4.** ERM-BD001b – accepted reference method and routine method data sets. Laboratory means with their standard deviations are represented as error bars. The bold line and the two dashed lines correspond to the mean of laboratory means ± $U_{CRM}$

European Commission

**EUR 30063 EN – Joint Research Centre – Directorate F – Health, Consumers and Reference Materials**
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