European Commission

ERM
European Reference Materials

CERTIFICATION REPORT

The certification of the mass concentration of beta-2-microglobulin in human serum: ERM-DA470k/IFCC

EUR 26972 EN - 2015

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JRC93350

EUR 26972 EN

ISBN 978-92-79-44555-2(PDF)
ISSN 1831-9424 (online)
doi:10.2787/45447

Luxembourg: Publications Office of the European Union, 2015

Printed in Belgium

Abstract
This report describes the additional certification of the mass concentration of beta-2-microglobulin (B2M) in ERM-DA470k/IFCC, a human serum material. The material was released in 2008 and was certified for the mass concentration of 12 proteins in human serum. The material is now additionally certified for B2M in accordance with ISO Guide 34:2009.
A full description of the processing steps used to produce the material can be found in the first report.
The between unit-homogeneity was quantified and the stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006.
The within-unit homogeneity was estimated to determine the minimum sample intake.
The material was characterised by an inter-laboratory comparison exercise performed by laboratories of demonstrated competence and with adherence to ISO/IEC 17025.
Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) and include uncertainties relating to possible lack of homogeneity, instability and to characterisation.
The material is intended for the calibration or control, for the certified proteins only, of immunoassay-based in-vitro diagnostic devices or control products. Any other calibrator should be verified as commutable. The material was produced in a similar manner as ERM-DA470, the use of which has led to a significant reduction in the between-method and between-laboratory variation for the proteins certified (B2M was not certified in this material) [,]. It was verified during the value assignment procedure that there were no significant matrix effects, and that different methods produced consistent results. The Certified Reference Material (CRM) is available in the lyophilised form of a 1.0 mL portion of serum with additives (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium azide, benzamidine hydrochloride, sodium chloride and aprotinin). The CRM is available in threaded glass bottles with rubber stoppers and polypropylene screw caps maintained under nitrogen gas. The water mass fraction of the sample is (4.3 ± 0.6) mg/g. The lyophilised powder has to be reconstituted with (1.00 ± 0.01) g of distilled water and the minimum amount of reconstituted sample to be used for B2M measurement is 2 µL.
The CRM including its B2M certified value was accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium..

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The certification of the mass concentration of beta-2-microglobulin in human serum: ERM-DA470k/IFCC

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Disclaimer

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 additional certification of the mass concentration of beta-2-microglobulin (B2M) in ERM-DA470k/IFCC, a human serum material. The material was released in 2008 and was certified for the mass concentration of 12 proteins in human serum. The material is now additionally certified for B2M in accordance with ISO Guide 34:2009 [1].

A full description of the processing steps used to produce the material can be found in the first report [2].

The between unit-homogeneity was quantified and the stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 [3]. The within-unit homogeneity was estimated to determine the minimum sample intake.

The material was characterised by an inter-laboratory comparison exercise performed by laboratories of demonstrated competence and with adherence to ISO/IEC 17025 [4].

Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [5] and include uncertainties relating to possible lack of homogeneity, instability and to characterisation.

The material is intended for the calibration or control, for the certified proteins only, of immunoassay-based *in-vitro* diagnostic devices or control products. Any other calibrator should be verified as commutable. The material was produced in a similar manner as ERM-DA470 [6], the use of which has led to a significant reduction in the between-method and between-laboratory variation for the proteins certified (B2M was not certified in this material) [7,8]. It was verified during the value assignment procedure that there were no significant matrix effects, and that different methods produced consistent results. The Certified Reference Material (CRM) is available in the lyophilised form of a 1.0 mL portion of serum with additives (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium azide, benzamidine hydrochloride, sodium chloride and aprotinin). The CRM is available in threaded glass bottles with rubber stoppers and polypropylene screw caps maintained under nitrogen gas. The water mass fraction of the sample is (4.3 ± 0.6) mg/g. The lyophilised powder has to be reconstituted with (1.00 ± 0.01) g of distilled water and the minimum amount of reconstituted sample to be used for B2M measurement is 2 µL.

The CRM including its B2M certified value was accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

The following value for B2M was assigned:

Protein in the reconstituted material (see section 9.3)	Mass concentration	
	Certified value [2] [mg/L]	Uncertainty [3] [mg/L]
Beta-2-microglobulin (B2M) [1]	2.17	0.07

1) B2M as measured by immunonephelometry, immunoturbidimetry, fluorometric enzyme immunoassay and chemiluminescent immunoassay using a pure protein solution as calibrant.

2) The value is the unweighted mean of 13 accepted mean values, independently obtained by 13 laboratories. The certified mass concentration is traceable to the SI, via calibration with a pure protein solution of B2M.

3) Expanded uncertainty *U* with a coverage factor *k* = 2, corresponding to a level of confidence of approxiamtely 95 %, estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM), ISO, 1995.

Table of contents

Glossary

ANOVA	Analysis of variance
b	Slope in the equation of linear regression $y = a + bx$
B2M	beta-2-microglobulin
BSA	Bovine serum albumin
c	Mass concentration $c = m / V$ (mass / volume)
CI	confidence interval
CLSI	Clinical and Laboratory Standards Institute
CRM	Certified reference material
EC	European Commission
$\boldsymbol{C_{TM}}$	Concentration of the target material
$\boldsymbol{C_{TM}(i)}$	Concentration of the target material in the dilution $\boldsymbol{i}$
ERM®	Trademark of European Reference Materials
GUM	Guide to the Expression of Uncertainty in Measurements [5]
$\boldsymbol{f_{M,i}}$	Dilution factor for the material for dilution i
$F_{TM}(i)$	Dilution factor for the target material for dilution i
HBV	Hepatitis B virus
HBs	Hepatitis B surface antigen
HCV	Hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HG-AFS	Hydride generation-atomic fluorescence spectrometry
HIV	Human immunodeficiency virus
HTLV	Human T-cell lymphotropic virus
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IRMM	Institute for Reference Materials and Measurements of the JRC
ISO	International Organization for Standardization
ISS	International staging system
IU	International units
IVD	In Vitro Diagnostics
JRC	Joint Research Centre of the European Commission
k	Coverage factor
m	Mass
MD	Medical devices
MRL	Maximum residue limit
MS	Mass spectrometry

$MS_{between}$	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_{ns}	Number of results from the normal stock
n_{ref}	Number of results from the reference stock
N	Number of samples (units) analysed
n.a.	Not applicable
n.c.	Not calculated
p	Number of accepted datasets
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference material
RSD	Relative standard deviation
RSE	Relative standard error (=RSD/$\sqrt{n}$)
r^2	Coefficient of determination of the linear regression
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
SDS	Sodium dodecyl sulphate
SI	International System of Units
s_{meas}	Standard deviation of measurement data; an additional index "rel" is added as appropriate
s_{ns}	Standard deviation of results of normal stock samples
s_{ref}	Standard deviation of results of normal stock samples
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_{sl}	Proposed shelf life
TM	target material
TF	Transfer factor
TRIS	Tris(hydroxymethyl)aminomethane
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_c	combined standard uncertainty; an additional index "rel" is added as appropriate
u_{cal}	Standard uncertainty of the calibrant
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_{Δ}	Combined standard uncertainty of measurement result and certified value
U_{Δ}	Combined expanded uncertainty of measurement result and certified value
u_{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
u_{meas}	Standard measurement uncertainty
U_{meas}	Expanded measurement uncertainty
u_{sts}	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
x_i	Result at time point i
$\bar{x}$	Mean of all results for all time points in the stability study
$\bar{x}_{ns}$	Mean of all results of normal stock samples
$\bar{x}_{ref}$	Mean of all results of reference stock samples
$\bar{y}$	Mean of all results in the homogeneity study
Δ_{meas}	Absolute difference between mean measured value and the certified value
$\nu_{s,meas}$	Degrees of freedom for the determination of the standard deviation s_{meas}
$\nu_{MSwithin}$	Degrees of freedom of MS_{within}

1 Introduction

1.1 Background

Serum beta-2-microglobulin (B2M) is an important marker used in the investigation of patients with multiple myeloma and lymphoma. The international staging system (ISS) [9] uses concentrations of serum albumin and B2M to classify patients into prognostic groups. The important values for B2M are below 3.5 mg/L for the "good" prognostic category and above 5.5 mg/L for the poorer prognostic category. Recent work has highlighted the discrepancies between results obtained in different laboratories for B2M measurements leading to difficulties in implementing the staging system [10]. Therefore, the Institute for Reference Materials and Measurements (IRMM) was asked by the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC) to develop a reference material to support the standardisation of B2M measurements.

The EU Directive on In Vitro Diagnostic Medical Devices (IVD-MD) (Directive 98/79/EC) [11] requires traceability of the assigned values of calibrants and control materials to reference measurement procedures and/or reference materials of higher order.

1.2 Design of the project

The material was characterised through a value transfer procedure [12]. In this procedure, a pure protein solution spiked into an appropriate background is used to calibrate of the measurements of the candidate reference material. Six dilutions of the pure protein solution were measured in parallel with six dilutions of ERM-DA470k/IFCC.

The techniques used to measure the protein concentrations were immunonephelometry and immunoturbidimetry, fluorometric enzyme immunoassay and chemiluminescent immunoassay. The measurements were performed with different instruments (Abbott Architect c8000, Beckman Coulter AU480, Hitachi 917, Hitachi 7180, Roche Cobas c501, Siemens BN ProSpec®, Tosoh AIA-2000 and Spherelight™ Wako) and reagents. In total thirteen laboratories participated in the value assignment. Most characterisation measurements were performed under an ISO 13485 quality system.

2 Participants

2.1 Project management and evaluation

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

2.2 Processing and homogeneity study

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

Siemens Healthcare Diagnostics Products GmbH , Marburg, DE

2.3 Stability study

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

Olympus Life and Materials Science, Clare, IE

Siemens Healthcare Diagnostics Products GmbH , Marburg, DE

2.4 Characterisation

Abbott Diagnostics Division, Irving, Texas, US

A&T Corporation, Kanagawa, JP

Centro Interdipartimentale per la Riferibilità Metrologica in Medicina di Laboratorio (CIRME), Universita degli Studi di Milano, Milano, IT (measurements under the scope of ISO/IEC 17025 accreditation ACCREDIA; 217 rev. 01)

DAKO Denmark A/S, Glostrup, DK

Denka Seiken Co., Ltd, Niigata, JP

Eiken Chemical Co., Ltd, Tokyo, JP

LSI Medience Corporation, Tokyo, JP

Nittobo Medical Co., Ltd, Fukushima, JP

Roche Diagnostics GmbH, Penzberg, DE

Sanyo Chemical Industries, Ltd., Kyoto, JP

St Georges Hospital, Protein Reference Unit, London, UK (measurements under the scope of Clinical Pathology Accreditation, United Kingdom Accreditation Services; CPA 1929)

Tosoh Corporation, Tokyo, JP

Wako Pure Chemical Industries, Ltd., Osaka, JP

3 Material processing and process control

The processing of the material is described in the first report for ERM-DA470k/IFCC [2].

4 Homogeneity

A key requirement for any certified reference material aliquoted into units is the equivalence between those units. It is important to establish whether the variation between units of the reference material is significant compared to the uncertainty of the certified value, although, it is not relevant if this variation between units is significant compared to the analytical variation. ISO Guide 34 requires RM producers to quantify the between unit variation. This aspect is covered by 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. Quantification of within-unit inhomogeneity is therefore necessary to determine the minimum sample intake.

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified value of the CRM was valid for all units of the material, within the stated uncertainty.

For the between-unit homogeneity assessment, the number of units selected corresponds to approximately the cubic root of the total number of the units produced and therefore forty units were selected using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. For this, the entire batch was divided into forty groups (containing a similar number of units) and one unit was selected at random from each group. Three independent samples were taken from each of these units, and analysed by immunonephelometry. The measurements were performed in a randomised manner to be able to separate a potential analytical drift from a trend in the filling sequence.

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 visible.

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 are 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 assessed using histograms and normal probability plots to determine if the data followed a unimodal distribution. Minor deviations from unimodality of the individual values do not significantly affect the estimate of between-unit standard deviations.

It should be noted that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and 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, $u^{*}_{bb,rel}$, the maximum inhomogeneity that could be hidden by method repeatability, was calculated as

described by Linsinger *et al.* [13]. $u^*_{bb,rel}$ and is comparable to the limit of detection 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 in Equations 1, 2 and 3:

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

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

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

where:

MS_{within}	mean square within a unit from an ANOVA
$MS_{between}$	mean 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 measurements are shown in Annex A. The results of the evaluation of the between-unit variation are summarised in Table 1.

Table 1: Results of the homogeneity study

	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]	$u_{bb,rel}$ [%]
B2M	2.90	n.c.[1)]	0.67	0.67

[1)] n.c.: cannot be calculated as $MS_{between} < MS_{within}$

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 used as uncertainty contribution to account for potential heterogeneity.

4.2 Within-unit homogeneity and minimum sample intake

The material is a solution and is not expected to have any heterogeneity. This assumption was confirmed by the homogeneity/stability/characterisation study, where sample intakes as low as 2 µL were found to give acceptable repeatability, demonstrating that there is no heterogeneity or contamination at a sample intake of 2 µL.

Therefore the minimum sample intake is 2 µL.

5 Stability

Time and temperature were regarded as the most relevant influences on the stability of the material.

Stability testing is necessary to establish the conditions for storage (long-term stability) as well as the conditions for dispatch of the material 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 materials are to be transported at ambient temperatures.

The stability studies were performed using an isochronous design [14]. In this approach, units were stored for a specified length of time at different temperatures whereupon the units were then moved to conditions where further degradation was 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, units of ERM-DA470k/IFCC were stored at -70 °C, -20 °C, 4 °C and 60 °C for 0, 1, 2 and 4 weeks. The reference temperature was set to -150 °C (above liquid nitrogen). Two units per storage time were selected using a random stratified sampling scheme. Three samples, taken from each unit, were analysed by immunonephelometry, under repeatability conditions, in a randomised sequence, to be able to separate a potential analytical drift from a trend over storage time.

The data were evaluated for each temperature individually. No outliers were detected when the results were screened using the single and double Grubbs test.

In addition, the data were evaluated against storage time and regression lines of B2M mass concentration versus time were constructed. The slopes of the regression lines were not significantly different from zero (on 99 % confidence level) for all temperatures when they were tested for significance (loss/increase due to shipping conditions).

The results of the measurements are shown in Annex B.

No technically unexplained outliers/statistical outliers were detected for B2M and none of the trends were statistically significant at a 99 % confidence level for any of the temperatures.

Therefore, the material was found to be stable at all temperatures for B2M. Nevertheless, for some other proteins certified in this material, some trends were found at 60 °C. Therefore, the material shall be shipped under cooled conditions.

5.2 Long-term stability study

For the long-term stability study, units were stored at -70 °C for 0, 2, 4, 8, 10 and 12 months and at -20 °C for 0, 2, 8 and 12 months. The reference temperature was set to -150 °C (above liquid nitrogen). two units per storage time were selected using a random stratified sampling scheme. Three samples were taken from each unit and were measured by immunonephelometry. 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 data were evaluated for each temperature individually. No outliers were detected when the results were screened for outliers using the single and double Grubbs test.

Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. Some significant (at 99 % confidence level) trends in the analytical sequence were observed (see Annex C - Figure C-1). As the analytical sequence and the unit numbers were not correlated, correction for these trends can improve the sensitivity of the subsequent statistical analysis through a reduction in analytical variation without masking potential between-unit heterogeneities. Therefore, trends in the analytical sequence were corrected as the trend was significant on at least a 99 % confidence level as shown in Equation 4.

$$corrected\ result = measured\ result - b \times i \qquad \textbf{Equation 4}$$

b = slope of the linear regression

i = position of the result in the analytical sequence

Furthermore, the data were plotted against storage time and linear regression lines of mass concentration versus time were calculated. The slope of the regression lines was tested for statistical significance (loss/increase due to storage conditions). For B2M, the slopes of the regression lines were not significantly different from zero (at a 99 % confidence level) for both -20 °C and -70 °C.

The results of the long term stability measurements are shown in Annex C.

No technically unexplained outliers were observed and none of the trends was statistically significant at a 99 % confidence level for any of the temperatures. As for the short-term stability, the storage temperature was based on all the parameters certified in the material. The material must therefore be stored at -70 °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 report that there was no detectable degradation during the time frame studied.

Uncertainties of stability during dispatch and storage were estimated, as described in [15]. 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} are calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as in Equation 5 and 6:

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

$$u_{lts,rel} = \frac{RSD}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{sl} \qquad \textbf{Equation 6}$$

RSD relative standard deviation of all results of the stability study

t_i time elapse at point *i*

$\bar{t}$ mean of all t_i

t_{tt} chosen transport time (1 week at 4 ºC)

t_{sl} chosen shelf life (12 months at -70 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the 4 °C studies. The uncertainty describes the possible change during a dispatch at 4 °C lasting for one week.
- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the -70 °C studies. The uncertainty contribution describes the possible degradation during 12 months storage at -70 °C.

The results of these evaluations are summarised in Table 2.

Table 2**:** Uncertainties of stability during dispatch and storage. $u_{sts,rel}$ was calculated for a temperature of 4 °C and 1 week; $u_{lts,rel}$ was calculated for a storage temperature of -70 °C and 1 year.

	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
B2M	0.27	0.62

After the certification study, the material will be included in IRMM's regular stability monitoring programme to determine its future stability.

The characterisation of B2M was performed later than the other certified proteins in this CRM and therefore the stability of the material beyond the established 12 months shelf life was assessed. This was performed by comparative analysis of two units of the CRM stored at the normal storage temperature (-70 °C) and two units stored at the reference temperature (-150 °C above liquid nitrogen) directly after the material processing. Storage of the reference samples at -150 °C is assumed to render the material degradation negligible. All the samples were analysed (in triplicate for each unit) by immunonephelometry.

The results of the normal stock samples (stored at -70 °C) were compared with the results for the reference stock within their respective uncertainties. The uncertainty of the dedicated measurements was established as the standard deviation divided by the square root of the number of measurements. A comparison of normalised results was performed to assess the consistency of the data and the assumption that the material is stable. The normalisation was performed as described below:

- the normal stock results were divided by the results obtained from analysis of the reference samples,
- the certified value was set to 1,
- the expanded standard uncertainty of the dedicated measurements was established as in Equation 7:

$$U_{meas} = 2 \cdot \frac{\overline{x_{ns}}}{x_{ref}} \cdot \sqrt{\left(\frac{\frac{s_{ns}}{\sqrt{n_{ns}}}}{\overline{x_{ns}}}\right)^2 + \left(\frac{\frac{s_{ref}}{\sqrt{n_{ref}}}}{\overline{x_{ref}}}\right)^2} \qquad \textbf{Equation 7}$$

where:

U_{meas} expanded standard measurement uncertainty with coverage factor, $k = 2$

s_{ns}, s_{ref} standard deviations of results of normal stock samples and reference samples, respectively

n_{ns}, n_{ref} number of results of normal stock samples and reference samples, respectively

$\overline{x}_{ns}$, $\overline{x}_{ref}$ mean of all results of normal stock samples and reference samples, respectively

There was no difference between the results from the normal stock and the results from the reference stock thus confirming the stability of ERM-DA470k/IFCC with regard to B2M mass concentration as can be seen in Table 3.

Table 3: Results of the stability test on ERM-DA470k/IFCC.

	$\bar{x}_{ns} \pm s_{ns}$ [mg/L] $n_{ns} = 6$	$\bar{x}_{ref} \pm s_{ref}$ [mg/L] $n_{ref} = 6$	Ratio ± U_{meas}
B2M	2.40 ± 0.05	2.38 ± 0.04	1.007 ± 0.021

6 Characterisation

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

This was based on an inter-laboratory comparison by expert laboratories, i.e. the mass concentration of beta-2-microglobulin in the material was determined in different laboratories which applied different measurement procedures to demonstrate the absence of a measurement bias. Due to the nature of the analyte however, all participants used immunoassays for the measurements. This approach aims to negate laboratory bias, which reduces the combined uncertainty.

6.1 Selection of participants

Thirteen laboratories were selected based on criteria that comprised both technical competence and quality management. Each participant laboratory was required to operate a quality system and to deliver documented evidence of proficiency in the field of protein measurements in human serum by submitting results for inter-laboratory comparison exercises or method validation reports. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 [4] was obligatory. Most measurements were performed under an ISO 13485 quality management system [16] and the certification numbers are stated in the list of participants (Section 2).

6.2 General principles

The characterisation of B2M in the human serum reference material ERM-DA470k/IFCC was performed according to the principles as documented in [12] and [17]. The characterisation was achieved by calibrating the measurements of the candidate reference material with a pure protein reference solution. The pure protein solution had been previously assigned a value for its B2M mass concentration. The practical transfer protocol is based on duplicate measurements of 6 dilutions of the pure protein solution and 6 dilutions of the candidate reference material under conditions as repeatable as possible. The transfer protocol requires several measurements a day (three in this study) repeated on several days (four in this study). An important prerequisite for this transfer study was that all reconstitutions and dilutions were controlled by weighing, thus reducing uncertainty in the transfer.

The following definitions apply ([12] and [17]) to the transfer procedure:

The reference preparation (pure protein solution) is defined as the protein preparation with known concentration value (Table 4) used as calibrant. The solution was prepared by extensive dialysis of lyophilised B2M reconstituted in 0.1 mol/L potassium chloride. The target concentration was approximately 1 g/L. The solution was assigned a value by amino acid analysis [18]. The results were confirmed by measurement of this solution by amino acid analysis in a second laboratory [19] and dry mass determination [20].

The purity of the protein was assessed by polyacrylamide gel electrophoresis and by capillary electrophoresis. No additional bands or peaks were detected thus giving an uncertainty for purity equal to the limit of detection of the individual methods. When comparing these limit of detection to $\boldsymbol{u_{cal,rel}}$, they were considered to be negligible and were not included in the uncertainty budget for the pure protein solution.

Table 4: Assigned value and expanded uncertainty of B2M in the pure protein solution

Assigned value [mg/L]	$u_{cal,rel}$ [%]
917	1.05

Target material (TM) is defined as the serum protein matrix material with unknown concentration value. In this case it is the matrix material ERM-DA470k/IFCC.

The transfer method should be based on well-established and recognised methods used in clinical chemistry. In this characterisation study, immunonephelometry, immunoturbidimetry, fluorometric enzyme immunoassay and chemiluminescent immunoassay were used.

Slight variations in the assay conditions, in the programming of the instruments or in the reagents may lead to different results. This has led to the prerequisite of method standardisation [21]. To minimise the factors contributing to variation, an optimised and practical transfer protocol was prepared with detailed instructions and emphasis on weighing all solutions used to prepare dilutions. Specific registration forms containing the intended dilution schemes were used to report the raw data. These forms were subsequently collected for treatment of the raw data.

In total, sixty different sets of the B2M dilution series (five increasing concentrations ranging from 1 to 5 mg/L and one blank solution) were prepared from the pure protein solution. Each laboratory received four different sets (each set with the six concentrations) chosen randomly.

Six dilutions of the target material (TM) were prepared on each day at each laboratory from a different ERM-DA470k/IFCC unit. The dilutions were performed by weighing to determine a gravimetric dilution factor. For this purpose, the dilution factors were calculated using the following densities: 1.0051 for saline solution, 1.0174 for saline solution containing 5 % (w/v) bovine serum albumin and 0.9982 for distilled water (water density at 20 °C). The concentration of the specific protein (although still unknown) in each dilution will be as in Equation 9:

$$\boldsymbol{C_{TM}(i) = F_{TM}(i) \times C_{TM}} \qquad \textbf{Equation 8}$$

where F_{TM} is the dilution factor of TM and (i) denotes the different dilutions (i =1-6).

Participating laboratories were instructed to use their common in-house calibrant to determine the dose-response function of the instrument.

A regression line was constructed comparing the gravimetrically calculated concentrations (as determined gravimetrically) in the dilutions of the pure protein solution to the measured concentrations.

The slope and intercept were determined for each of the runs and used to interpolate the measured value of the dilutions of the target material.

The initial concentration in the target material is then calculated using the dilution factor F_{TM}. Dilutions 1 and 2 for the target material were outside the range of the regression line obtained with the pure protein solution dilutions and were therefore not used for calculations. The eight remaining values (four dilution points measured in duplicate) were then used to determine the concentration in the target material in each run.

On each of the four days, a new set of vials of the reference preparation (blank and five different concentrations) and one new unit of the target material were used and a new set of dilutions prepared. Each day three calibrations were performed together with determinations of samples and controls (i.e., 3 runs).
The methods including instruments, dilution buffers, antibodies and reagents and laboratories are listed in Annex D.

6.3 Calculation of the dilutions

The liquids used for the reconstitution of the materials and for the preparation of the dilutions were weighed to a standard deviation of maximum 0.0001 g (with the exception of one laboratory).

The density of the solutions were either provided by the participating laboratories or measured, at IRMM, on a DMA 4500 M densitometer (Anton Paar, Graz, AT).

The following mass corrections were applied:

For the reconstitution of ERM-DA470k/IFCC with ultrapure water, as in Equation 10:

$$\boldsymbol{f_{M,i} = \frac{m_{intended}}{m_{M,i}}} \qquad \textbf{Equation 9}$$

where $m_{intended}$ is the mass intended to be added (1.0000 g), and $m_{M,i}$ is the measured mass of water added to the vial.

For the dilutions as in Equation 11:

$$\boldsymbol{f_{D,ij} = \frac{\frac{m_{DM,i}}{\rho_{DM,i}}}{\frac{m_{DM,i}}{\rho_{DM,i}} + \frac{m_{DD,i}}{\rho_{DD,i}}}} \qquad \textbf{Equation 10}$$

For the dilution j of the material i with $m_{DM,i}$ and $\rho_{DM,i}$ corresponding respectively to the mass and to the density of the reconstituted material, and $m_{DD,i}$ and $\rho_{DD,i}$ corresponding respectively to the mass and to the density of the diluent for dilution j.

The concentration c_{ij} (with c = m/V (mass / volume) and the unit (mg/L)) of B2M in the dilutions is calculated via Equation 12:

$$\boldsymbol{c_{ij} = c_{0i} \times f_{M,i} \times f_{P,i} \times f_{D,ij}} \qquad \textbf{Equation 11}$$

With c_{0i} being the concentration of B2M in material i and c_{ij} the concentration of B2M in dilution j of the material i.

6.4 Evaluation of results

The characterisation study resulted in thirteen datasets. All individual results of the participants are shown in tabular form in Annex E.

6.4.1 Technical evaluation

The data were first evaluated for compliance with the analysis protocol provided to each participating laboratory and for their technical validity. The following criteria were considered during the evaluation:

The following analyses were performed on all data:

- test for normality (visual inspection and normal probability plot)
- test for linearity (visual inspection and evaluation of r^2)

The following general acceptance criteria were applied to the datasets:

- r^2 of the regression must be above 0.98 for all measurement systems

- data from at least four dilutions must be available
- The dataset from any one day must contain at least 50 % of the data generated on that day, otherwise all the data generated on that day are declared non-valid
- At least two daily value assignments must be valid
- The day-to-day variation (RSD) of valid datasets for a particular laboratory must be equal to or below 5 %

Rationale for the acceptance criteria (see also reference [12]):

- Overall $r^2 < 0.98$ of regression indicates quality problems in the measurements (scattering, outliers, run-to-run variation).

Laboratory 2 found one vial from the set of dilutions of the reference solution to be broken upon arrival and was therefore not analysed for day 1. The analytical runs on that day were therefore performed with one blank and four concentrations which still covered the range between 1 and 5 mg/L.

Despite these considerations, all the data sets were retained. The characterisation study resulted in thirteen datasets covering fifty-two days and hundred and fifty-six runs.

6.4.2 Statistical evaluation

The datasets accepted in section 6.4.1 were evaluated for normality of dataset means using kurtosis/skewness tests and normal probability plots. They were assessed for outlying means using the Grubbs test and using the Cochran test for outlying standard deviations, (both at a the 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 Table 5.

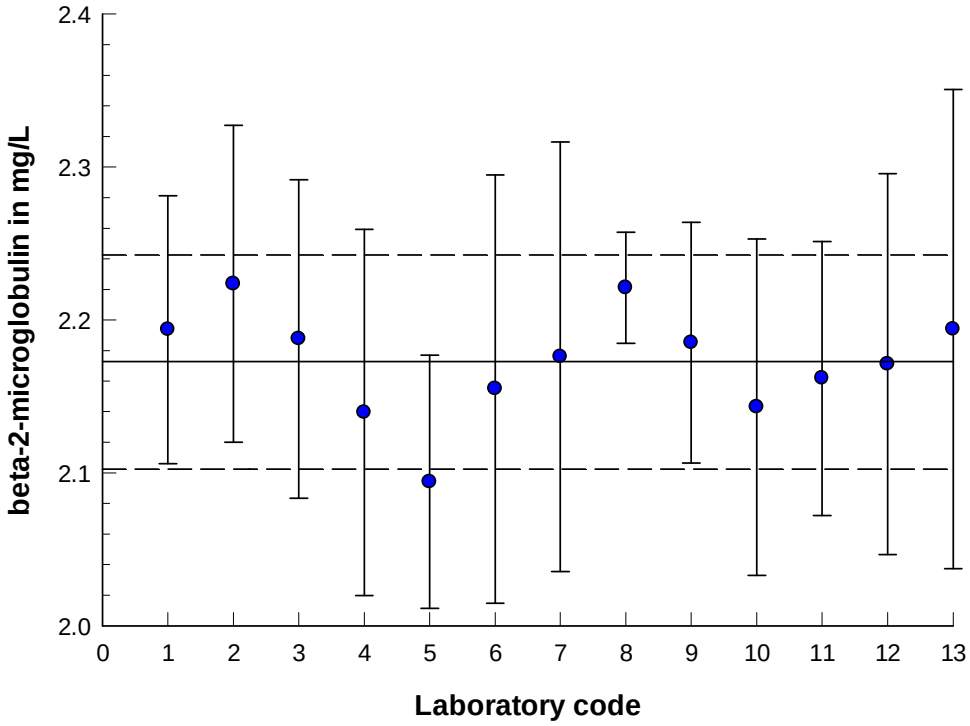
Table 5: Statistical evaluation of the technically accepted datasets for B2M. *p*: number of technically valid datasets

	p	Outliers		Normally distributed	Statistical parameters			
		Means	Variances		Mean [mg/L]	*s* [mg/L]	$s_{between}$ [mg/L]	s_{within} [mg/L]
B2M	13	1	0	Yes	2.17	0.04	0.04	0.10

The laboratory means followed normal distributions and none of the data contains outlying variances. Laboratory L5 was flagged as an outlier for the mean by the Nalimov test. This result was compared to the rest of the data using the principles described in the Application Note 1 [22]. For this, the uncertainties were estimated respectively as the relative standard deviation of the laboratory L5 and the relative standard deviation of the combined results from the thirteen other laboratories. The conclusion was that there was no significant difference between the individual result and the rest of the datasets. The datasets were therefore consistent and the mean of laboratory means was a good estimate of the true value.

The results of the characterisation campaign are presented in Figure 1.

Figure 1: Results of the characterisation measurements for B2M. The bars represent the laboratory means ± 2s. The full line represents the mean of the means and the dotted lines represent the mean of means ± 2s.



The uncertainty for the characterisation exercise was estimated as the relative standard uncertainty of the mean of laboratory means, i.e., with *s* the relative standard deviation of the mean of laboratory means and p the number of datasets, converted into a relative value: $u_{char,\ rel}$ = 0.45 %.

7 Value Assignment

During this study, a certified value was assigned.

Certified values are values that fulfil the highest standards of accuracy. Full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [5] were established.

7.1 Certified values and their uncertainties

The unweighted mean of the means of the accepted datasets as shown in Table 5 was assigned as certified value for B2M.

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

$$U_{CRM,rel} = k \cdot \sqrt{u^2_{char,rel} + u^2_{cal,rel} + u^2_{bb,rel} + u^2_{sts,rel} + u^2_{lts,rel}} \qquad \textbf{Equation 12}$$

- $u_{char,rel}$ was estimated as described in Section 6

- $u_{cal,\ rel}$ is the relative standard uncertainty from the pure protein solution as described in section 6
- $u_{bb,\ rel}$ was estimated as described in Section 4.1.
- $u_{sts,\ rel}$ was estimated as described in section 5.3
- $u_{lts,\ rel}$ was estimated as described in Section 5.3.

Because of the sufficient numbers of the degrees of freedom of the different uncertainty contributions, a coverage factor *k* of 2 was applied, to obtain the expanded uncertainties. The certified values and their uncertainties are summarised in Table 6.

Table 6: Certified values and their uncertainties for B2M in ERM-DA470k/IFCC

	Certified value [mg/L]	$u_{char,\ rel}$ [%]	$u_{cal,\ rel}$ [%]	$u_{bb,\ rel}$ [%]	$u_{sts,\ rel}$ [%]	$u_{lts,\ rel}$ [%]	$U_{CRM,\ rel}$ [%]	U_{CRM} [mg/L] [2)]
B2M[1)]	2.17	0.45	1.05	0.67	0.27	0.62	2.95	0.07

[1)] When reconstituted according to the specified procedure (see section 9.3)

[2)] Expanded (k = 2) and rounded uncertainty.

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

Beta-2-microglobulin protein in the pure protein solution used for calibration is a clearly defined analyte whose identity was confirmed by Size Exclusion Chromatography, semi-native gel electrophoresis, 2-D gel electrophoresis and immunoassay response. The measurand in the pure protein solution is therefore structurally defined and independent of the measurement method.

The participating laboratories used different methods for the final determination, different dilution buffers, antibodies and reagents, demonstrating absence of measurement bias. However, the characterisation of the matrix material ERM-DA470k/IFCC was performed using only immunoassay-based methods and therefore the measurand is defined as beta-2-microglobulin as measured by the immunoassays used in the presented procedures.

Quantity value

Only validated methods were used for the determination of the assigned value. The value assigned to the pure protein solution is traceable to the SI. All relevant input parameters were calibrated with this material. The individual results are therefore traceable to the SI, as was confirmed by the agreement among the technically accepted datasets. As the assigned values are combinations of consensus results individually traceable to the SI, the assigned quantity values themselves are also traceable to the SI.

8.2 Commutability

Many measurement procedures include one or more steps, which are selecting specific (or specific groups) of analytes from the sample for the subsequent steps of the 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 the 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 CSLI Guideline C-53A [23] 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 in such cases, the results from routinely used methods cannot be legitimately compared with the certified value to determine whether a bias exists in calibration, nor can the CRM be used as a calibrant. For instance, CRMs intended to be used to establish or verify metrological traceability of routine clinical measurement procedures must be commutable for the routine clinical measurement procedures for which they are intended to be used.

For beta-2-microglobulin, ERM-DA470k/IFCC and its dilutions prepared using the assay diluent were shown to be commutable for combinations of the following platforms: Beckman Coulter Immage with Beckman Coulter reagents; Beckman Coulter AU640 with Beckman Coulter reagents and Siemens BN ProSpec with Siemens reagents. The dilutions of the pure protein solution were proven to be commutable with the following platforms: Beckman Coulter AU480 with Beckman Coulter reagents, Hitachi 917 with DAKO reagents and Siemens BN ProSpec with Siemens reagents. If ERM-DA470k/IFCC is used for the calibration of other beta-2-microglobulin assays other than the ones used during the certification study it should be verified by the user that the material or its dilution used is commutable for that particular method in combination with other methods.

9 Instructions for use

9.1 Safety information

The usual laboratory safety measures apply.

Avoid swallowing as well as contact with skin. Do not discharge the waste into the drain.

Each portion of donated blood used in the production of the material has been tested for the presence of HBs antigen, HCV, HIV1/HIV2, and HTLV1 antibodies and found to be negative. However, the product must be handled with adequate care as any material of human origin. It is intended for *in vitro* analysis only.

9.2 Storage conditions

Unopened vials should be stored at - (70 ± 5) °C. Under the condition that any microbial contamination during the reconstitution procedure has been excluded, the solution of ERM-DA470k/IFCC can be used for one week. It is advisable to cover the vial with the original seal after use and to store it at 2 to 8 °C.

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 of opened vials.

9.3 Intended use and instructions for use

The material is primarily intended to be used for the calibration of immunoassay-based *in vitro* diagnostic devices or control products for the proteins certified. As for any calibrator it should be verified that it is commutable. The material was produced in a similar manner as ERM-DA470, the use of which has led to a significant reduction in the between-method and between-laboratory variation for the proteins certified [8, 9]. It was verified during the value

assignment procedure that there were no significant matrix effects, and that different methods produced consistent results. However, when the material is used as a calibrator, the commutability should be verified for the particular assay concerned.

The entire content of the vial must be reconstituted.

Reconstitution of the material

To make it ready for use, the material has to be reconstituted according to the following procedure:

- Remove the vial from the freezer during the afternoon of the day before use and place the vial for 1 hour in the room where the balance is located.
- After 1 hour tap the bottom of the vial gently on the surface of the table. Make sure that all the material has settled down on the bottom of the vial. Remove the screw cap.
- Weigh the vial together with the rubber stopper. Note the mass or press the “TARE” button on the balance. Lift the rubber stopper with care until air is allowed to enter the vial and the groove in the rubber stopper becomes accessible.
- Add 1.00 mL of ultrapure water through the groove, and press the rubber stopper back into place. Weigh the vial and note the mass. If you have used the “TARE” function, the value can be used directly for the mass m. Otherwise the first mass must be subtracted from the second to obtain m.
- The concentration of B2M in the solution, corrected for the reconstitution mass, can be obtained by multiplying the certified value for that protein with $m_{intended}$ / m, with $m_{intended}$ the mass intended to be added (1.000 g).
- Leave the vial at room temperature for one hour, then invert it carefully at least five times (do not shake it) during the next hour.
- Leave the vial at room temperature overnight. On the day of use invert the vial carefully five times during one hour.

9.4 Minimum sample intake

The minimum volume of reconstituted sample to be used is 2 µL.

9.5 Use of the certified value

The material is primarily intended to be used to calibrate serum-based protein standards and control materials of organisations that offer such preparations for the quantification of B2M by immunoassay.

Use as a calibrant

The uncertainty of the certified value shall be taken into account in the estimation of the measurement uncertainty.

Comparing an analytical result with the certified value

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, www.erm-crm.org [22].

For assessing the method performance, the measured values of the CRMs are compared with the certified values. The procedure is described here in brief:

- Calculate the absolute difference between mean measured value and the certified value (Δ_{meas}).
- Combine 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_{Δ}) from the combined uncertainty ($u_{\Delta,}$) using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %.
- If $\Delta_{meas} \leq U_{\Delta}$ no significant difference between the measurement result and the certified value, at a confidence level of about 95 % exists.

Use in quality control charts

The materials can be used for quality control charts. Different CRM-units will give the same result as inhomogeneity was included in the uncertainties of the certified values.

10 Acknowledgments

The authors would like to acknowledge the support received from Maria Concepcion Contreras Lopez and Hilde de Schrijver from IRMM in the set-up of the required isochronous studies, from Andres Perez from IRMM for organising the additional stability study and from Olivier de Rudder from IRMM for the density measurements.

Furthermore, the authors would like to thank Tsevetelina Gerganova and Jean Charoud-Got (IRMM) for the reviewing of the certification report, as well as the experts of the Certification Advisory Panel "Biological Macromolecules and Biological/Biochemical Parameters", H. Hird (The Food and Environment Research Agency, UK), M. Wagner (University for Veterinary Medicine Vienna, AT) and L. Siekmann (University of Bonn, DE) for their constructive comments.

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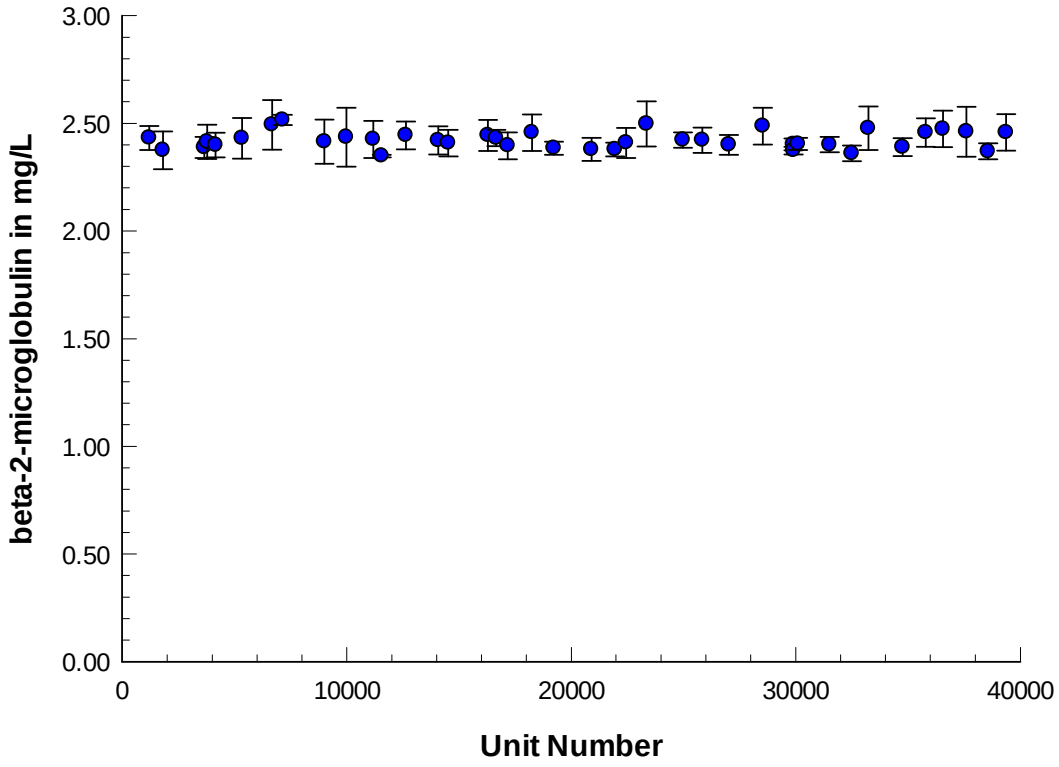
Annexes

Annex A: Results of the homogeneity measurements

Figure A-1: Concentration of B2M in 40 units of ERM-DA470k/IFCC determined during the homogeneity study (standard deviation for the three replicates shown).



Annex B: Results of the short-term stability measurements

Figure B-1: Concentration of B2M in ERM-DA470k/IFCC stored at 60 °C during the short-term stability study

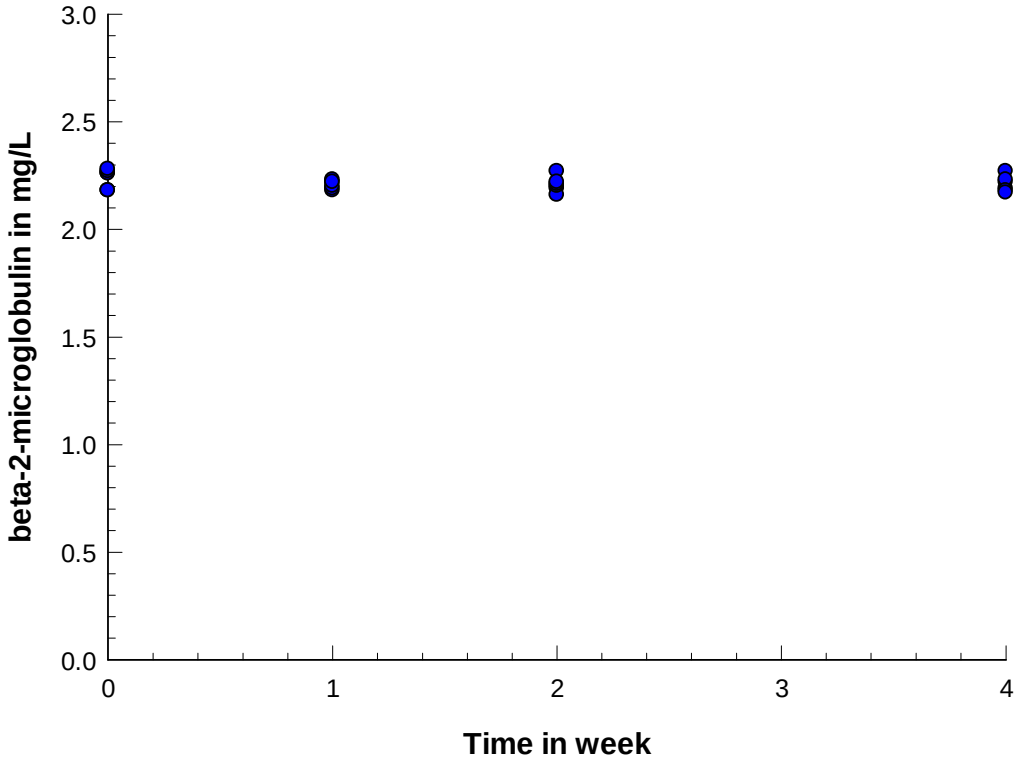


Figure B-2: Concentration of B2M in ERM-DA470k/IFCC stored at 4 °C during the short-term stability study

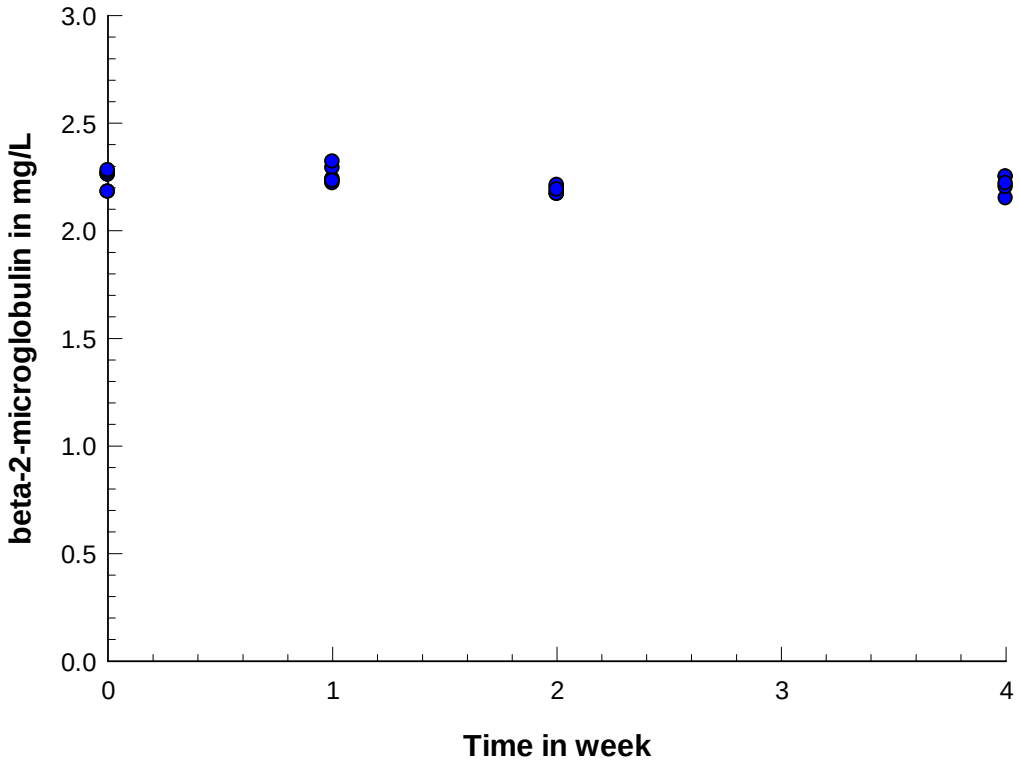


Figure B-3: Concentration of B2M in ERM-DA470k/IFCC stored at -20 °C during the short-term stability study

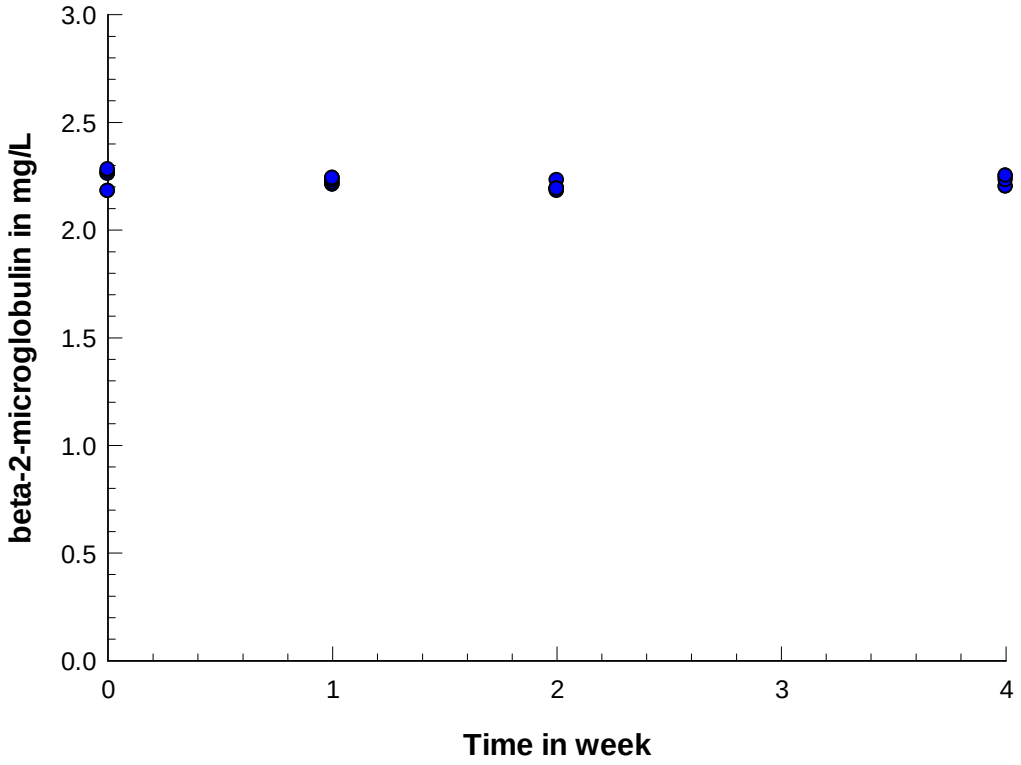
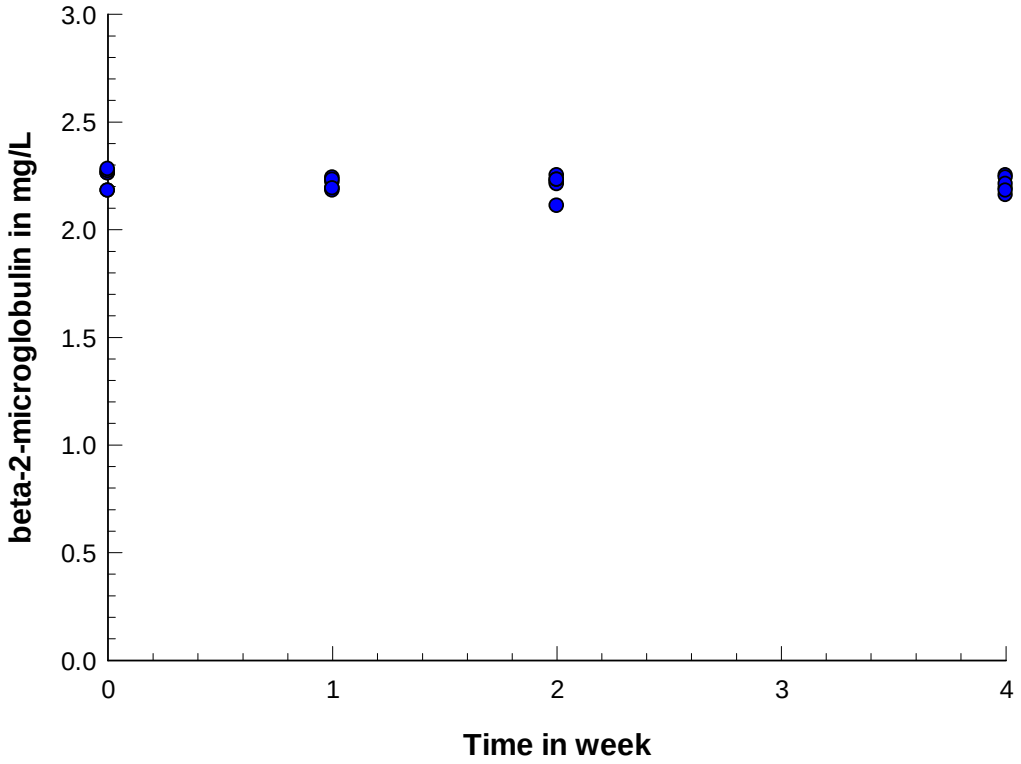


Figure B-4: Concentration of B2M in ERM-DA470k/IFCC stored at -70 °C during the short-term stability study



Annex C: Results of the long-term stability measurements

Figure C-1: Regression analysis of the results in the analytical sequence order (before trend correction)

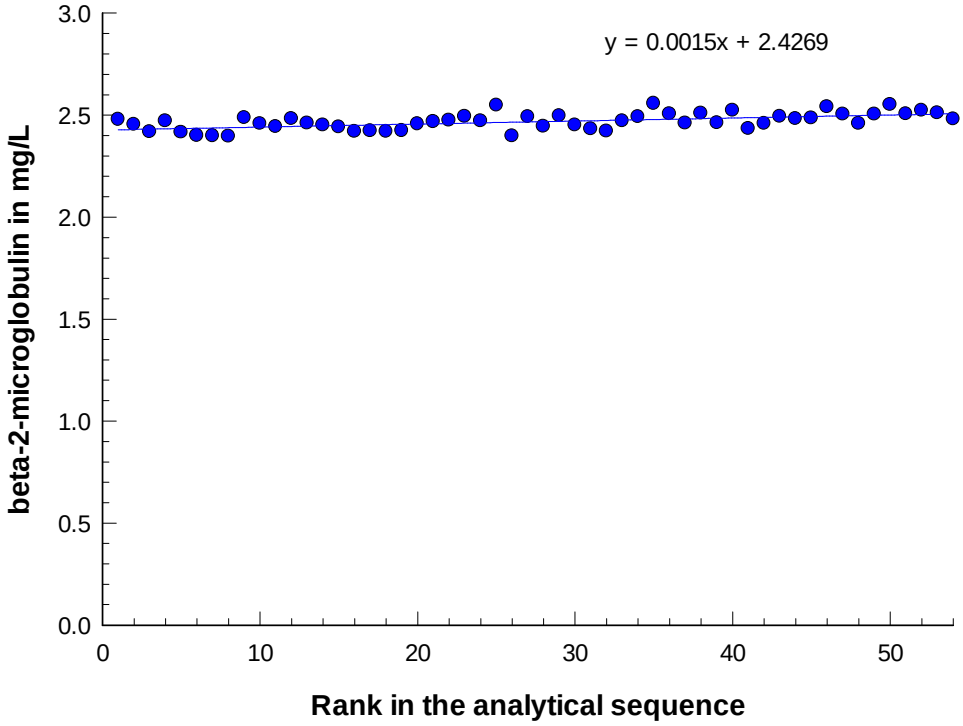


Figure C-2: Concentration of B2M in ERM-DA470k/IFCC stored at -20 °C during the long-term stability study

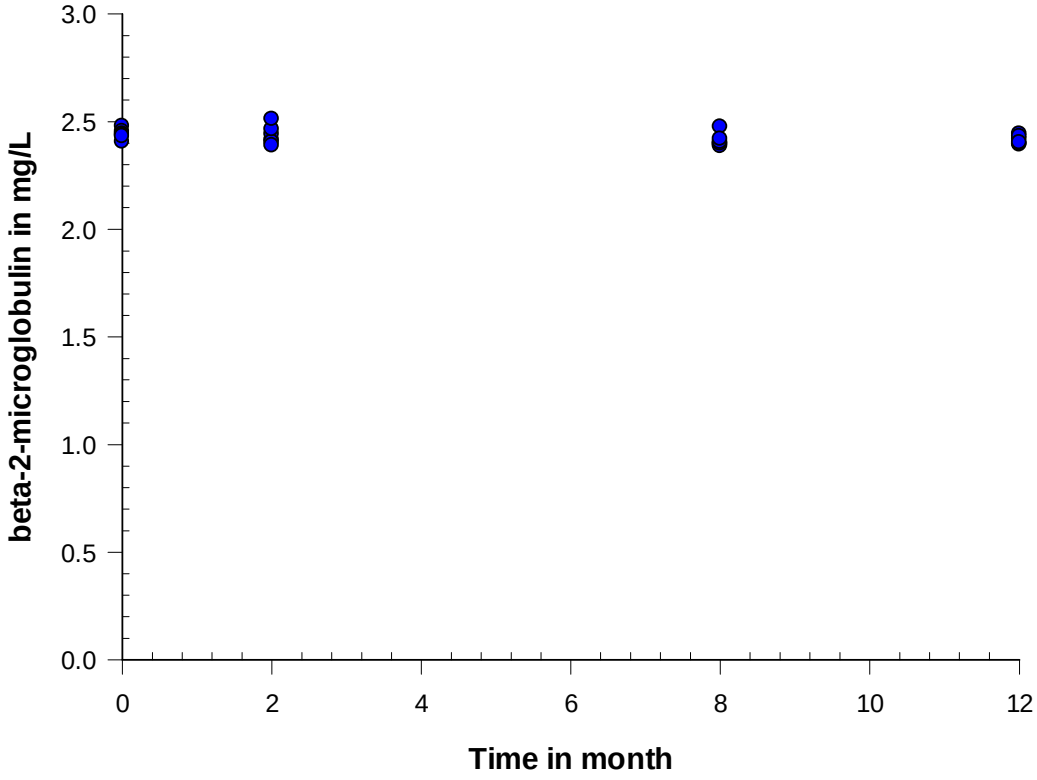
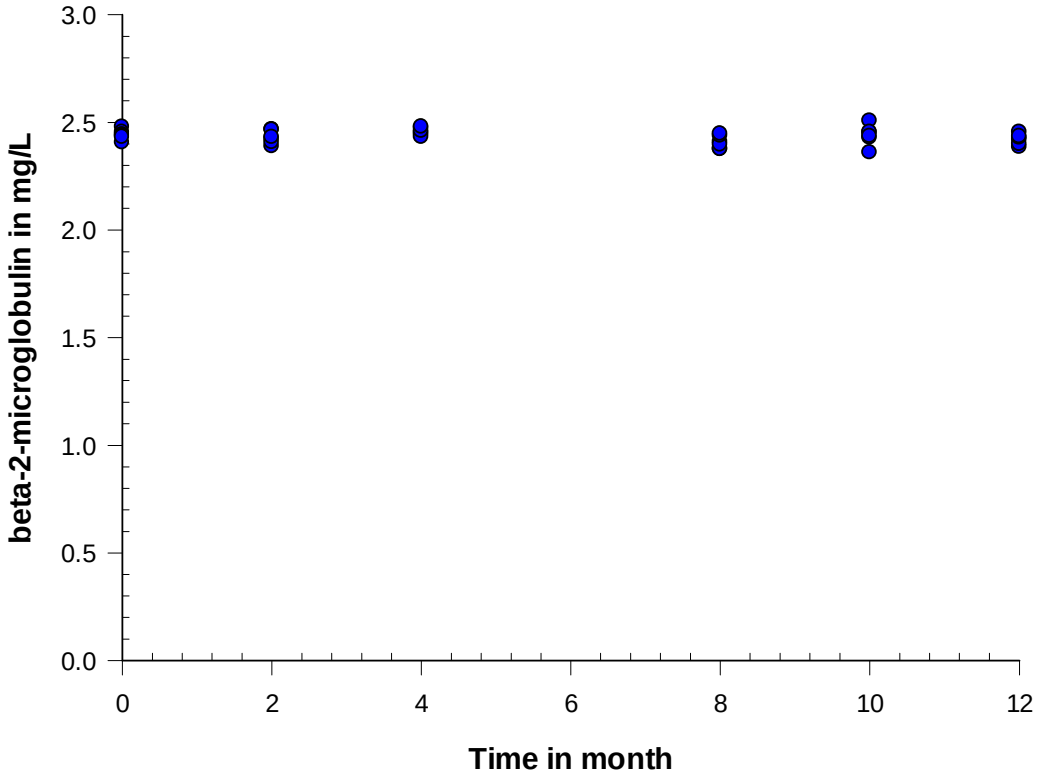


Figure C-3: Concentration of B2M in ERM-DA470k/IFCC stored at -70 °C during the long-term stability study



Annex D: Summary of methods used in the characterisation study

Laboratory	Method	Instrument	Dilution Buffer	Antibody	Reagent
L1	Immunoturbidimetry	Abbott Architect® c8000	Saline solution	Abbott polystyrene latex particles coated IgG anti-human B2M (rabbit)	Phosphate Buffer + Bovine Serum Albumin
L2	Fluorometric enzyme immunoassay	TOSOH AIA-2000	TOSOH AIA-Pack BMG Kit containing all the reagents		
L3	Immunoturbidimetry	Roche Cobas c501	Saline	Roche latex particles coated with polyclonal α-human-β-2-microglobulin antibody	TRIS/HCl Buffer with NaCl and EDTA
L4	Immunoturbidimetry	Hitachi 917	Saline	BMG-Latex X1 "SEIKEN" Kit containing antibody and reagent	
L5	Immunoturbidimetry	Hitachi 7180	Saline with 5 % BSA	A&T Immunoticles Auto β2-m R-2	A& T Immunoticles Auto β2-m R-1
L6	Immunoturbidimetry	Hitachi 7180	Saline	LZ TEST 'EIKEN' β2m	LZ TEST 'EIKEN' β2m
L7	Immunonephelometry	Siemens BN ProSpec	Siemens N-Diluent	Siemens N Latex β2-microglobulin	Siemens N Reaction Buffer
L8	Chemiluminescent immunoassay	Spherelight™ Wako	Wako Spherelight™ Dilution Buffer	Wako Spherelight™ β2-m Kit	
L9	Immunoturbidimetry	Hitachi 7180	Nittobo BMG dilution buffer	Nittobo N-Assay LA β2-MG-HII	
L10	Immunoturbidimetry	Beckman Coulter AU480	Distilled water	Beckman Coulter latex particles coated with rabbit IgG anti-human β-2-microglobulin	Beckman Coulter β-2-microglobulin reagent
L11	Immunoturbidimetry	Hitachi 7180	Saline	Superior BMG-II (DKL/LSI Medience)	
L12	Immunoturbidimetry	Hitachi 7180	Saline	Wako β2m-Latex Reagent	Wako β2m-LT Buffer
L13	Immunoturbidimetry	Hitachi 917	MilliQ water	Dako B2M 1a	Dako B2M

Annex E: Results of the characterisation measurements in mg/L

Laboratory	Day	Run 1	Run 2	Run 3
L1	1	2.16	2.28	2.26
	2	2.17	2.20	2.22
	3	2.20	2.20	2.23
	4	2.10	2.11	2.18
L2	1	2.11	2.16	2.17
	2	2.28	2.22	2.23
	3	2.26	2.23	2.26
	4	2.24	2.24	2.27
L3	1	2.16	2.23	2.22
	2	2.17	2.12	2.15
	3	2.23	2.25	2.28
	4	2.14	2.14	2.16
L4	1	2.19	2.18	2.15
	2	2.19	2.17	2.17
	3	2.15	2.17	2.17
	4	2.05	2.06	2.05
L5	1	2.06	2.04	2.06
	2	2.13	2.14	2.13
	3	2.08	2.07	2.05
	4	2.13	2.13	2.12
L6	1	2.18	2.19	2.20
	2	2.24	2.23	2.21
	3	2.10	2.10	2.19
	4	2.08	2.06	2.07
L7	1	2.02	2.15	2.11
	2	2.18	2.25	2.25
	3	2.11	2.17	2.14
	4	2.21	2.28	2.24
L8	1	2.25	2.25	2.22
	2	2.21	2.22	2.18
	3	2.22	2.25	2.22
	4	2.24	2.19	2.20

L9	1	2.14	2.17	2.12
	2	2.24	2.26	2.20
	3	2.16	2.17	2.16
	4	2.19	2.19	2.20
L10	1	2.15	2.05	2.09
	2	2.22	2.19	2.23
	3	2.17	2.15	1.98
	4	2.17	2.13	2.18
L11	1	2.17	2.16	2.22
	2	2.18	2.13	2.08
	3	2.15	2.06	2.15
	4	2.26	2.19	2.20
L12	1	2.20	2.19	2.19
	2	2.24	2.24	2.24
	3	2.15	2.16	2.15
	4	2.10	2.09	2.09
L13	1	2.24	2.23	2.24
	2	2.19	2.18	2.16
	3	2.29	2.26	2.24
	4	2.08	2.10	2.10

European Commission

EUR 26972 EN – Joint Research Centre – Institute for Reference Materials and Measurements

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Luxembourg: Publications Office of the European Union

2015 –34 pp. – 21.0 x 29.7 cm

EUR – Scientific and Technical Research series – ISSN 1831-9424

ISBN 978-92-79-44555-2

doi:10.2787/45447

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LA-NA-26972-EN-N

Publications Office

doi:10.2787/45447

ISBN: 978-92-79-44555-2