

TRACEABILITY OF RESULTS: DEVELOPMENT OF A SECONDARY CERTIFIED REFERENCE MATERIAL FOR SOMATIC CELL COUNTING FOR MILK QUALITY CONTROL.

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ABSTRACT

The nutritional value of milk, its quality and acceptance by consumers depend on its composition. Its analysis is essential to support livestock and dairy production, since it provides information on its quality, composition, and safety, and in several countries, determines the payment for quality to the producer. For this reason, laboratories must be technically competent and ensure the validity of their results through metrological traceability to a stable and common international reference, which guarantees the comparability and global acceptance of measurement results. To achieve this, the use of Certified Reference Materials (CRM) is key, since it allows the calibration of rapid measurement equipment, widely used by the industry to obtain results in less time without compromising accuracy. This requires the development of regional capabilities to produce CRM in the dairy sector, contributing to obtaining valid results and improving competitiveness in global markets. In 2020, the Joint Research Center launched the CRM ERM®-BD001, with two levels of Somatic Cell Count (SCC) in spray-dried cow milk in an inert gas atmosphere, which is metrologically traceable to the International System of Units (SI) and ensures global analytical equivalence.

In this framework, the Laboratorio de Materiales de Referencia of INTI-Argentina used ERM®-BD001 to produce secondary CRMs (INTI-MRC002) in a matrix similar to natural milk, achieving metrological traceability of the assigned SCC values to the SI through the certified values of the primary CRM. The INTI-MRC002 met the requirements of ISO 17034:2017 and its associated guidelines/standards, ensuring the suitability of the production, packaging and storage process, and ensuring its fitness for use. Recognition of the quality management system as producers of reference materials was achieved before the QSTF of the SIM. This advancement has strengthened the metrological traceability chain in Latin America, ensuring the comparability of the results and their acceptance at an international level.

Keywords: *certified reference material (CRM), somatic cell count (SCC), metrological traceability.*

INTRODUCTION

Milk is one of the most versatile and valuable foods in the food industry and has been known as the most complete food in nature for millennia (Linehan K. *et. al.*, 2024). According to the Food and Agriculture Organization (FAO), approximately 80% of the annual milk production comes from cows, with the remainder from other dairy animals such as buffalo, goats, camels, and sheep.

The composition of milk determines its nutritional quality, its value as a raw material for the production of food products, and its acceptance by consumers. Its analysis is essential to support livestock and dairy production, as it provides information on its quality, composition, and safety. Furthermore, it enables the identification of factors affecting production, the optimization of livestock feeding and management, and the improvement of production efficiency.

In many countries, programs include the physicochemical, microbiological, and sanitary analysis of raw milk, enabling the implementation of payment systems based on its quality. These systems represent one of the approaches used by dairy companies and industries to raise awareness and incentivize milk producers to improve milk quality. They play a crucial role in determining the final price paid to producers, which is based on the levels of specific milk components, such as proteins, somatic cell count, total bacterial count, among others (Revelli, G. R., (2011); Busanello, M., (2020); Resolución Conjunta N° 739 y 495 de 2011).

For this reason, testing laboratories associated with this activity must be technically competent and ensure the validity of their results through metrological traceability. Traceability to a common and stable international reference guarantees the comparability and global acceptance of measurement results (Murcia-Rubiano, F. *et. al.*, 2021).

Metrological traceability is defined through the International Vocabulary of Metrology (VIM) as property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributes to the measurement uncertainty (Joint Committee for Guides in Metrology [JCGM], 2012).

Comparability of measurements is an essential feature of an international system within measurement results can be universally accepted and can only be guaranteed if those results can be linked to internationally recognized references and comparison through their relationship to that common stable reference (Centro Nacional de Metrología [CENAM], 2002).

Comply with this, laboratories and industries design and implement different tools of ensuring the validity of its results, among which are, without limiting, participation in proficiency testing and interlaboratory comparisons, internal quality control, analysis of spiked samples and

use of reference material. The latter is particularly effective for evaluating and demonstrating the validity of analytical data (Barwick, V. *et. al.*, 2001).

The ISO Guide 30 (2015) defines a reference material (RM) as a material that is sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process. Furthermore, defines a Certified reference material (CRM) is a reference material characterized by a metrologically valid procedure for one or more specified properties, accompanied by a reference material certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability.

Padua-Gandra *et. al.* (2016) highlights the relevance of method validation processes and the development of internal quality, and traceability of measurements to international systems. Thus, it is necessary to build capabilities within the country for the development and production of CRM that can be used in the food sector. This contribution ensures the validity of results in laboratories and, consequently, enhances competitiveness in the market by achieving valid outcomes that support products in global markets.

The production of RM and CRM is governed by international standards, such as ISO 17034 (2016) and its associated guidelines and is carried out by various institutions, including National Metrology Institutes (NMIs) like the Instituto Nacional de Tecnología Industrial (INTI).

In Argentina, Laboratorio de Materiales de Referencia of INTI-Lácteos headquarters in Rafaela, through its Sistema Centralizado de Calibración (SICECAL®), designs, produces and distributes RM and CRM of different dairy matrices. These materials are acquired and used by national and Latin American laboratories that analyze milk, dairy products and derivatives for calibration, control of their measuring equipment and evaluation of analytical and instrumental

methods, ensuring the validity of the results they obtain. An example of this is the CRM of somatic cell count in milk.

The somatic cell count (SCC) in milk is used a crucial diagnostic tool to evaluate udder health status and ensure milk quality and safety. High SCC levels are generally associated with mastitis, an infection of the mammary gland that impacts milk quality and profitability, leading to losses due to reduced production, lower quality and safety, and increased veterinary costs. (Schukken *et al.*, 2003; International Dairy Federation [IDF], 2021, p.15; Baltián, L. R. *et al.*, 2023).

The importance of raw milk quality is undeniable in dairy production, since it is closely linked to flavor, sensory and physicochemical aspects, as well as hygiene, nutrition and safety of the final dairy products (Ntuli *et. al.*, 2023).

Different methods are used to determine SCC in milk. There are "direct" methods, which involve counting particles or cells, and "indirect" methods, which estimate the number of cells based on reactions revealing cellular components. The direct microscopic counting is considered the reference method by the International Organization for Standardization (ISO) y la International Dairy Federation (IDF) according to ISO 13366-1:2008/Cor 1: 2009 | IDF 148-1:2008/Cor 1: 2009. This method is laborious and requires a very well-trained analyst. In contrast, among the indirect methods, one of the automated SCC methods are based on flow cytometry, which stains cells and detects the stained particles using light energy emitted by them. In these methods, a sample is mixed with a buffer and a dye, then transferred to a rotating disk illuminated by a Zenon lamp. The light emitted by the cells is filtered and detected by a photomultiplier, generating an electrical pulse that is amplified (Di Marzo, L., 2016). According to the provisions of the reference standard ISO 13366-2:2006 | IDF 148-2:2006, periodic calibration of these devices is essential to guarantee

their precision. This method is widely used by dairy laboratories, allowing large-scale counts at low cost and with considerably greater precision. However, said reference standard raises the issue of the lack of availability of a CRM that is an integral part of a reference system for SCC in milk.

Since 2008, la IDF, International Committee for Animal Recording (ICAR) and European Commission's Joint Research Centre (EC JRC) have closely collaborated in a joint project to develop solutions and tools to promote a better global equivalence in SCC in milk.

Thus, in 2020, the JRC launched an CRM (CRM ERM®-BD001) with 2 levels of SCC in milk powder in an inert gas atmosphere. As SCC is a method-dependent measurand, the characterization of this CRM was conducted through a network of laboratories that applied the same reference measurement procedure (ISO 13366-1:2008/Cor 1: 2009 | IDF 148-1:2008/Cor 1: 2009).

Furthermore, the producer of the CRM declares that the certified value is traceable to SI units, which is based on the traceability of all relevant input factors, such as the calibrated micropipette used for milk sampling or the slide pre-marked with an outline shape (circular or rectangular) with a defined area. Since the assigned values are a combination of consistent results individually traceable to the SI, the assigned quantity values themselves are also traceable to the SI. The use of CRM allows a direct connection to be established to the SI, ensuring analytical equivalence on a global scale and providing an anchor point to which secondary reference materials can be linked (EC Joint Research Centre, 2020)¹.

In this context, the Laboratorio de Materiales de Referencia of INTI-Argentina used ERM®-BD001 to produce secondary CRMs (INTI-MRC002) in a matrix similar to natural milk,

¹ Metrological traceability of the primary reference material ERM®-BD001 declared in the certification report by the producer. Doi:10.2760/681742

achieving the metrological traceability of the assigned SCC values to the SI through the certified values of the primary CRM. The expectation for this was to promote better equivalence of SCC results following the metrological traceability chain.

MATERIALS AND METHODS

Selection of raw material

To achieve 5 candidate CRM with low, medium and high SCC values covering a range between 50 000 cells/ml and 1 200 000 cells/ml established by the reference standard, raw milk from authorized dairy farms and industries was acquired, and selected once the SCC and its suitability for use were determined.

Analytical Method

The analytical methods utilized for assigning values adhere to the ISO-IDF Standards.

- ISO 13366-1:2008/Cor 1: 2009 | IDF 148-1:2008/Cor 1: 2009. Milk – Enumeration of somatic cells – Part 1: Microscopic method (Reference method). The Laboratory is accredited by the Organismo Argentino de Acreditación (OAA) for its application of this method.
- ISO 13366-2:2006 | IDF 148-2:2006. Milk – Enumeration of somatic cells – Part 2: Guidance on the operation of fluoro-opto-electronic counters. These measurements are conducted in external subcontracted laboratories, accredited by the OAA.

CRM Preparation

After selecting the raw material, the necessary mixtures were prepared to create 5 sublots each falling within the following ranges: 50 000 cells/ml and 200 000 cells/ml, 201 000 cells/ml

and 400 000 cells/ml, 401 000 cells/ml and 600 000 cells/ml, 601 000 cells/ml and 800 000 cells/ml and 801 000 cells/ml and 1 200 000 cells/ml. These sublots were then analyzed using the BacSomatic™ FOSS analyzer, which conforms to the method described in ISO 13366-2:2006 | IDF 148-2:2006. Each subplot was labeled as INTI-MRC002-G2304 from 1 to 5.

Each subbatch of candidate CRM was filtered to remove any suspended “macroparticles”. Subsequently, it was transferred to glass jars and subjected to heat treatment using a flowing steam autoclave to eliminate bacterial flora.

The conditioned candidate CRM was homogenized in a thermostated water bath with gentle stirring by inversion. Working in an aseptic environment, 2-bromo-2-nitro-1,3-propanediol (Bronopol) was added as a preservative and the mixture was divided into sterile 50 ml plastic bottles, each labeled accordingly.

Homogeneity Study

Following the recommendations of ISO Guide 35 (2017), a homogeneity study was conducted, involving the analysis from each subplot, a subset of 5 units, employing a simple random sampling strategy. The determination of SCC was then performed on these units in a single measurement run, in a completely random order, using the BacSomatic™ FOSS analyzer.

The analytical results obtained were evaluated using the Grubbs test for outlier detection. Trend analysis of the results was conducted regarding the packaging order and the order of analysis execution, followed by the assessment of intra-unit and inter-unit homogeneity through one-way analysis of variance (ANOVA), applying F-test at a significance level of 0,05.

The homogeneity uncertainties for each subplot were estimated following the guidelines of ISO Guide 35 (2017), applying the formulas (1), (2), (3), and (4):

$$u_{hom} = u_{bb} = u'_{bb} \quad (1)$$

$$u_{bb} = s_{bb} \quad (2)$$

$$S_{bb}^2 = \frac{MS_{bb} - MS_w}{n} \quad (3)$$

$$u_{bb}^2 = \frac{MS_w}{n} \sqrt{\frac{2}{V_{MS_w}}} \quad (4)$$

s_{bb} Between-unit component of variance from a homogeneity study, expressed as a standard deviation.

V_{MS_w} Within-unit mean squares degrees of freedom.

n Number of observations per unit.

MS_{bb} Mean square or variance between units.

MS_w Mean square or variance within the unit.

u_{bb} o u'_{bb} Standard uncertainty associated with between-unit variability.

Stability Study

A long-term stability study was conducted, performing 2 measurements at each time point (1, 8, 15, 22, 29, 36, 43, 50, 57, and 62 days), under storage conditions of $4\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$. For the statistical analysis of the results, a linear regression analysis of variance was conducted, considering the case of linear behavior and applying Fisher's F-test at a significance level of 0,05.

Stability uncertainties were estimated for each subplot following the guidelines of ISO Guide 35 (2017), applying formula (5).

$$u_{est} = s_{\beta_1} \times (t_{m1} + t_{cert}) \quad (5)$$

- $s_{\beta 1}$ Standard deviation associated with the slope $\beta 1$ of the regression (standard error for the estimated slope)
- t_{m1} Time interval between value assignment and the initial stability monitoring point in days.
- t_{cert} Period of validity of certificate issued.

Characterization

To conduct the analytical measurement, individual samples of CRM candidates are randomly selected.

Characterization was performed using the reference measurement procedure at the Laboratorio de Materiales de Referencia and the fluoro-opto-electronic method was employed in multiple accredited laboratories.

Characterizations uncertainty was estimated by the weighed the standard uncertainty of the reference method results (u_{LMR}) with the standard uncertainty of the fluor-opto-electronic method results (u_{LS}), following formula (6).

$$u_{char} = 0,5 \times u_{LMR} + 0,5 \times u_{LS} \quad (6)$$

Primary Reference Material Used for Trueness Assessment

The CRM ERM®-BD001, traceable to the International System of Units (SI), was used as the primary reference material to evaluate the trueness of the reference measurement procedure. For this purpose, it was prepared and analyzed by reference, according to the supplier's instructions.

Trueness was evaluated by comparing the result obtained by the laboratory with the value assigned to the CRM ERM®-BD001 declared in its certificate, using the formula (7) as the acceptance criterion.

$$|x_{med} - x_{MRC}| \leq k \sqrt{u_{med}^2 + u_{MRC}^2} \quad (7)$$

x_{med} Average value obtained in the CRM ERM®-BD001 measurement.

x_{MRC} SCC certified value.

u_{med} Standard uncertainty associated with the average value obtained by measuring the SCC of the CRM ERM®-BD001.

u_{MRC} Standard uncertainty associated with the certified value of SCC.

Value Assignment and Uncertainty Estimation

Each assigned value was calculated by weighed the averages of the results obtained according to both the reference method ($\overline{x_{LMR}}$) and the routine method ($\overline{x_{LS}}$), after evaluating the presence of outliers according to formula (8).

$$SCC \text{ assigned value} = 0,5 \times \overline{x_{LMR}} + 0,5 \times \overline{x_{LS}} \quad (8)$$

The uncertainty of the assigned value was estimated by a quadratic combination of the standard uncertainty from the characterization (u_{char}), the standard uncertainty provided by the homogeneity study (u_{hom}) and the standard uncertainty provided by the long-term stability study (u_{lts}). A coverage factor $k = 2$ (95% confidence) was applied to obtain the expanded uncertainties, as indicated by the following formula (9).

$$U = 2 \times \sqrt{(u_{char})^2 + (u_{hom})^2 + (u_{lts})^2} \quad (9)$$

RESULTS AND DISCUSSION

Homogeneity Study

The results for the homogeneity study were subjected to the Grubbs test, to detect outlier values in the dataset. In the analyzed batch, no outlier values were detected, which allowed the initial dataset to be used for subsequent statistical analysis

The absence of trends in the packaging and in the measurement sequence was verified through a linear regression study, where it was established as an acceptance criterion that the confidence interval of the slope of the data included the value zero. Table 1 presents the results of the trend analysis for each subplot (1 to 5), where the absence of significant trends can be observed both during the packaging of the production batch and in the material analysis sequence.

Finally, the homogeneity of the data was verified through one-way ANOVA. Table 1 presents the results of the analysis for each of the sublots. In all cases, the $F_{\text{Calculated}}$ value was lower than the F_{Critical} value, allowing us to confirm that the means of the SCC values of the 5 analyzed containers are equal. This confirms the homogeneity of the SCC parameter in the material.

Table 1.*Results of the trend analysis and homogeneity for the 5 sublots.*

ANALYZED TREND					TEST ANOVA		UNCERTAINTY
Measurement trends (analyzed)		Processing trends (packaging)					
slope interval		slope interval			$F_{\text{Calculated}}$	F_{Critical}	u_{hom} (cells/ml)
Lower	Upper	Lower	Upper				
1	-668,6	479,8	-712,5	425,8	0,330	4,387	1 906
2	-1762,0	1531,3	-1574,4	1721,3	0,124	4,387	5 843
3	-2266,4	1476,1	-1139,7	2517,3	0,142	4,387	6 659
4	-2306,6	2474,4	-3092,4	1567,9	1,397	4,387	5 018
5	-4765,0	2030,8	-4206,0	2786,4	1,017	4,387	1 673
No significant trends were observed		No significant trends were observed			$F_{\text{Calculated}} < F_{\text{Critical}}$		

Stability Study

Following the guidelines of the reference guide for data evaluation, a regression analysis of SCC in contrast to time and a one-way analysis of variance were conducted, with the acceptance criterion that the $F_{\text{Calculated}}$ value should be lower than the critical value of $F_{(1,n-2,\alpha)}$. Table 2 presents the results of the variance study, while Figure 1 illustrates the absence of trends during the evaluated period and conditions. As a result, no significant degradation was observed, demonstrating the material's stability under these conditions. Additionally, the uncertainties associated with long-term stability are depicted.

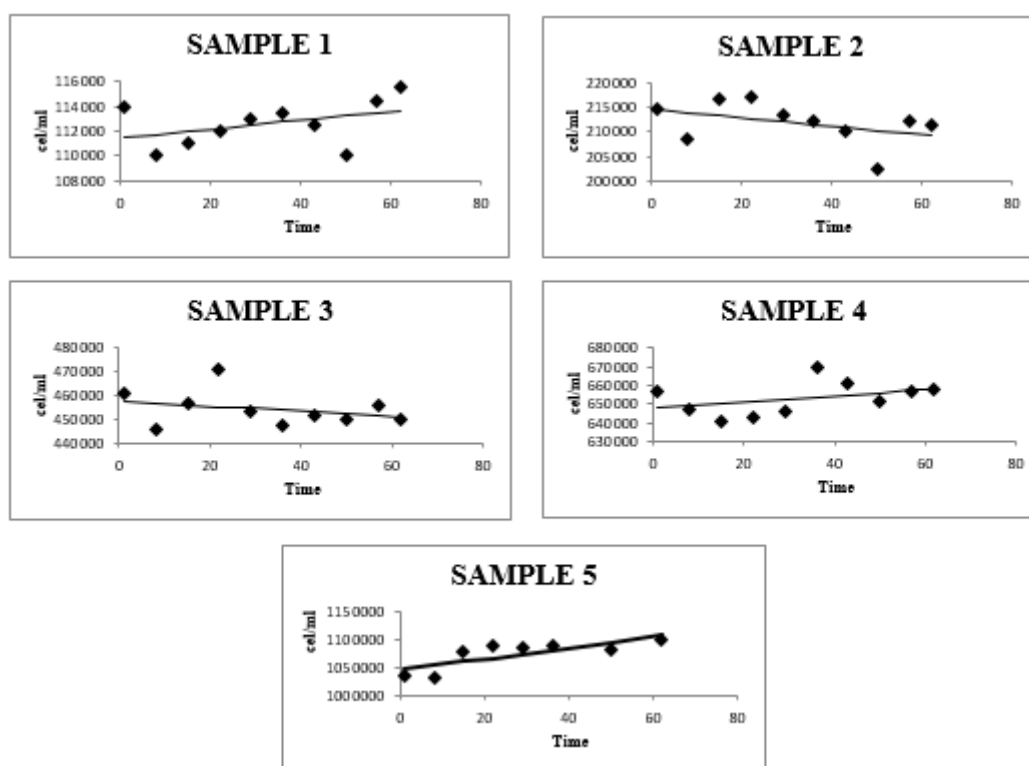
Table 2.

Data obtained from the analysis of variance for the study of stability.

SAMPLE	REGRESSION DATA		u_{Its} (cells/ml)
	$F_{Calculated}$	$F_{(1,n-2, \alpha)}$	
1	1,546	5,320	1 796
2	1,703	5,320	4 022
3	0,850	5,320	7 376
4	1,597	5,320	8 472
5	2,289	6,610	45 925

Figure 1.

Long-term stability study.



Trueness Assessment

Table 3 presents the results obtained by the laboratory during the analysis of the CRM ERM®-BD001, including the assigned value reported in the certificate and the assessment of its precision.

Measurements and property values are demonstrated to be mutually consistent within their respective uncertainties.

Table 3.

Trueness Assessment.

(cells/ml)				
Reference Value $\pm U^*$	Laboratory Value $\pm U^*$	Bias	Reproducibility Limit	$ x_{med} - x_{MRC} \leq k \sqrt{u_{med}^2 + u_{MRC}^2}$
64 000 \pm 8 000	75 000 \pm 31 228	11 000	35 160	11 000 \leq 32 236
	58 000 \pm 31 228	-6 000	27 261	6 000 \leq 32 236
1 202 000 \pm 121 000	1 135 000 \pm 31 228	-67 000	441 967	67 000 \leq 14 965
	1 112 000 \pm 31 228	-90 000	433 126	90 000 \leq 124 965

Note. *Expanded uncertainty

Material Value Assignment and CRM Uncertainty Estimation

Table 4 presents the expanded uncertainties of the CRMs estimated by combining the uncertainties from the studies mentioned above and using a coverage factor ($k = 2$) with a significance level of 95 %.

Table 4.*CRM Uncertainty Estimation.*

Sample	(cells/ml)				
	Characterization Uncertainty	Homogeneity Uncertainty	Stability Uncertainty	Combined Uncertainty	Expanded Uncertainty
1	15 125	1 906	1 795	15 349	30 698
2	32 107	5 843	4 022	32 881	65 762
3	35 931	6 659	7 376	37 280	74 560
4	42 183	5 018	8 472	43 317	86 634
5	64 378	1 673	19 374	67 251	134 502

Finally, the reference value of the CRM in SCC assigned through the characterization study was 104 056 cells/ml \pm 30 698 cells/ml, 356 041 cells/ml \pm 65 762 cells/ml, 493 864 cells/ml \pm 74 560 cells/ml, 711 346 cells/ml \pm 86 634 cells/ml y 905 160 cells/ml \pm 134 502 cells/ml for CRM 1, 2, 3, 4 y 5 respectively, completing the pack provided by the Laboratorio de Materiales de Referencia.

Figure 2:*CRM of SCC produced by the Laboratorio de Materiales de Referencia, INTI.*

CONCLUSIONS

Based on the results, the Laboratorio de Materiales de Referencias of INTI has achieved the development of a secondary CRM in SCC, achieving metrological traceability of the values assigned to the SI through the certified values of the primary CRM ERM®-BD001.

The laboratory followed the guidelines set by ISO 17034:2017 and its associated standards/guidelines, which allowed the developed CRM INTI-MRC002-G2304 to meet the requirements for homogeneity and long-term stability, thus confirming the suitability of the production, packaging, and storage processes.

By demonstrating the trueness of the microscopic method, it was confirmed that the results obtained using this method were metrologically traceable to the same reference as the property values of the primary CRM, in this case to the SI.

The Quality Management System supporting measurement services: Somatic Cell Count, milk (Certified Reference Materials), as defined in a motion of the Quality System Task Force (QSTF), has been reviewed and found to be in conformance with the requirements of ISO 17034:2016 under the mandate of the Inter-American Metrology System in support of the expectations of the CIPM Mutual Recognition Arrangement.

This development has enabled reaching a higher common link in the metrological traceability chain in Latin America, allowing comparability of results and achieving universal acceptance. The Laboratorio de Materiales de Referencia, by producing repeated batches, will ensure the continuous availability of these secondary CRMs for use. Thus, SICECAL® user laboratories employing our secondary CRM for the calibration of their fluoro-opto-electronic equipment will obtain measurement results traceable to the SI, which can be compared with results from other laboratories, enhancing acceptance and mutual trust in the dairy sector.

The use of INTI-MRC002 represents a key tool for ensuring reliable and metrologically traceable measurements. This allows producers to meet industry demands and access fairer economic compensation for their production. This contributes to the sustainable development of dairy farms and the improvement of the quality of milk used for product manufacturing, boosting the competitiveness of the sector.

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