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CERTIFICATION REPORT

The certification of anti-myeloperoxidase immunoglobulin G in human serum ERM® - DA476/IFCC

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Abstract
This report describes the production and certification of ERM-DA476/IFCC, a new serum protein reference material intended for the standardisation of measurements of anti-myeloperoxidase immunoglobulin G (anti-MPO IgG) antibodies. The material was produced according to ISO Guide 34:2009 . The raw material used to prepare ERM-DA476/IFCC was a plasmapheresis material containing a high concentration of anti-MPO IgG. After a thorough commutability study lyophilised serum was selected as the format for the candidate reference material. Serum processing was performed based on the procedure used for the reference material ERM-DA470k/IFCC . The plasma was converted into serum which was then delipidated. After the addition of preservatives the processed serum was diluted with plasmapheresis solution containing albumin, prior to the transfer of 1 mL aliquots to glass vials. The serum was then lyophilised and the vials closed with rubber stoppers and screw caps under nitrogen atmosphere prior to storage at -70 °C.
The 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 exercise performed by laboratories of demonstrated competence and with adherence to ISO/IEC 17025, using a purified anti-MPO IgG preparation as calibrant. This was achieved using a value transfer protocol previously used in the characterisation of ERM-DA470k/IFCC [3]. Technically invalid results were removed. However no other outliers were eliminated on statistical grounds only.
Uncertainties of the certified values were calculated in accordance to the Guide to the Expression of Uncertainty in Measurement (GUM) and include uncertainties relating to possible lack of homogeneity, instability and characterisation.
The material is intended for the calibration of methods and quality control. As any reference material, it can also be used for control charts or validation studies. The CRM is available in glass vials containing the lyophilised residue of 1 g serum. The minimum amount of reconstituted sample to be used is 10 µL.
The CRM was accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

CERTIFICATION REPORT

The certification of anti-myeloperoxidase immunoglobulin G in human serum ERM® - DA476/IFCC

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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 and certification of ERM-DA476/IFCC, a new serum protein reference material intended for the standardisation of measurements of anti-myeloperoxidase immunoglobulin G (anti-MPO IgG) antibodies. The material was produced according to ISO Guide 34:2009 [1].

The raw material used to prepare ERM-DA476/IFCC was a plasmapheresis material containing a high concentration of anti-MPO IgG. After a thorough commutability study lyophilised serum was selected as the format for the candidate reference material [2]. Serum processing was performed based on the procedure used for the reference material ERM-DA470k/IFCC [3]. The plasma was converted into serum which was then delipidated. After the addition of preservatives the processed serum was diluted with plasmapheresis solution containing albumin, prior to the transfer of 1 mL aliquots to glass vials. The serum was then lyophilised and the vials closed with rubber stoppers and screw caps under nitrogen atmosphere prior to storage at -70 °C.

The between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 [4].

The material was characterised by an inter-laboratory comparison exercise performed by laboratories of demonstrated competence and with adherence to ISO/IEC 17025 [5], using a purified anti-MPO IgG preparation as calibrant. This was achieved using a value transfer protocol previously used in the characterisation of ERM-DA470k/IFCC [3]. Technically invalid results were removed. However no other outliers were eliminated on statistical grounds only.

Uncertainties of the certified values were calculated in accordance to the Guide to the Expression of Uncertainty in Measurement (GUM) [6] and include uncertainties relating to possible lack of homogeneity, instability and characterisation.

The material is intended for the calibration of methods and quality control. As any reference material, it can also be used for control charts or validation studies. The CRM is available in glass vials containing the lyophilised residue of 1 g serum. The minimum amount of reconstituted sample to be used is 10 µL.

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

The following value was assigned:

	Mass Concentration	
	Certified value [2)] [mg/L]	Uncertainty [3)] [mg/L]
anti-MPO IgG[1)]	84	9

1) Anti-myeloperoxidase immunoglobulin G as measured by immunoassays

2) Unweighted mean value of the means of accepted data sets each set obtained in a different laboratory and/or with a different method of determination. The certified mass concentration and its uncertainty are traceable to the stated value of the mass concentration in United States National Reference Preparation (USNRP) 12-0575C (Reimer et al., Am. J. Clin. Pathol. 77 (1982) 12-19)

3) The certified uncertainty 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 minimum sample intake is 10 µL

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Glossary

ANCAs	Anti-neutrophil cytoplasmic antibodies
Anti-MPO	Anti-myeloperoxidase
ANOVA	Analysis of variance
b	Slope in the equation of linear regression $y = a + bx$
c	Mass concentration $c = m / V$ (mass / volume)
c_{ij}	Concentration of the protein in dilution *j* of material *i*
c_{0i}	Concentration of the protein in material *i*
$c_{anti\text{-}MPO\ IgG}$	Concentration of the purified anti-MPO IgG
$c_{ERM\text{-}DA476/IFCC}$	Concentration of anti-MPO IgG in the ERM-DA476/IFCC
CLSI	Clinical and Laboratory Standards Institute
CRM	Certified Reference Material
C_R	Mass concentration of the protein in the calibrant
C_s	Signals of unknown test samples
C_T	Mass concentration of the protein in the target material
df	Degrees of freedom
EC	European Commission
ELISA	Enzyme-linked immunosorbent assay
EQAS	External Quality Assurance Scheme
ERM®	Trademark of European Reference Materials
EU	European Union
F_R	Dilution factor of the calibrant
F_T	Dilution factor of TM
GUM	Guide to the Expression of Uncertainty in Measurements
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
i	Different dilutions of TM
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IgG	Immunoglobulin G
ISO	International Organization for Standardization
IU	International units
IVD-MD	In Vitro Diagnostic - Medical Devices
JRC-IRMM	Joint Research Centre – Institute of Reference Materials and Measurements
j	Dilutions of the calibrant
k	Coverage factor

M	Molar mass
$m_{intended}$	Mass intended to be added
$m_{DD,j}$	Mass of diluent for dilution j
$m_{DM,i}$	Mass of the reconstituted material
$m_{M,i}$	Measured mass of water added
$m_{PD,i}$	Mass of the diluent for the predilution
$m_{PR,i}$	Mass of the reconstituted material
MS	Mean of squares
$MS_{between}$	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.c.	Not calculated
PES	Polyethersulfone
QC	Quality Control
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference Material
RSD	Relative standard deviation
RT	Room Temperature
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
SEC	Size Exclusion Chromatography
s_{ijk}	Signal of the k^{th} measurement of material *i* within dilution *j*
SS	Sum of squares
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 elapse at time point *i*
$\overline{t}$	Mean of all t_i
$t_{\alpha,\,df}$	Critical *t*-value for a *t*-test, with a level of confidence of 1-α and df degrees of freedom
t_{sl}	Chosen shelf life
t_{tt}	Chosen transport time

TIU	Trypsin inhibitor unit
TM	Target material
TF	Transfer factor
TRIS	Tris(hydroxymethyl)aminomethane
u	Standard uncertainty
U	Expanded uncertainty
u_{b}	Standard uncertainty of the slope
u_{bal}	Standard uncertainty related to the balance; an additional index "rel" is added as appropriate
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
$u_{char,cal}$	Standard uncertainty of the calibrant characterisation; 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
$u_{ERM\text{-}DA470k/IFCC}$	Standard uncertainty of the certified value for ERM-DA470k/IFCC
u_{Δ}	Combined standard 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_{pur}	Standard uncertainty for the purity of the calibrant; 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
USNRP	United States National Reference Preparation
UV	Ultraviolet
V	Volume
$\overline{y}$	Mean of all results of the homogeneity study
α	Significance level
Δ_{meas}	Absolute difference between mean measured value and the certified value

$\nu_{MSwithin}$	Degrees of freedom of MS_{within}
WG-HAT	Working Group – Harmonisation of Autoantibody Tests

1. Introduction

1.1 Background

Autoimmune antibodies are important analytes in laboratory medicine. The measurement of their concentrations is used in routine medical evaluations and in a large number of specific indications, and can be performed repeatedly without (major) injury to the patient.

In 2009 the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) formed a new working group with a mandate for the Harmonisation of Autoantibody Tests (known as WG-HAT). The detection and quantification of IgG antibodies to autoantigens are important for the diagnosis and monitoring of a number of autoimmune diseases. For every autoantibody in routine use, there is currently marked diversity in the response of methods available for analysis and the materials used for assay calibration. There are materials designated as standards for some of these methods, however they are not fully characterised and often used inconsistently. This generates large variability in the analysis results (as shown in EQAS results) and a potential delay in the diagnosis and subsequent follow-up of the disease.

The immunoassays used for the measurement of autoimmune antibodies can be sensitive and specific, and are convenient in a clinical setting because they give fast results. The signal is dependent on a large number of factors such as antibody specificity, reaction kinetics and equilibria, multimeric state of the proteins, complex matrix effects, etc. Therefore the quantification with immunoassays requires the use of a proper calibrant.

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

A calibrant is required to have an assigned value that is metrologically traceable, and accompanied by an uncertainty statement. The stability and homogeneity with respect to the certified property must be verified, and the calibrant must be commutable, i.e. resemble the patient samples [7]. These attributes are particularly challenging for serum protein calibrants, as they form a mixture of interacting proteins with different isoforms and complexes.

1.2 Choice of the material

One of the type of antibodies selected by IFCC for quantification and standardisation are the antibodies against the neutrophil enzyme myeloperoxidase. These antibodies are detected as perinuclear anti-neutrophil cytoplasmic antibodies (ANCAs) and represent the cornerstones of the diagnosis of small vessel associated vasculitis. Microscopic polyangiitis and eosinophilic granulomatosis with polyangiitis are disorders mostly associated with ANCA antibodies directed against the naturally occurring myeloperoxidase [8].

The requirements for a material to be used as a reference material for the calibration of immunoassay-based *in vitro* diagnostic devices or control products for anti-MPO IgG quality control are, next to requirements for homogeneity, stability, traceability and commutability:

- The concentration of the target protein in the final material should be high enough so that dilutions of the material can cover the relevant part of the measurement interval of the methods.
- There must be continuity of the measurement results from methods calibrated against consecutive reference materials. This is an important issue in clinical chemistry, as the use of reference ranges and decision limits requires that measurement results are comparable over long time scales.

According to the data received and analysed, the raw material selected for anti-MPO IgG is sufficiently commutable to lead to a considerable reduction of inter-assay variances. Therefore it was decided to produce the new material according to procedures described below.

1.3 Design of the project

After a thorough commutability study a plasmapheresis material with a high concentration of anti-MPO IgG autoantibodies was selected as the starting material [2]. It was converted into serum and processed according to the procedure used for the reference material ERM-DA470k/IFCC. The procedure consists of a delipidation step followed by the addition of preservatives (Section 3.2). The processed serum was then transferred to vials (1 mL serum per vial) and lyophilised. The vials were closed under nitrogen with rubber stoppers and screw caps and were then stored at -70 °C.

The homogeneity, short term and long term stability of the material were assessed for anti-MPO IgG.

A calibration solution was prepared and characterised to facilitate the characterisation of ERM-DA476/IFCC. Anti-MPO IgG was purified from plasmapheresis material by a combination of affinity chromatography and size exclusion chromatography. A value for the IgG concentration in the calibrant was assigned using 5 routine methods selective for total IgG (based on either turbidimetry or nephelometry). The value assignment was performed using ERM-DA470k/IFCC as a calibrant. The value obtained by immunoassays was consistent with that obtained by UV measurements.

A value was assigned to the ERM-DA476/IFCC by using the purified anti-MPO IgG calibrant spiked into human serum and routine anti-MPO IgG procedures (ELISA, chemiluminescent and fluoroenzyme immunoassays). This was achieved using a value transfer protocol that can be considered as reference procedure [3]. In the value transfer procedure 6 dilutions of the target material were measured in parallel next to 6 dilutions of the calibrant. The concentration of the target material was determined against the calibrant solutions (Figure 1). The procedure is described in detail in Chapter 6.2.

The characterisation measurements were either performed under an ISO/IEC 17025 accreditation or within the scope of an ISO 13485 quality system.

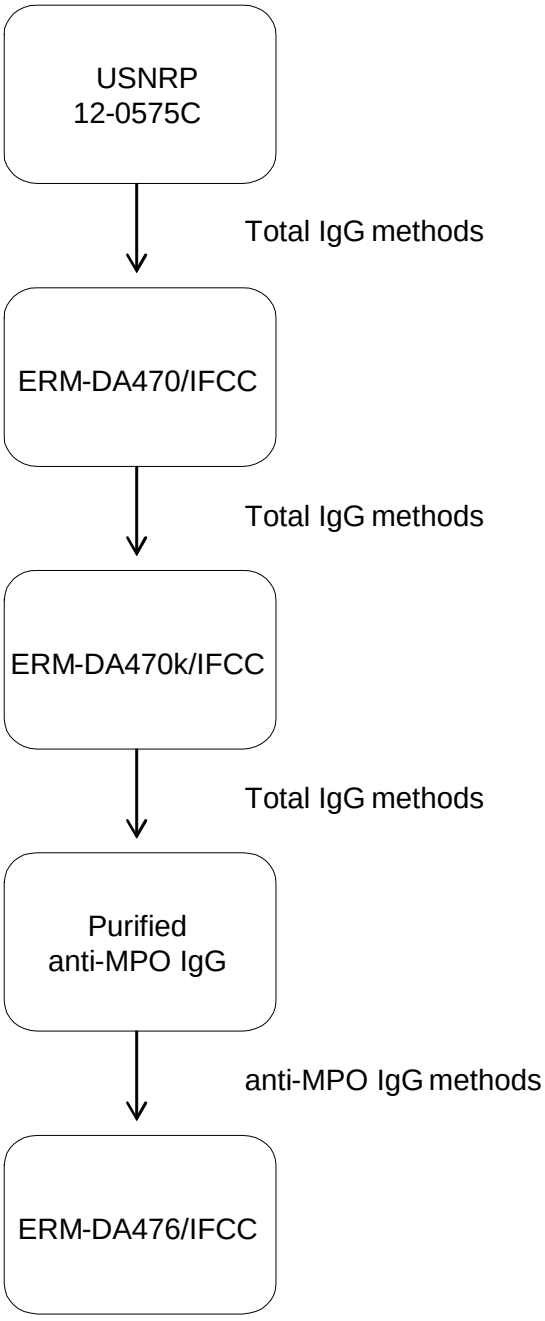

Figure 1: Traceability chain

2. Participants

2.1 Provision of raw materials

Statens Serum Institute, Amager, DK

2.2 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)

2.3 Processing

- 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

2.4 Homogeneity and stability studies

INOVA Diagnostics, INC., San Diego, US

2.5 Characterisation

- AESKU Diagnostics GmbH & Co., Wendelsheim, DE
- Bio-Rad Laboratories INC., California, US
- DAKO Denmark, Glostrup, DK
- EUROIMMUN Medizinische Labordiagnostika AG, Dassow, DE
- EuroDiagnostica AB, Malmö, SE
- 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)
- IMMCO Diagnostics, Buffalo, US
- INOVA Diagnostics, INC., San Diego, US
- Lund University, Lund, SE
- Protein Reference Unit, St. Georges Hospital, London, UK (certified to UKAS CPA 1929)
- Phadia / Thermo Fisher Scientific, Freiburg, DE
- Roche Diagnostics GmbH, Penzberg, DE
- Siemens Healthcare Diagnostics Products GmbH, Marburg, DE

3. Material processing and process control

3.1 Origin of the starting material

The raw material was a plasmapheresis material collected from patients diagnosed with an autoimmune, non-infectious disease, provided by Statens Serum Institute (DK). It was tested and found to be negative for Hepatitis B surface antigen, HIV 1&2, HIV antigen and Hepatitis C antibodies.

3.2 Processing and processing control

3.2.1. Plasma conversion into serum

An aliquot (1 L) of the plasmapheresis material was thawed and warmed up to 37 °C. A 1/100 volume of 5 mg/mL protamine sulphate solution (Sigma-Aldrich, DE) was then added and the material was stirred for 10 minutes at 37 °C. After incubation for one hour at room temperature (RT) the material was then incubated at 4 °C for 40 hours to allow the formation of fibrin. The fibrin was then removed by centrifugation at 10000 rpm for 10 min whereupon the supernatant was filter sterilized using a 0.22 µm PES filter (Corning Incorporated, USA).

3.2.2. Serum processing

The procedure for the processing of serum was based on that used for ERM-DA470k/IFCC [3]. Defibrinated serum was treated with NaCl (final concentration 50 g/L, Sigma-Aldrich, DE) and the pH adjusted to 8.5 with a saturated TRIS solution (Sigma-Aldrich, DE). The lipids present in the serum were removed by incubation for 1 h at 4 °C under slow stirring (100 rpm) with synthetic amorphous silica. Protein precipitates and silica particles were removed by centrifugation at 10000 rpm for 30 min. The clear supernatant was dialysed against nine changes of isotonic NaCl solution (0.9 % w/v) (Sigma-Aldrich, DE) over 24 hours. The pH was then adjusted to 7.2 with a 100 mmole/L HEPES solution (Sigma-Aldrich, DE) and the following preservatives added: sodium azide (final concentration of 0.95 g/L), aprotinin (final concentration of 61.5 TIU/mL) and benzamidine hydrochloride monohydrate (final concentration of 1 mmole/L). All three preservatives were purchased from Sigma-Aldrich (DE). The serum was then filter sterilised through a 0.22 µm PES filter (Corning Incorporated, USA) prior to dilution 1:6 with Zenalb® 4.5 (Bio Products Laboratory, UK).

3.2.3. Filling

1 mL serum was transferred to each vial under clean-room conditions. Colourless threaded siliconised glass vials and white screw caps were used.

Lyophilisation was started within one hour after filling the vials. Closing the bottles using rubber stoppers was performed under a low-pressure nitrogen atmosphere. Filling and lyophilisation were carried out with the local defined processes in accordance with LP-00614 (Filling and Labelling) and LP-00619 (Lyophilisation).

4. Homogeneity

A key requirement for any certified reference material aliquoted into units is the 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. Consequently, ISO Guide 34 requires RM producers to quantify the between unit variation. This aspect is covered in between-unit homogeneity studies.

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified value of the CRM was valid for all vials 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 units produced and therefore 20 units were selected using a random stratified sampling scheme, covering the whole batch. For this, the batch was divided into twenty groups (with a similar number of units) and one unit was selected at random from each one. Three independent samples were taken from each of these units, and analysed by an ELISA (QUANTA Lite™ MPO IgG). The measurements were performed under repeatability conditions and in a randomised manner so as 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 were observed.

The dataset was assessed for consistency using Grubbs outlier tests with a confidence level of 99 % on the individual results and the unit means. No outlying individual results or outlying unit means were detected.

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 visually assessed 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 the statistical evaluation of the homogeneity studies at a 95 % confidence level show that the material is homogeneous.

It should be noted that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and are 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.* [9]. u^*_{bb} 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:

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

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

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

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 from the homogeneity studies are shown in Annex A. The results of the evaluation of the between-unit variation are summarised in Table 1. The values from the equations above were converted into relative uncertainties. The uncertainty contribution for homogeneity was determined by the method repeatability.

Table 1: Results of the homogeneity study

CRM	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]
ERM-DA476/IFCC	3.09	n.c.[1]	0.85

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

As u^*_{bb} sets the limits of the study to detect inhomogeneity it was adopted as the uncertainty contribution to account for potential inhomogeneity.

4.2 Within-unit homogeneity and minimum sample intake

The minimum sample intake is the minimum amount of sample which is shown to be representative for the whole unit and thus can be used in an analysis. The within-unit homogeneity is correlated to the minimum sample intake and therefore it follows that individual aliquots of a material below the minimum sample intake will not contain the same amount of analyte. Sample sizes equal or above the minimum sample intake guarantee the certified value within its stated uncertainty.

The smallest sample intake tested was determined using the method information supplied by the participants, from the results of the homogeneity/stability experiments and from the characterisation study. In all cases ELISA based methods were used. The smallest sample intake used was 10 µL, which was henceforth established as the minimum sample intake.

The standard deviation within a bottle (s_{wb}) is lower than the expected method variability, so there is no indication of intrinsic heterogeneity or contamination at a sample intake of 10 µL.

5. Stability

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 material is to be transported at ambient temperatures.

The stability studies were performed using an isochronous design [10]. In this approach, units were stored for a specified length of time at different temperature 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. The data were analysed by calculating the regression line for protein concentration in relation to time, and determining whether it is significantly different from zero.

5.1 Short-term stability study

During the short-term stability study, units were stored at -20 °C, 4 °C and 18 °C for 0, 1, 2 and 4 weeks. The reference temperature was set at -150 °C. Two units per storage time were selected using a random stratified sampling scheme. Three samples, taken from each unit, were analysed by anti-MPO BioFlash ELISA under repeatability conditions in a randomised sequence, to be able to separate a potential analytical drift from a trend over storage time. The values were corrected for the variable reconstitution volume.

The data were evaluated for each temperature individually. The results were screened for outliers using the single and double Grubbs test. For every temperature there were 24 measurements performed.

Furthermore, the data were evaluated against storage time and regression lines of protein concentration versus time were calculated. The slope of the regression line was tested for statistical significance (loss/increase due to shipping conditions) and it was found to be significantly different from 0 when the absolute value of the slope b divided by its uncertainty u_b ($|b/u_b|$) is larger than $t_{0.05,\ 22}$= 2.07. There were no indications of instability at any of the temperatures for measurements performed with the BioFlash assay.

Since no technical reason for the outliers was found, all data were retained for statistical analysis.

The results of the short-term stability studies are shown in Annex B.

No technically unexplained outliers/Statistical outliers were detected and thus all data were retained for the estimation of u_{sts}. None of the trends were statistically significant at a 95 % confidence level for any of the temperatures.

From this study it was concluded that for the shipment (up to 2 weeks) the possible contribution of the transport to the uncertainty of the certified values is negligible compared to the overall uncertainty. For practical reasons and for retaining the uncertainty values as low as possible it is strongly recommended to ship the material on dry ice and within one week.

5.2 Long-term stability study

During the long-term stability study, samples were stored at -20 °C and -70 °C for 0, 8, 16 and 24 months. The reference temperature was set at -150 °C. Two units per storage time were selected using a random stratified sampling scheme. Three samples from each unit were analysed by a BioFlash ELISA immunoassay 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 found when the results were screened using the single and double Grubbs test. Furthermore, the data were plotted against storage time and linear regression lines of protein activity (U/mL) fraction versus time were calculated. The slope of the regression lines was assessed for statistical significance (loss/increase due to storage conditions). The slopes of the regression lines were not significantly different from zero (at a 95 % confidence level) for either temperature.

No technically unexplained outliers were observed and none of the trends was statistically significant at a 95 % confidence level for any of the temperatures. The material can be stored at both -20°C and -70 °C. However, as it is envisaged to be stored for long periods of time, it should be preferably kept at -70 °C.

5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results, no study can 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 timeframe studied.

Uncertainties of stability during dispatch and storage were estimated as described in [11]. 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:

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

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

RSD relative standard deviation of all results of the stability study

t_i time elapse at time point *i*

$\bar{t}$ mean of all t_i

t_{tt} chosen transport time (1 week at -20 ºC)

t_{sl} chosen shelf life (24 months at -70 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the -20 °C studies. The uncertainty describes the possible change during a dispatch at -20 °C lasting for one week.
- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the -70 °C study. The uncertainty contribution describes the possible degradation during 24 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 storage at -20 °C for 1 week; $u_{lts,rel}$ was calculated for storage at -70 °C for 2 years.

CRM	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
ERM-DA476/IFCC	0.66	1.95

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

6. Characterisation

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

The characterisation of both the anti-MPO IgG serving as calibrant and of the candidate CRM was based on an inter-laboratory comparison of expert laboratories, i.e. the protein concentration of the material was determined in different laboratories who applied their own methodology and instrumentation. This approach aims to negate the laboratory bias, which reduces the combined uncertainty.

In the case of the value assignment for the calibrant, the participant laboratories used turbidimetry and nephelometry for their measurements. For the characterisation of the ERM-DA476/IFCC all participants used ELISA-type immunosorbent assays.

6.1 Selection of participants

Six laboratories were selected for the calibrant material and 10 for the target material based on criteria that comprised both technical competence and quality management. Each participant was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field of *in vitro* diagnostics in relevant matrices by submitting results for intercomparison exercises or method validation reports. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was. The accreditation numbers for the accredited laboratory are stated in the list of participants (Section 2).

6.2 General principles of the value assignment

The laboratories were provided with detailed protocols and reporting sheets, as well as with vials of the materials to be analysed. Laboratories were asked to specify the platform and reagents used, and the order in which the measurements were performed. Both procedures required that the material was reconstituted the day before the measurements, according to the procedure described in the certificate of ERM-DA470k/IFCC and in the present report (Chapter 9.3).

Principle of the analysis

The aim of the value transfer is to determine the transfer factor (TF)

$$TF = C_T/C_R \quad (1)$$

where C_T and C_R are the mass concentration of the protein in the target material (TM, ERM-DA476/IFCC) and in the calibrant (purified anti-MPO IgG), respectively.

Six different dilutions of the calibrant are prepared. The mass concentration of the protein in each dilution will be:

$$C_R(i) = F_R(i) \times C_R \quad (2)$$

where F_R is the dilution factor of the control and (i) denotes the different dilutions (i = 1 to 6).

The concentration of the protein in the dilutions of the target material will be:

$$C_S(j) = F_T(j) \times C_T \quad (3)$$

Where F_T is the dilution factor of TM and (j) denotes the different dilutions of TM (j = 1 to 6).

For the transfer procedure a calibration run is made, and the calibration curve is constructed by plotting the signals against the different dilutions of RM. In a measurement run the different dilutions of TM are assayed. The signals of these unknown test samples $C_S(j)$ are interpolated on the calibration curve, and are measured in relative concentrations of RM:

$$C_S(j) = F_R(j) \times C_R \quad (4)$$

A combination of (3) and (4) gives:

$$C_S(j) = F_T(j) \times C_T = F_R(j) \times C_R$$

$$F_R(j) = C_T/C_R \times F_T(j) \quad (5)$$

which is the equation of a straight line through the origin (y = TF x).

The different dilution factors $F_R(j)$ obtained by interpolation are plotted against the different dilution factors $F_T(j)$ used for the initial dilution of TM. Since all dilutions are controlled by weighing, the uncertainty of $F_T(j)$ is negligible compared to that of the measurement results.

Calculation of the dilutions

The precision of the values for the dilutions were considerably improved by weighing all the liquids used for the reconstitution of the materials and for the preparation of the dilutions to a precision of at least 0.0001 g.

The following mass corrections were applied:

For the reconstitution:

$$f_{M,i} = \frac{m_{intended}}{m_{M,i}}$$

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

For eventual predilutions:

$$f_{P,i} = \frac{m_{PR,i}}{m_{PR,i} + m_{PD,i}}$$

where $m_{PR,i}$ and $m_{PD,i}$ are the masses of the of the reconstituted material and of the diluent for the predilution

For the dilutions:

$$f_{D,ij} = \frac{m_{DM,i}}{m_{DM,i} + m_{DD,i}}$$

for the dilution *j* of the material *i*, with $m_{DM,i}$ the mass of the reconstituted (eventually prediluted) material, and $m_{DD,i}$ the mass of the diluent for dilution j.

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

$c_{ij} = c_{0i} \times f_{M,i} \times f_{P,i} \times f_{D,ij}$

with c_{0i} of the protein in material *i*, and c_{ij} the concentration of the protein in dilution *j* of material *i*.

Determination of the transfer factors (TFs)

During the transfer procedure the dilutions of the reference material (either ERM-DA470k/IFCC in case of the characterisation of the calibrant or of anti-MPO IgG in the case of the characterisation of ERM-DA476/IFCC) were used to construct the calibration curve. Based on this curve and the known concentration of the spiked material, the values of the target material were calculated. The relative concentrations were corrected for the masses of the dilutions (and predilution).

During the value transfer procedure the laboratories measured the 6 dilutions of the target (Y1-Y6) and the calibrant (X1-X6) in triplicate on each day. For both materials, single measurement results S_{ijk} (signal of the k^{th} measurement of material *i* within dilution *j*, in g/L) were plotted in scatter plots $S_{ijk} = f(c_{ij})$ so as to evaluate outliers. A linear regression with intercept was performed on the means of the S_{ijk} in function of the concentration c_{ij}.

The following analyses were performed on all data:

- Evaluation of the studentised residuals for the identification of outliers
- Test for the homoscedasticity of the residuals (whether the variances are homogeneous) via a *t*-test
- Test for normality (visual inspection and normal probability plot)
- Test for linearity (visual inspection and evaluation of R^2)
- Test for outliers of the regression model according to the method of Lund [12] (only in case of homoscedasticity)
- Verification that the intercept ± 4 times the *s* covers the origin.

The TFs were calculated as the ratio of the slopes of the linear regression lines for the candidate reference material and for the calibrant.

The following general acceptance criteria were applied to the datasets:

1. R^2 of the regression must be above 0.99 for data of all platforms
2. Data from at least 4 dilutions must be available
3. The dataset from any one day must contain at least 50 % of the data generated. on that day, otherwise all of the data generated on that day are declared non-valid
4. At least two daily value assignments must be valid
5. The day-to-day variation (RSD) of valid datasets for the protein and laboratory must be below 15 %

Rationale for the acceptance criteria:

- Overall $R^2 < 0.99$ of regression indicates quality problems in the measurements (scattering, outliers, run-to-run variation).

- During the data analysis, the results where the linear regression went through 0 ($y = bx$) were compared to those when linear regressions were performed with an intercept ($y = a+bx$). The mean of means of the TFs between laboratories were comparable. However, since only measured data were used and no model other than the linearity within the actual measurement interval was assumed it was decided to process the data allowing for intercepts in the linear regression, provided that the intercept ± 4 s covers the origin. As a consequence, the criterion for the acceptance of datasets was adapted, and a (within laboratory) day-to-day RSD of 15 % was defined.

6.3 Characterisation of the calibrant

During the characterisation of the calibrant (anti-MPO IgG), each laboratory received one vial of purified anti-MPO IgG and five vials of its dilution prepared in JRC-IRMM as well as one vial of ERM-DA470k/IFCC and five vials of its dilution. They were required to provide 18 independent results, per material (three per dilution). Participating laboratories were instructed to use their in-house calibrant to determine the dose-response function of the instrument. The units for material characterisation were selected using a random stratified sampling scheme and covered the whole batch. The techniques used to measure the protein concentration of the calibrant were nephelometry, turbidimetry and spectrophotometry. The measurements were performed with different platforms (Annex C, Table C1) and reagents. In total six laboratories participated in the characterisation study.

6.3.1. Production and physico-chemical characterisation of the calibrant

The anti-MPO IgG was purified from human serum (provided by Statens Serum Institute) in a three step purification process. Firstly, total IgG was separated by affinity chromatography using a Protein A column. Secondly, the specific anti-MPO IgG was separated from all other IgGs eluting from the protein A column using an in-house prepared Hi-trap column prepared using commercially available purified human myeloperoxidase (Scipac, UK) according to the protocol provided by the manufacturer. All anti-MPO IgG containing fractions were pooled, concentrated to a final volume of 500 µL using Amicon Ultra centricons with a 30 KDa cut-off (Milipore, USA) and subsequently applied on a Superdex 200 10/300 GL column (GE

Healthcare, SE). The purity of the final material with respect to IgG selectivity for MPO was assessed by affinity chromatography. The in-house prepared anti-MPO Hi-Trap column described above was used for this purpose. In the chromatograph only one peak was eluted under the acidic conditions of the eluting buffer (pH 2.7) and no peaks were observed during the elution with the binding buffer (pH 7.0). The background noise was taken into consideration for the ratio to the peak for anti-MPO IgG. The uncertainty of the purity of the material was set as three times this ratio. The calculated uncertainty was equal to 0.5 % (Table 3).

6.3.2. Value assignment of the calibrant

The concentration of the calibrant was measured by nephelometry or turbidimetry (Annex C, Table C1). The participating laboratories provided us with concentration values for both the ERM-DA470k/IFCC and the purified anti-MPO IgG solution based on their in-house calibrant. Regression lines were constructed comparing these measured concentrations to the gravimetrically calculated dilution factors of these samples. The slope ratio of the ERM-DA470k/IFCC to the purified anti-MPO IgG was used to determine the value of the dilutions of the target material.

The data were assessed for both their compliance with the analysis protocol and for their validity based on technical reasons. The following criteria were considered during the evaluation:

- Compliance with the analysis protocol: sample preparations and measurements performed on a minimum of two days, and the analytical sequence.
- Method performance, i.e. agreement of the measurement results with the assigned value of their in-house QC sample.
- R^2 of the regressions must be above 0.99 for data of all platforms.

Based on the criteria above, one dataset was rejected as not technically valid. The results provided from this laboratory did not comply with the criteria for the correlation coefficients, showing R^2 values < 0.99. In another laboratory (L1, Annex C, Table C1), the higher concentration analysed was not taken into account while plotting the results as it was outside the laboratory's working range of the method.

The 5 datasets accepted based on technical reasons were assessed for the normal distribution of dataset means using normal probability plots and were assessed for outlying means using the Grubbs test and using 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 the individual laboratories are given in Table C1 of the Annex C.

The uncertainty relating to the characterisation of the calibrant (purified anti-MPO IgG) was estimated as the standard error of the mean of laboratory means (Table 3). The uncertainty of the reference material used for the calibration (ERM-DA470k/IFCC) was taken into account as well the uncertainty of the purification of the material. The uncertainty deriving from the gravimetric preparation of the dilutions was found to be very low and according to GUM specifications it was not taken into account for the final uncertainty of the calibrant ($u_{bal,rel}$ = 0.005 %).

Table 3: Uncertainty budget for purified anti-MPO IgG.

Analyte	*p*	Mean concentration [mg/mL]	*s* [mg/mL]	$u_{ERM\text{-}DA470k,rel}$ [%]	$u_{char,\ cal\ rel}$ [%]	$u_{pur,rel}$ [%]
anti-MPO IgG	5	1.52	0.03	0.98	0.88	0.43

These different contributions were combined to estimate the relative uncertainty of the concentration value of the calibrant ($u_{anti\text{-}MPO\ IgG,\ rel}$) as:

$$u_{anti\text{-}MPO\ IgG} = \sqrt{u^2_{char\,cal,rel} + u^2_{ERM\text{-}DA470k/IFCC} + u^2_{pur,rel}} \qquad \textbf{Equation 6}$$

- $u_{char,cal}$ was estimated from the data in Annex C, Table C1.
- $u_{ERM\text{-}DA470k/IFCC}$ was estimated in the report of the material [3]
- u_{pur} was estimated as described in Section 6.3.1.

The concentration of the material was additionally determined by UV spectrophotometry at 280 nm using an absorption coefficient of 1.36 $(g/L)^{-1}cm^{-1}$ (210000 $M^{-1}cm^{-1}$) [13]. The concentration which was measured on 4 separate days, in triplicate, was equal to 1.43 mg/mL. This value was not used for the calculation of the certified value of the candidate reference material nor in the calculation of the uncertainties.

6.4 Characterisation of ERM-DA476/IFCC

During the target material characterisation each laboratory received four vials of ERM-DA476/IFCC and four vials of anti-MPO spiked into human serum. They were required to perform six dilutions from these vials and to provide 72 independent results, three per vial per day, for each material. The vials for the materials characterisation were selected using a random stratified sampling scheme and covered the whole batch. The sample preparations and measurements had to be spread over four days to ensure intermediate precision conditions. For the spiked material (calibrant), participants were required to treat them as they would any other sample. The dilution protocol was the same for both materials (6.67, 10, 15, 20, 50 and 100 (m/m) % of the reconstituted materials).

The techniques used to measure the protein concentration were traditional ELISAs and in some cases its variations (chemiluminescent and fluoroenzyme immunoassays) specific for IgG anti-MPO. In total 10 laboratories participated in the value assignment, using 9 different kits (Annex C, Table C2).

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

- Compliance with the analysis protocol (sample preparations and measurements performed on a minimum of two days) and the analytical sequence.
- Method performance, i.e. agreement of the measurement results with the assigned value of their in-house QC sample

- R^2 of the regressions either linear or logarithmic must be above 0.99 for data of all platforms

Linear regressions were used for the analyses of the data provided by laboratories 1, 3, 5-10 (Annex C, Table C2). However, in the case of laboratories 2 and 4 the data provided were following a logarithmic distribution and had to therefore be treated accordingly.

Based on the above criteria, the results obtained from laboratory 10 were completely omitted and not used for the certification. In particular, the R^2 values for all days were much below the set limit of 0.99 whilst at the same time there was a 35 % day-to-day variation. Additionally for laboratory 3, two out of 4 days had correlation coefficients below 0.99 and thus those two days were not taken into account for the final value assignment.

The valid datasets were assessed for the normal distribution of the dataset means using normal probability plots and were assessed for outlying means using the Grubbs test and using 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 laboratory means follow normal distributions. None of the data contains outlying means and variances. The datasets were therefore consistent and the mean of laboratory means was a good estimate of the true value. The value assignment data of the individual laboratories are shown in Annex C, Table C3.

7. Uncertainty budget and certified values

Certified values are values that fulfil the highest standards of accuracy. Procedures at IRMM require no less than 6 valid datasets to assign certified values. Full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [6] were established. A certified value was assigned to ERM-DA476/IFCC.

The assigned uncertainty consists of uncertainties relating to the characterisation of the target material, u_{char} (Section 6), to the combined uncertainty of the characterisation of the calibrant $u_{char,cal}$ (Section 6), to the potential between-unit inhomogeneity, u_{bb} (Section 4) and to the potential degradation during transport (u_{sts}) and long-term storage, u_{lts} (Section 5). The uncertainty relating to the use of analytical balance during the gravimetric preparation of the dilutions of both the calibrant and the target material was found to be negligible (equal to 0.005 and 0.006 % respectively) [4]. The uncertainty relating to possible degradation during transport and long-term storage is included in the results for u_{sts} and u_{lts}. The uncertainty of between-unit inhomogeneity was found to be too low to be calculated and thus the maximum hidden heterogeneity was eventually used for the calculation of the uncertainty of the certified value. These different contributions were combined to estimate the expanded, relative uncertainty of the certified value ($U_{CRM,\,rel}$) with a coverage factor k as:

$$U_{CRM,rel} = k \cdot \sqrt{u^2_{char,rel} + u^2_{char,cal,rel} + u^2_{bb,rel} + u^2_{sts,rel} + u^2_{lts,rel}} \qquad \textbf{Equation 7}$$

- u_{char} was estimated as described in Section 7.1
- $u_{char,cal}$ was estimated as described in Section 6.3.2
- u_{bb} was estimated as described in Section 4.1.

- u_{sts} was estimated as described in section 5.3
- u_{lts} was estimated as described in Section 5.3.

7.1 Certified value

The certified value was calculated from the average of the TF (Annex C, Table C3) and the value of the calibrant (Table 3, 3rd column) according to:

$c_{ERM\text{-}DA476/IFCC} = TF_{average} * c_{anti\text{-}MPO\ IgG}$

Where $c_{anti\text{-}MPO\ IgG}$ is the concentration of anti-MPO IgG (calibrant) spiked gravimetrically into human serum.

The uncertainty related to the characterisation of the ERM-DA476/IFCC is estimated as the standard error of the mean of laboratory means (Table 4).

Table 4: Value assignment for ERM-DA476/IFCC

CRM	*p*	Mean [mg/L]	*s* [mg/L]	$u_{char,\ rel}$ [%]
ERM-DA476/IFCC	9	84.20	10.69	4.25

7.2 Estimation of uncertainty

The relative expanded uncertainty was calculated from the relative combined standard uncertainty $u_{CRM,rel}$ by multiplication with a coverage factor k ($U_{CRM}=u_{CRM}$*k). This coverage factor was taken as 2 as there is a sufficient numbers of degrees of freedom of the different uncertainty contributions. The relative expanded uncertainty was multiplied by the mean of dataset means to obtain the expanded uncertainty $U_{CRM.}$

The uncertainty budget after taking into consideration the various uncertainty contributions and the relative combined uncertainty ($u_{CRM,rel}$) is shown in Table 5.

Table 5: Uncertainty budget for ERM-DA476/IFCC

CRM	$u_{anti\text{-}MPO\ IgG\ rel}$ [%]	$u_{char,\ rel}$ [%]	$u_{bb,\ rel}$ [%]	$u_{sts,\ rel}$ [%]	$u_{lts,\ rel}$ [%]	$u_{CRM,rel}$ [%]	U_{CRM} [mg/L]
ERM-DA476/IFCC	1.38	4.25	0.84	0.66	1.95	4.99	8.40

Finally the certified value for the ERM-DA476/IFCC is equal to 84 mg/L and the expanded uncertainty of this value is equal to 9 mg/L.

8. Metrological traceability and commutability

8.1 Metrological traceability

Identity

The mass concentration of anti-MPO IgG in ERM-DA476/IFCC is defined by the immunoassay procedures used to characterise it. The assigned value is therefore operationally defined by *method*.

Quantity value

The measurements of anti-MPO IgG were calibrated with ERM-DA470k/IFCC by applying the value transfer procedure described in this report. The value transfer measurements were strictly controlled with respect to adherence to the procedure and the adequate functioning of equipment and reagents was verified. Different combinations of reagents and platforms were used, which gave consistent results. Therefore the certified value is not dependent on the individual methods.

The certified values for anti-MPO IgG in ERM-DA476/IFCC were obtained by calibration with purified anti-MPO.

The traceability chain is based on the use of calibrated balances and a thorough control of the weighing procedure. The certified mass concentration and its uncertainty are traceable to the stated value of the mass concentration in USNRP 12-0575C [14] applying the procedures described in the certification report of ERM-DA470k/IFCC [3] and in the present report.

8.2 Commutability

Many measurement procedures include one or more steps, which select specific analytes from the sample for the subsequent steps of the measurement process. 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 expressing this concept. For instance, the CSLI Guideline C-53A [7] 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, thus, is a crucial characteristic for the application of different measurement methods. When 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 does not exist 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.

Different formats of candidate reference materials, all based on the same raw material as ERM-DA476/IFCC, have been tested and found to be commutable for combinations of seven methods giving correlating results (Annex D, Table D1). Therefore ERM-DA476/IFCC is expected to be commutable for the majority of IgG anti-MPO methods [2]. However, if

another method is used other than those included here then commutability should be verified.

9. Instructions for use

9.1 Safety information

The usual laboratory safety measures apply. Do not discharge the waste into the drain. Each portion of donated blood used in the production of the material has been tested and found negative for Hepatitis B surface antigen, HIV 1&2, HIV antigen and Hepatitis C antibodies. 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 both -20°C and -70 °C. However, as it is envisaged to be stored for long periods of time, it should be preferably kept at -70 °C. If microbial contamination has been excluded during the reconstitution procedure, the solution of ERM-DA476/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.

9.3 Preparation and use of the material/Reconstitution

The material must be reconstituted according to the following procedure:

- Remove the vial from the freezer or refrigerator during the afternoon of the day before use and place the vial in the room where the balance is located for 1 hour.
- 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 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 a particular protein 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 before use.

9.4 Minimum sample intake

The minimum representative sample intake is 10 µL. The entire content of the vial must be reconstituted.

9.5 Use of the certified value

The main purpose of this material is to be used for the calibration of immunoassay-based *in vitro* diagnostic devices or control products for anti-MPO IgG. As any reference material, it can also be used for control charts or validation studies.

When the material is used as a calibrant in a particular assay the commutability should be verified for the assay concerned.

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 [15].

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.

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Annexes

Annex A: Results of the homogeneity measurements

Source of Variation	*SS*	df	*MS*	*s*	F	F-crit 95 %	F-crit 99 %
Between Units	37.6	19	1.98	$MS_B<MS_W$	0.67	1.9	2.4
Within Units	118.3	40	2.96	1.72			
Total	155.88	59					
Homogeneity Results	Weight.Avg. [mg/L]	S_{bb} [mg/L]	S_{bb} [%]	S_{wb} [mg/L]	S_{wb} [%]	U_{bb}* [mg/L]	u_{bb}*[%]
	55.76	$MS_B<MS_W$	$MS_B<MS_W$	1.72	3.08	0.47	0.84

Table A1: Analysis of Variance of the homogeneity results

Annex B: Results of the short-term stability measurements

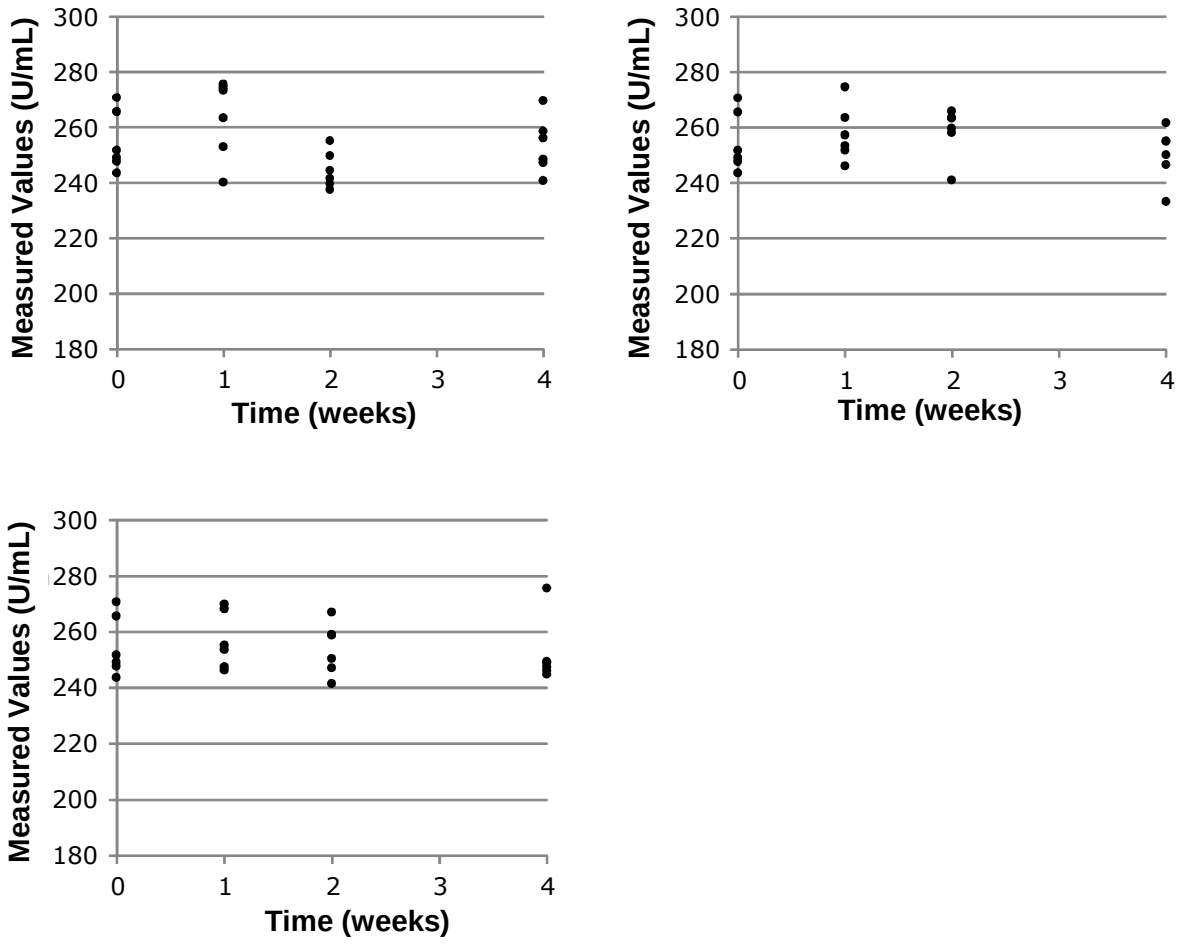

Fig. B1: Short-term stability data (results of individual replicates) of ERM-DA476/IFCC; Measured values by an ELISA immunoassay (U/mL), when stored for several weeks at -20 °C (a), 4 °C (b) and 18 °C (c), results at time point 0 weeks correspond to units that were stored at the reference temperature of -20 °C.

CRM	$u_{sts,}$ 1 week [%]			$u_{sts,}$ 2 weeks [%]		
ERM-DA476/IFCC	-20 °C	4 °C	18 °C	-20 °C	4 °C	18 °C
	0.66	0.54	0.55	1.32	1.07	1.09

Table B1: Uncertainties of stability during storage at different temperatures for 1 and 2 weeks

Annex C: Calibrant characterisation

anti-MPO IgG

Laboratory	Platform	Method	Concentration [mg/mL]
L1	Immage	Nephelometry	1.47
L2	Cobas 6000/8000	Turbidimetry	1.51
L3	BN Prospec	Nephelometry	1.53
L4	Modular P	Turbidimetry	1.55
L5	Cobas c 501	Turbidimetry	1.52

Table C1: All laboratories giving acceptable results, together with the platforms they used and the principles of the methods that these platforms employ are listed.

ERM-DA476/IFCC

Laboratory	Method	Principle	Concentration [mg/L]
L1	Wieslab® Capture MPO-ANCA	ELISA	92
L2	ImmuLisa ™	ELISA	68
L3	Autoimmune EIA Anti-Myeloperoxidase	ELISA	99
L4	Anti-Myeloperoxidase ELISA (IgG)	ELISA	75
L5	Wieslab® Capture MPO-ANCA	ELISA	88
L6	Wieslab® MPO-ANCA	ELISA	73
L7	QUANTA Flash MPO	Chemiluminescent immunoassay	78
L8	EliA MPO^{S}	Fluoro-enzyme immunoassay	93
L9	AESKULISA MPO	ELISA	91
L10	QUANTA Lite™ MPO IgG	ELISA	-

Table C2: All laboratories participating in the value assignment of the target material. The methods they used and the principles that these methods employ are listed. On the last column the average concentrations of the ERM-DA476/IFCC are presented.

ERM-DA476/IFCC

Laboratory code	Day 1 [slope ratio]	Day 2 [slope ratio]	Day 3 [slope ratio]	Day 4 [slope ratio]	TF [mean slope ratio]	Day-to-Day variation [%]
L1	0.962	0.860	0.827	0.809	0.864	7.91
L2	0.70	0.63	0.61	0.61	0.639	6.37
L3	[1)]	0.918	[1)]	0.941	0.930	1.75
L4	0.751	0.697	0.660	0.721	0.707	5.46
L5	0.723	0.866	[1)]	0.857	0.815	9.82
L6	0.674	0.728	0.712	0.589	0.676	9.19
L7	0.720	0.622	0.738	0.785	0.717	9.60
L8	no data	0.812	0.889	0.866	0.856	10.69
L9	0.747	0.771	0.898	0.925	0.835	10.69

[1)]: results excluded for technical reasons

Table C3: Individual results for the value assignment for the characterisation of the target material, ERM-DA476/IFCC. The relative uncertainties per laboratory and their day-to-day variation are presented.

Annex D: Commutability

Laboratory	Method	Principle
L1	Varelisa™ MPO ANCA	ELISA
L2	EliA MPO^{S}	Fluoro-enzyme immunoassay
L3	Wieslab® Capture MPO-ANCA	ELISA
L4	Wieslab® Anti-MPO ELISA	ELISA
L5	Anti-MPO ELISA	ELISA
L6	QUANTA Lite MPO IgG ELISA	ELISA
L7	Anti-MPO ORG 519	ELISA

Table D1: Laboratories that participated in the commutability studies and the methods they used

European Commission

EUR 27092 EN – Joint Research Centre – Institute for Reference Materials and Measurements

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Author(s): Evanthia Monogioudi, Dana P. Hutu, Gustavo Martos, Emma Tuddenham, Joanna Sheldon, Heinz Schimmel, Stefanie Trapmann, Pierluigi Meroni, Hendrik Emons and Ingrid Zegers

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