European Commission

ERM
European Reference Materials

CERTIFICATION REPORT

The certification of the mass concentration of immunoglobulin G proteinase 3 anti-neutrophil cytoplasmic autoantibodies (IgG PR3 ANCA) in human serum: ERM® - DA483/IFCC

EUR 28537 EN - 2017

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

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

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

JRC106170

EUR 28537 EN

ISBN 978-92-79-66974-3 (PDF)
ISSN 1831-9424 (online)
doi:10.2760/373057

Luxembourg: Publications Office of the European Union, 2017

Printed in Belgium

Abstract

This report describes the production and certification of ERM-DA483/IFCC, a serum protein reference material intended for the standardisation of measurements of immunoglobulin G proteinase 3 anti-neutrophil cytoplasmic autoantibodies (IgG PR3 ANCA). The material was produced according to ISO Guide 34:2009 [ ] and is certified in accordance with ISO Guide 35:2006.
The raw material used to prepare ERM-DA483/IFCC was a plasmapheresis material containing a high concentration of IgG PR3 ANCA. After a prior commutability study lyophilised serum was selected as the best format for the reference material. The processing of the serum was 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 buffer containing albumin, prior to the transfer of 1 mL aliquots to glass vials. The serum was then lyophilised and the vials were closed with rubber stoppers and screw caps under argon atmosphere prior to storage at -70 °C.
The between unit-homogeneity was quantified and stability during dispatch and storage was assessed in accordance with ISO Guide 35:2006.
The material was characterised by an inter-laboratory comparison exercise performed by laboratories of demonstrated competence, using a purified IgG PR3 ANCA preparation as calibrant. This was achieved by applying a value transfer protocol previously used in the characterisation of ERM-DA470k/IFCC. Technically invalid results were removed, but no outliers were eliminated on statistical grounds alone.
The uncertainty of the certified value was estimated in accordance to the Guide to the Expression of Uncertainty in Measurement (GUM) and included components relating to possible lack of homogeneity, stability and the property value measured during characterisation.
The main purpose of this material is to be used for the calibration of immunoassay-based in vitro diagnostic devices or control products for IgG PR3 ANCA measurements. As any reference material, it can also be used for control charts or validation studies. The CRM is available in glass vials containing approximately 0.1 g of dried powder. The minimum sample intake to be used after reconstitution of the material is 5 µL.
The CRM was accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

The certification of the mass concentration of immunoglobulin G proteinase 3 anti-neutrophil cytoplasmic autoantibodies (IgG PR3 ANCA) in human serum: ERM® - DA483/IFCC

Evanthia Monogioudi [1)], Dana Petronela Hutu [1)], Jean Charoud-Got [1)], Joanna Sheldon [2)], Heinz Schimmel [1)], Stefanie Trapmann [1)], Pier Luigi Meroni [3)], Hendrik Emons [1)] and Ingrid Zegers [1)]

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

2) Protein Reference Unit and Immunopathology Department,St Georges' Hospital, London (UK)

3) Department of Clinical Sciences and Community Health, University of Milan & IRCCS Istituto Auxologico Italiano, Milan (IT)

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 production and certification of ERM-DA483/IFCC, a serum protein reference material intended for the standardisation of measurements of immunoglobulin G proteinase 3 anti-neutrophil cytoplasmic autoantibodies (IgG PR3 ANCA). The material was produced according to ISO Guide 34:2009 [1] and is certified in accordance with ISO Guide 35:2006 [2].

The raw material used to prepare ERM-DA483/IFCC was a plasmapheresis material containing a high concentration of IgG PR3 ANCA. After a prior commutability study lyophilised serum was selected as the best format for the reference material. The processing of the serum was 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 buffer containing albumin, prior to the transfer of 1 mL aliquots to glass vials. The serum was then lyophilised and the vials were closed with rubber stoppers and screw caps under argon atmosphere prior to storage at -70 °C.

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

The material was characterised by an inter-laboratory comparison exercise performed by laboratories of demonstrated competence, using a purified IgG PR3 ANCA preparation as calibrant. This was achieved by applying a value transfer protocol previously used in the characterisation of ERM-DA470k/IFCC [3]. Technically invalid results were removed, but no outliers were eliminated on statistical grounds alone.

The uncertainty of the certified value was estimated in accordance to the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and included components relating to possible lack of homogeneity, stability and the property value measured during characterisation.

The main purpose of this material is to be used for the calibration of immunoassay-based *in vitro* diagnostic devices or control products for IgG PR3 ANCA measurements. As any reference material, it can also be used for control charts or validation studies. The CRM is available in glass vials containing approximately 0.1 g of dried powder. The minimum sample intake to be used after reconstitution of the material is 5 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]
IgG PR3 ANCA [1]	270	29

1) Proteinase 3 anti-neutrophil cytoplasmic antibodies as measured by immunoassays

2) Unweighted mean value of the means of 10 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 uncertainty is the expanded uncertainty of the certified value 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

Table of contents

Glossary

ANCA	Anti-neutrophil cytoplasmic antibodies
ANOVA	Analysis of variance
b	Slope in the equation of linear regression $y = a + bx$
$c_{IgG\ PR3\ ANCA}$	Concentration of the purified IgG PR3 ANCA
$c_{ERM\text{-}DA483/IFCC}$	Concentration of IgG PR3 ANCA in ERM-DA483/IFCC
CLSI	Clinical and Laboratory Standards Institute
CRM	Certified Reference 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
GUM	Guide to the expression of uncertainty in measurements
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	Joint Research Centre
k	Coverage factor
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
PES	Polyethersulfone
PR3 ANCA	Proteinase 3 anti-neutrophil cytoplasmic antibodies
QC	Quality control
rel	Index denoting relative figures (uncertainties etc.)
RLU	Relative Light Units
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 elapsed 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 relating to the balance; an additional index "rel" is added as appropriate
u^*_{bb}	Standard uncertainty relating 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 relating 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
$\overline{y}$	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.

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 (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 analysis methods 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 are often used inconsistently. This generates large variability in the analyses results (as shown in the external quality assurance scheme (EQAS) results), possible misdiagnosis and a potential delay in the diagnosis or in the follow-up of the disease.

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

One type of antibodies selected by IFCC for quantification and standardisation are the antibodies associated with an autoimmune condition known as small vessel vasculitis. This condition is mediated by antibodies against the neutrophil enzyme proteinase 3 (PR3) and myeloperoxidase (MPO). IgG PR3 ANCA are found in about 80 % of patients with Wegener's granulomatosis, and in about 35 % of patients with microscopic polyangiitis, Churg-Strauss syndrome, and renal-limited rapidly progressive glomerulonephritis [5, 6].

These antibodies are detected as anti-neutrophil cytoplasmic antibodies (C-ANCA) and represent the cornerstones of the diagnosis of small vessel-associated vasculitis together with MPO ANCA [7]. JRC has already developed and produced a certified reference material (CRM), against IgG MPO ANCA [8]. This new material, presented in the current report, against IgG PR3 ANCA, aims to fill the gap in the standardisation of measurements of IgG ANCA for small vessel-associated vasculitis.

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 must have an assigned value that is metrologically traceable, and accompanied by an uncertainty statement. The stability and homogeneity of the material with respect to the certified property must be verified, and the calibrant must be commutable, i.e. resemble the patient samples [9]. 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

The requirements for a material to be used as a reference material for the calibration of immunoassay-based *in vitro* diagnostic devices or for analysis products for IgG PR3 ANCA are, in addition to requirements for homogeneity, stability, value 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.
- For the successful reproduction of the material, materials produced consecutively must be consistent. 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 periods of time.

According to a prior commutability study performed (Section 8) the raw material selected for IgG PR3 ANCA was sufficiently commutable to lead to a considerable reduction of inter-assay variability. Therefore it was decided to produce the new material according to the procedure described below.

1.3. Work-flow

After a thorough commutability study a plasmapheresis material with a high concentration of IgG PR3 ANCA was selected as the starting material. It was converted into serum and processed according to the procedure used for the reference material ERM-DA470k/IFCC. The procedure consists of delipidation 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 argon 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 the mass concentration of IgG PR3 ANCA.

A calibration solution was prepared and characterised so as to allow the characterisation of ERM-DA483/IFCC. IgG PR3 ANCA was purified from plasmapheresis material, the same material used for preparing the certified reference material (CRM), by a combination of affinity chromatography and size exclusion chromatography (Section 6.3). A value for the IgG mass concentration in the calibrant was assigned using three routine methods selective for total IgG (based on either turbidimetry or nephelometry) repeated in two separate days. The value assignment of the calibration solution was performed using ERM-DA470k/IFCC as a calibrant.

A property value was assigned to ERM-DA483/IFCC by using this purified IgG PR3 ANCA calibration solution and routine IgG PR3 ANCA procedures (ELISA, chemiluminescent and fluorescence immunoassays). This was achieved by using a value transfer protocol that can be considered as reference procedure [10]. Therein eight dilutions of the target material were measured in parallel with eight dilutions of the calibrant. The concentration of the target material was determined against the calibrant solution (Figure 1). The procedure is described in detail in Section 6.2. The traceability chain is described in Section 8.

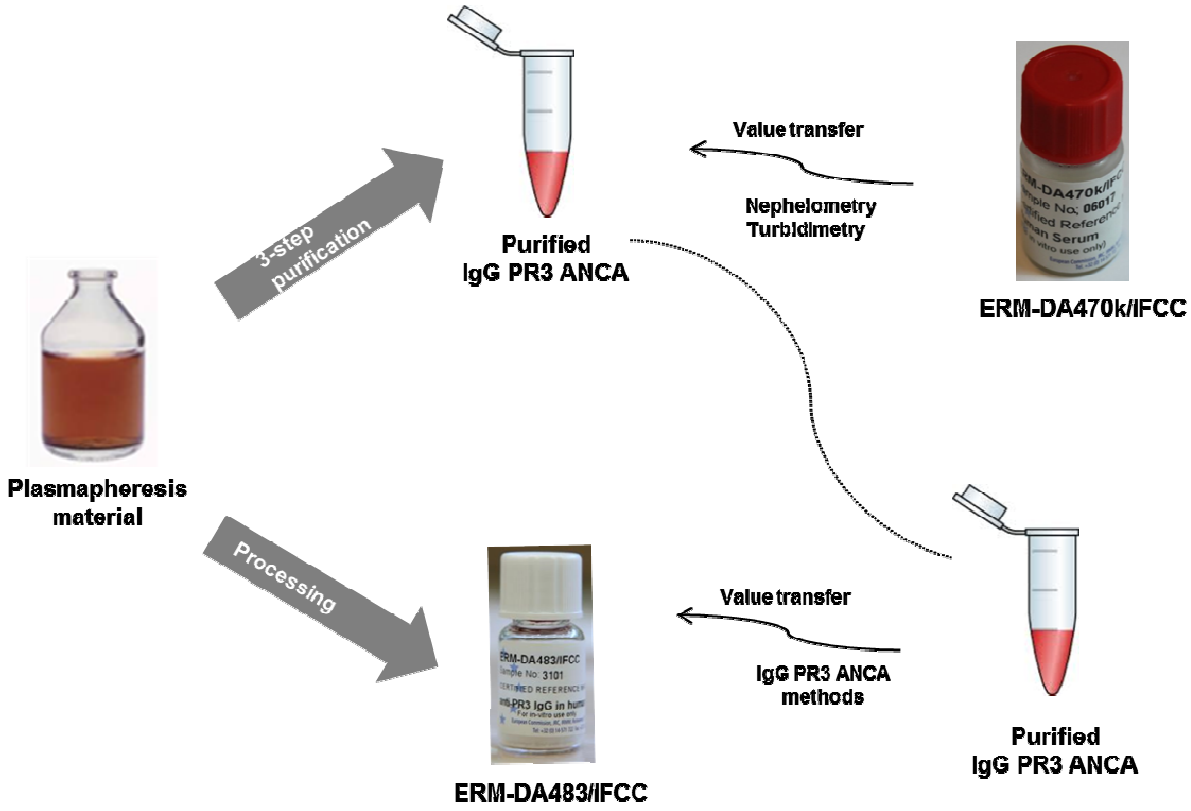

Figure 1: Scheme for the production and characterisation of the calibrant (purified IgG PR3 ANCA) and of the CRM (ERM-DA483/IFCC).

2. Participants

2.1. Provision of raw materials

- Statens Serum Institute, Amager, DK

2.2. Project management, evaluation and processing

- European Commission, Joint Research Centre, Directorate for Health, Consumers & Reference Materials, Reference Materials Unit (F.6), Geel, BE (accredited according to ISO Guide 34 BELAC 268-RM)

2.3. Homogeneity and stability studies

- European Commission, Joint Research Centre, Directorate for Health, Consumers & Reference Materials, Reference Materials Unit (F.6), Geel, BE (accredited according to ISO/IEC 17025 BELAC 268-RM)
- INOVA Diagnostics, INC., San Diego, US
- ORGENTEC Diagnostika GmbH, Mainz, DE

2.4. Characterisation

- AESKU Diagnostics GmbH & Co., Wendelsheim, DE
- Bio-Rad Laboratories INC., California, US
- CERBA specimen services, Saint-Ouen l'Aumône, FR (accredited to ISO 15189:2012, COFRAC, N 8-0945 rev. 6)
- EUROIMMUN Medizinische Labordiagnostika AG, Dassow, DE
- EuroDiagnostica AB, Malmö, SE
- IMMCO Diagnostics, Buffalo, US
- INOVA Diagnostics INC., San Diego, US
- Lund University, Lund, SE
- ORGENTEC Diagnostika GmbH, Mainz, DE
- Protein Reference Unit, St. Georges Hospital, London, UK (accredited to ISO 15189:2012, 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 used to produce ERM-DA483/IFCC 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 antibodies, 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 to 37 °C. A volume of 10 mL (5 mg/mL) protamine sulfate solution (Sigma-Aldrich, DE) was then added and the material stirred for ten min at 37 °C. Following an incubation of one hour at room temperature (RT) the material was further incubated at 4 °C for 40 h to allow the formation of fibrin. Fibrin was then removed by centrifugation at 16880 g for ten min whereupon the supernatant was filter-sterilized through a 0.22 µm PES filter (Corning Inc., US).

3.2.2. Serum processing

The procedure for the processing of serum was based on that used for ERM-DA470k/IFCC [11]. Defibrinated serum was treated with NaCl (final mass 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 one hour at 4 °C with slow stirring (3087 g) with synthetic amorphous silica (430 mg/g of protein). Protein precipitates and silica particles were removed by centrifugation at 16880 g for 30 min. The clear supernatant was dialysed nine times against an isotonic NaCl solution (0.9 % w/v) (Sigma-Aldrich, DE) over 24 h. The pH was then adjusted to 7.2 with a 100 mmol/L HEPES solution (Sigma-Aldrich, DE) and the following preservatives were 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 mmol/L). All three preservatives were purchased from Sigma-Aldrich (DE). The serum was then filter-sterilised through a 0.22 µm PES filter (Corning Inc., US).

3.2.3. Filling

The serum was processed as a single batch at JRC. Firstly, 1 mL of serum was transferred to 2.5 mL siliconised hydrolytic class III clear glass vials using a Rota ampouling machine R910 PA (Rota, Wehr, DE). This process was completed in four hours. The ampouling machine and the continually stirred ice-cooled serum were placed in a movable class 1000 clean room (Terra Universal Inc., Fullerton, California, US). The speed of the ampouling machine was optimised to minimise the formation of foam and achieve the accurate transfer of serum to the vials. Immediately after filling, lyophilisation inserts were manually placed in the neck of the vials. All vials were then loaded into a freeze dryer Epsilon2-100D (Martin Christ, Osterode, DE) for freeze-drying. After completion of the freeze-drying programme, the vials were filled with argon and the inserts were fully pressed down. Vials were manually capped, labelled and stored at -70 °C.

4. Homogeneity

A key requirement for any CRM 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, but it is not relevant if this variation between units is significant compared to the analytical variation. Consequently, ISO Guide 34 requires reference material (RM) producers to quantify the between unit variation. This aspect is covered in between-unit homogeneity studies. For the evaluation of the homogeneity of the material, the values obtained from the one-year long-term stability study (at -70 °C) were used. In the following chapters, data evaluation during a homogeneity study is described.

4.1. Between-unit homogeneity

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

For the between-unit homogeneity assessment, the number of units selected corresponds to the approximate cubic root of the total number of units produced and therefore 14 units were selected using a random stratified sampling scheme, covering the whole batch (the same samples used for long-term stability, Section 5.2). Three independent samples were taken from each of these units, and analysed by a chemiluminescent immunoassay (QuantaFlash

PR3) on the same day. The measurements were performed under repeatability conditions in one day in a randomised manner, thereby facilitating the separation of a potential analytical drift from a trend in the filling sequence.

Regression analyses were performed to evaluate potential trends in the filling sequence (Figure A1) as well as trends in the analytical sequence (Figure A2). 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 sufficiently homogeneous for its intended use.

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.* [12]. 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:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\overline{y}}$$ **Equation 1**

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

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

MS_{within}	mean square within a unit from an ANOVA
$MS_{between}$	mean squares between-unit from an ANOVA
$\overline{y}$	mean of all results of the homogeneity study
n	mean number of replicates per unit
$\nu_{MSwithin}$	degrees of freedom of MS_{within}

The results from the homogeneity studies are shown in Annex A, Table A1. For the calculation of the overall uncertainty, the s_{bb} value was used. 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.

Table 1: Results of the homogeneity study

CRM	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]
ERM-DA483/IFCC	1.87	0.96	0.56

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 can thus be used in an analysis. The within-unit homogeneity is correlated with the minimum sample intake and therefore sample sizes equal or above the minimum sample intake guarantee the certified value within its stated uncertainty.

The smallest sample intake tested was identified after consideration of the method information supplied by the participants, 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 5 µL and was established as the minimum sample intake, as it resulted in consistent measurement results.

The standard deviation within a bottle (s_{wb}) is lower than the expected method variability (i.e. < 5 % as set in our selection criteria), so there is no indication of intrinsic heterogeneity at a sample intake of 5 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, ambient temperatures can reach 60 °C and therefore stability under these conditions must be demonstrated if the material is to be transported at ambient temperatures without additional cooling.

The stability studies were performed using an isochronous design [13]. In this approach, units were stored for a specified length of time at different temperatures whereupon the units were 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 mass concentration in relation to time, and determining whether the slope 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 whereupon they were moved to the reference temperature of at most -150 °C, where further degradation is avoided. Two units per storage time were selected using a random stratified sampling scheme. Three samples, taken from each unit, were analysed by ORG 618 anti-PR3 hs 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 mass concentration versus time were calculated. The slope of the regression line was tested for statistical significance (loss/increase due to shipping conditions) and was found not to be significantly different from zero. However, the u_{sts} calculated from the data was rather large.

Therefore the data from the long-term stability study performed at -70 °C (Section 5.2) were used for the assessment of the uncertainty relating to transport (u_{sts}). During shipment at temperatures of -70 °C or below, the uncertainty associated with the short-term stability for a period of one month was 0.1 %, which is negligible with respect to other uncertainties. Therefore the material can be safely shipped at -70 °C or below.

5.2. Long-term stability study

During the long-term stability study, samples were stored at -20 °C and -70 °C for 0, 4, 8 and 12 months, whereupon they were moved to the reference temperature of at most -150 °C. Two units per storage time were selected using a random stratified sampling scheme. Three samples from each unit were analysed by a QuantaFlash PR3 chemiluminescent 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 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. Ideally the material should be stored for long periods of time at -70 °C. However, within the validity of the present certificate, the material can also be safely stored at -20 °C.

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

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 timeframe studied.

Uncertainties of stability during dispatch and storage were estimated as described in [14]. 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 (one week at -70 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 -70 °C studies. The uncertainty describes the possible change during a dispatch at -70 °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 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 dispatch for one week; $u_{lts,rel}$ was calculated for storage for one year at -70 ºC

CRM	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
ERM-DA483/IFCC	0.09	1.14

Following the conclusion of the certification study, the material will be included in JRC'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 IgG PR3 ANCA solution serving as calibrant and of the candidate CRM was based on an inter-laboratory comparison of expert laboratories, i.e. the protein mass concentration of the material was determined in different laboratories that used their own methodology and instrumentation. This approach aims to negate the laboratory bias, thereby reducing the combined uncertainty.

During the value assignment for the calibrant, the participant laboratories used turbidimetry and nephelometry for their measurements. For the characterisation of ERM-DA483/IFCC all participants used ELISA-type immunosorbent assays.

6.1. Selection of participants

Five laboratories were selected for the measurement of calibrant material and eight for the candidate CRM based on criteria that comprised of technical competence. Each participant was required to operate a quality system and to deliver documented evidence of proficiency in the field of *in vitro* diagnostics in relevant matrices by submitting results for intercomparison exercises or method validation reports. Holding a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 [15] or ISO 15189 [16] was.

6.2. General principles of the value assignment

The laboratories were provided with detailed protocols in regard to the dilutions to be performed, 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. The freeze-dried material of ERM-DA483/IFCC was reconstituted the day before the measurements, according to the procedure described in the certificate of ERM-DA470k/IFCC and in the present report (Section 9.3).

Determination of the transfer factors

During the transfer procedure calibration curves were constructed from the corresponding reference material or antibody preparation: ERM-DA470k/IFCC for the characterisation of the calibrant and purified IgG PR3 ANCA for the characterisation of ERM-DA483/IFCC. Based on these curves and the known concentration of the spiked material, the values of the target material were calculated. The relative mass concentrations were corrected for the masses of the dilutions (and predilution).

During the value transfer procedure, the laboratories measured the eight dilutions of the target (Y0-Y7) and the calibrant (X0-X7) 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 $s_{ijk} = f(c_{ij})$ to facilitate the identification of 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 normality (visual inspection and normal probability plot)

- Test for linearity (visual inspection and evaluation of R^2)
- Verification that the intercept ± four times the *s* covers the origin

The transfer factors (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 0.98
2. Data from at least four 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 null and void
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 %

6.3. Production of the calibrant

6.3.1. Purification and physico-chemical characterisation of the calibrant

The IgG PR3 ANCA was purified from human serum (provided by Statens Serum Institut, DK) in a three-step purification process. Firstly, total IgG was separated by affinity chromatography using a Protein A column. Secondly, the specific IgG PR3 ANCA was separated from all other IgGs eluting from the protein A column using an in-house tailored HiTrap column prepared using commercially available purified human proteinase 3 (Sigma-Aldrich, US) and a HiTrap NHS activated HP column (GE Healthcare, SE) according to the protocol provided by the manufacturer. All IgG PR3 ANCA containing fractions were pooled, concentrated to a final volume of 500 µL using Amicon Ultra centricons with a 30 kDa cut-off (Millipore, US) and subsequently separated on a Superdex 200 10/300 GL column (GE Healthcare, SE). The purity of the final material with respect to IgG selectivity for PR3 was assessed by affinity chromatography using an in-house prepared IgG PR3 ANCA HiTrap column as described above. In the chromatograph a single fraction of protein was eluted under the acidic conditions of the eluting buffer (pH 2.7). A small fraction of the protein eluted in the binding buffer (pH 7.0). The area of this peak corresponding to IgG not specific for PR3 ANCA was taken into consideration for the calculation of concentration of IgG PR3 ANCA in the calibrant and its uncertainty. The uncertainty value finally used with regard to the purity of the purified protein was equal to 1.6 %, corresponding to 50 % of the value for the impurity (IgG not selective for PR3 ANCA, chromatogram and peak areas not shown) (Table 3, Section 6.3.2).

6.3.2. Value assignment of the calibrant

During the characterisation of the calibrant (IgG PR3 ANCA), each laboratory received six vials containing various dilutions of purified IgG PR3 ANCA prepared at the JRC as well as six vials with dilutions of ERM-DA470k/IFCC for each day. Identical measurements were to be repeated over two days. The laboratories were therefore required to provide 36

independent results per material (three per dilution per day). 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. Measurements were performed with different platforms (Annex C, Table C1) and reagents. In total five laboratories participated in the characterisation study.

The concentration of the calibrant was measured by nephelometry or turbidimetry (Annex C, Table C1). The participating laboratories provided the mass concentration measured using their in-house calibrant for both, ERM-DA470k/IFCC and the purified IgG PR3 ANCA solution. Regression lines were constructed comparing these measured mass concentrations to the gravimetrically calculated dilution factors of these samples. The slope ratio of the ERM-DA470k/IFCC to the purified IgG PR3 ANCA was used to determine the value of the dilutions.

The data were assessed for both their compliance with the analysis protocol and for their validity based on technical issues. 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 regression lines must be above 0.98

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 < 0.98. Another dataset was also rejected as the samples were analysed after long storage at -20 °C, a temperature at which the protein is known to be unstable.

The results of the individual accepted laboratories are given in Table C1 of the Annex C.

The uncertainty relating to the characterisation of the calibrant (purified IgG PR3 ANCA) was estimated from the standard error of the mean of laboratory means (Table 3); the uncertainty of the reference material value used for the calibration (ERM-DA470k/IFCC) as well the uncertainty of the purity of the material. The uncertainty resulting from the gravimetric preparation of the dilutions was found to be insignificant and was not taken into account for the final uncertainty of the calibrant ($u_{bal,rel}$ = 0.008 %). The value given for the mean mass concentration of the purified IgG PR3 ANCA is the corrected value after taking the purity assessment into account.

Table 3: Uncertainty budget for purified IgG PR3 ANCA mass concentration

Analyte	*p*	Mean Mass Concentration [mg/L]	*s* [mg/L]	$u_{ERM\text{-}DA470k,rel}$ [%]	$u_{char,\ cal\ rel}$ [%]	$u_{pur,rel}$ [%]	$u_{IgG\ PR3\ ANCA,rel}$ [%]
IgG PR3 ANCA	3	288.96	5.99	0.98	1.99	1.6	2.75

These different contributions were combined to estimate the relative uncertainty of the mass concentration value of the calibrant ($u_{IgG\ PR3\ ANCA,\ rel}$) as:

$$u_{\mathrm{IgG\,PR3\,ANCA}} = \sqrt{u^2_{\mathrm{char\,cal,rel}} + u^2_{\mathrm{ERM\text{-}DA470k/IFCC}} + u^2_{\mathrm{pur,rel}}} \qquad \textbf{Equation 6}$$

- $u_{char,cal}$ was estimated from the data in Annex C, Table C1
- $u_{ERM\text{-}DA470k/IFCC}$ was estimated during its certification [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 $M^{-1}cm^{-1}$) [17]. The concentration which was measured on four separate days, in triplicate, was equal to 301.17 mg/L. This value was not used for the value assignment of the candidate reference material nor in the calculation of the uncertainties, as the absorption coefficient -used for the calculations is based on an absorption coefficient determined in 1955 [17], and its determination is not sufficiently documented regarding its traceability.

6.4. Characterisation of ERM-DA483/IFCC

During the characterisation of the target material each laboratory received four vials of ERM-DA483/IFCC and four vials of IgG PR3 ANCA. The laboratories were required to perform eight dilutions from each of these vials and to provide a total of 96 independent results, three per dilution, for each material. The vials for the material characterisation were selected using a random stratified sampling scheme and covered the whole batch. The sample preparations and measurements were to be spread over four days to ensure intermediate precision conditions. The dilution protocol was the same for both materials (5, 10, 20, 30, 40, 50, 75 and 100 (m/m) % of the reconstituted materials).

The techniques used to measure the protein concentration were traditional ELISA and in some cases its variations (chemiluminescent and fluorescence immunoassays) specific for IgG PR3 ANCA. In total eight laboratories participated in the value assignment, using ten different types of kits (Annex C, Table C2).

The data were first assessed for compliance with the mandatory analysis protocol and for their validity based on technical issues. 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 above 0.98 for data on all platforms

Linear regressions were used for the analyses of the data provided by the methods 1-4 and 6-8 (Annex C, Table C2). However, for methods 5, 9 and 10 the data provided were following a non-linear distribution and were therefore treated accordingly.

Based on the criteria above, data derived from the second day for method 1, and the first day for method 5, were not taken into consideration as their R^2 values were below the set limit of 0.98. Additionally for laboratory 6, two out of the four days had correlation coefficients below 0.98 and thus the data from those two days were not taken into account for the final value assignment.

The accepted datasets were tested for normality of dataset means using normal probability plots and were tested for outlying means using the Grubbs test and for outlying standard

deviations using the Cochran test (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 contained outlying means and variances. The datasets were therefore consistent and the mean of laboratory means was a good estimate of the true value. Standard deviations between laboratories are considerably larger than the standard deviation within laboratories, showing that confidence intervals of replicate measurements are unsuitable as estimate of measurement uncertainty.

It was verified however, that there was an overlap between the mean of all means plus/minus the standard deviation and the mean for individual laboratories, plus/minus the standard deviation for the individual laboratories (Figure C1).

The value assignment data of the individual laboratories are shown in Annex C, Table C3.

6.4.1. Mass concentration and characterisation uncertainty

The mass concentration of IgG PR3 ANCA in ERM-DA483/IFCC was calculated from the average of the TF (Annex C, Table C3) and the protein concentration obtained for the calibrant (Table 3, 3rd column) according to:

$$c_{ERM\text{-}DA483/IFCC} = TF_{average} \cdot c_{IgG\ PR3\ ANCA}$$

Where $c_{IgG\ PR3\ ANCA}$ is the concentration of IgG PR3 ANCA (calibrant) and $TF_{average}$ is the mean of means for all four analyses days and for all participating laboratories as described in Section 6.4.

The uncertainty relating to the characterisation of the mass concentration for IgG PR3 ANCA in ERM-DA483/IFCC was estimated as the standard error of the mean of laboratory means (Table 4).

Table 4: Value assignment for mass concentration for IgG PR3 ANCA in ERM-DA483/IFCC

CRM	*p*	Mean [mg/L]	*s* [mg/L]	$u_{char,\ rel}$ [%]
ERM-DA483/IFCC	10	270.27	36.88	4.32

7. Value assignment

Certified values are values that fulfil the highest standards of accuracy. Internal quality management procedures require no less than six technically valid datasets to assign certified values. Full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [7] were established. A certified value for the mass concentration of IgG PR3 ANCA was assigned to ERM-DA483/IFCC.

7.1. Certified values and their uncertainties

The mass concentration of IgG PR3 ANCA in ERM-DA483/IFCC was calculated as described in section 6.4.1. It was derived from the unweighted mean of the means of the TFs of the individual laboratories (Annex C, Table C3) and the IgG concentration for the calibrant (Table 3, 3rd column).

The assigned uncertainty consists of uncertainties relating to the characterisation of the target material, u_{char} (Section 7), 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) as selected at -70 °C. The uncertainty relating to the use of analytical balance during the gravimetric preparation of the dilutions of the calibrant was found to be negligible (equal to 0.008 %) [3]. The 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

The relative expanded uncertainty was calculated from the relative combined standard uncertainty $u_{CRM,rel}$ by multiplication with a coverage factor k ($U_{CRM} = u_{CRM} * k$). A coverage factor of two was taken as there are sufficient degrees of freedom for 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 the IgG PR3 ANCA mass concentration in ERM-DA483/IFCC

CRM	$u_{IgG\ PR3\ ANCA\ rel}$ [%]	$u_{char,\ rel}$ [%]	$u_{bb,\ rel}$ [%]	$u_{sts,\ rel}$ [%]	$u_{lts,\ rel}$ [%]	$u_{CRM,rel}$ [%]	U_{CRM} [mg/L]
ERM-DA483/IFCC	2.75	4.32	0.97	0.09	1.14	5.33	29

Finally, the certified value for the IgG PR3 ANCA mass concentration in ERM-DA483/IFCC and its accompanying uncertainty are shown in Table 6.

Table 6: Mass concentration of IgG PR3 ANCA in ERM-DA483/IFCC

	Certified value [mg/L]	Uncertainty [mg/L]
IgG PR3 ANCA	270	29

8. Metrological traceability and commutability

8.1. Metrological traceability

Identity

The identity of IgG PR3 ANCA measured in ERM-DA483/IFCC is defined by the immunoassay procedures used to characterise it. The assigned property value is therefore operationally defined by *method*.

Quantity value

The certified value for the IgG PR3 ANCA mass concentration in ERM-DA483/IFCC was obtained by using different immunoassays specific for IgG PR3 ANCA and purified IgG PR3 ANCA used as calibrant (Figure 2). The value for the total IgG concentration in this calibrant was previously obtained using immunoassays for total IgG mass concentration by calibrating with ERM-DA470k/IFCC. The mass concentration of total IgG present in ERM-DA470k/IFCC was certified using total IgG immunoassays using a certified reference material (ERM-DA470/IFCC) as calibrant. Finally, the total IgG present in this last material and its uncertainty are traceable to the stated value of the mass concentration of total IgG in USNRP 12-0575C [18]. The procedures applied are described in the certification report of ERM-DA470k/IFCC [19] and in the present report (Figure 2).

The certified value for the IgG PR3 ANCA mass concentration in ERM-DA483/IFCC is traceable to stated value of the mass concentration of total IgG in USNRP 12-0575C [18]. 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 for ERM-DA483/IFCC is not dependent on the individual methods used.

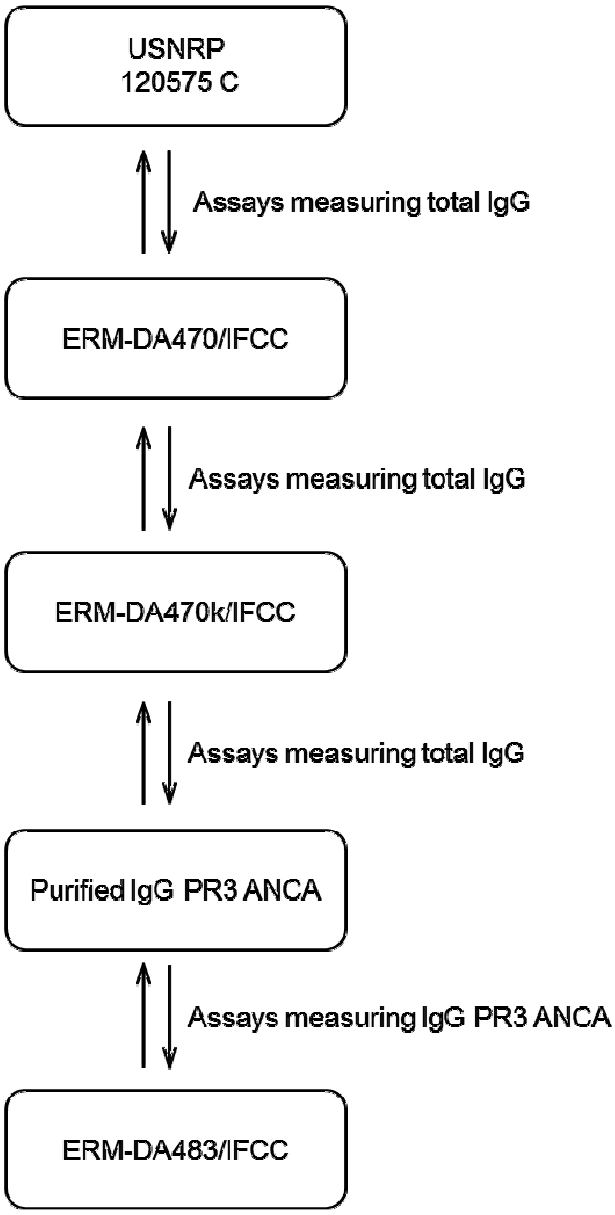

Figure 2: Reference materials and methods used for establishing the metrologically traceability chain (arrows facing upwards) and calibration sequence (arrows facing downwards)

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 [11] 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 the commutability of a CRM is not established, the results from routinely used methods cannot be legitimately compared with the certified value to determine whether a bias in calibration exists or not, nor can the CRM be used as a calibrant. For instance, CRMs intended to be used for establishing or verifying metrological traceability of measurement results for routine clinical measurement procedures must be commutable for the routine clinical measurement procedures for which they are intended to be used.

Different formats of materials, all based on the same raw material as ERM-DA483/IFCC, have been tested and found to be commutable for combinations of six methods giving correlating results (Annex D, Table D1). Therefore ERM-DA483/IFCC is expected to be commutable for the majority of IgG PR3 ANCA methods. 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 laboratory safety measures apply. The plasmapheresis material used in the production of the material has been tested and found negative for Hepatitis B surface antigen, HIV 1&2 antibodies, HIV antigen and Hepatitis C antibodies. However, the product must be handled with 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 either -20 °C or -70 °C, as the material has been shown to be stable at both temperatures for up to one year. If microbial contamination has been excluded during the reconstitution procedure, the solution of ERM-DA483/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 occur during storage of the material at the customer's premises.

9.3. Reconstitution and use of the material

The material must be reconstituted according to the following procedure:

- Remove the vial from the freezer and place it in the room where the balance is located one hour before reconstitution.
- Prior to reconstitution, 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 carefully the rubber stopper until the groove.

- Add 1.00 mL of milli Q 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 (1.0000 g).
- Leave the vial at RT for one hour, then gently mix by inversion at least five times (do not shake it) during the next hour.
- Leave the vial at room temperature overnight. The following day, gently mix by inversion five times in a period of one hour, prior to starting the analysis.

9.4. Minimum sample intake

The entire contents of the vial must be reconstituted. The minimum sample intake after reconstitution is 5 µL.

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 IgG PR3 ANCA measurements. As any reference material, it can also be used for control charts or validation studies.

When the material is used as a calibrant 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 [20].

To assess 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 thank Alan Wiik and Niels Rasmussen from the Statens Serum Institute (Amager, DK) for the raw materials, and would also like to acknowledge the support received from Håkan Emteborg and Katharina Teipel in relation to the processing of this CRM and from Maria Concepción Contreras López from JRC concerning the set-up of the isochronous studies.

Furthermore, the authors would like to thank Reinhard Zeleny and James Snell (JRC) for reviewing the certification report, as well as the experts of the Certification Advisory Panel "Biological Macromolecules and Biological/Biochemical Parameters", Hez Hird (Low Marishes, UK), Martin Wagner (University for Veterinary Medicine Vienna, AT) and Lothar Siekmann (University of Bonn, DE), for their constructive comments. The authors are grateful for the active involvement of the laboratories participating in the certification study.

11. References

1. ISO Guide 34, General requirements for the competence of reference materials producers, International Organization for Standardization, Geneva, Switzerland, 2009
2. ISO Guide 35, Reference materials – General and statistical principles for certification, International Organization for Standardization, Geneva, Switzerland, 2006
3. Certification report, Certification of proteins in the human serum. Certified Reference Material ERM® - DA470k/IFCC, European Commission. Joint Research Centre, Institute of Reference Materials and Measurements, EUR 23431 EN - 2008
4. ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement, (GUM 1995), International Organization for Standardization, Geneva, Switzerland, 2008
5. Hoffman, G. S. and U. Specks, Antineutrophil cytoplasmic antibodies, Arthritis & Rheumatism, 1998, 41:1521-1537
6. F.J. van der Woude, N. Rasmussen, S. Lobatto, A. Wiik, H. Permin, L.A. van Es, M. van der Giessen, G.K. van der Hem, T.H. The, Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis, The Lancet, 1985, 325:425-429
7. R. Goldschmeding, C.E van der Schoot, D. ten Bokkel Huinink, C.E. Hack, M.E. van den Ende, C.G. Kallenberg A.E. von dem Borne, Wegener's granulomatosis autoantibodies identify a novel diisopropylfluorophosphate-binding protein in the lysosomes of normal human neutrophils, J Clin Invest, 1989, 84: 1577-1587
8. Certification report, The certification of anti-myeloperoxidase immunoglobulin G in human serum ERM® - DA476/IFCC, European Commission. Joint Research Centre, Institute of Reference Materials and Measurements, EUR 27092 EN - 2015
9. H. Vesper, H. Emons, M. Gnezda, C. P. Jain, W. G. Miller, R. Rej, G. Schumann, J. Tate, L. Thienpont, J. E. Vaks, Characterization and Qualification of Commutable Reference Materials for Laboratory Medicine; Approved Guideline, CLSI document C53-A, Clinical and Laboratory Standards Institute, Wayne, PA, US, 2010
10. S. Blirup-Jensen, A.M. Johnson, M. Larsen, Protein standardization IV: Value transfer procedure for the assignment of serum protein values from a reference preparation to a target material, Clin Chem Lab Med, 2001, 39:1110-1122
11. I. Zegers, W. Schreiber, S. Linstead, M. Lammers, M. McCusker, A. Muñoz, Y. Itoh, G. Merlini, S. Trapmann, H. Emons, J. Sheldon, H. Schimmel, Development and preparation of a new serum protein reference material: feasibility studies and processing, Clin Chem Lab Med, 2010, 48:805-813
12. T.P.J. Linsinger, J. Pauwels, A.M.H. van der Veen, H. Schimmel, A. Lamberty, Homogeneity and stability of reference materials, Accred Qual Assur, 2001, 6:20-25
13. A. Lamberty, H. Schimmel, J. Pauwels, The study of the stability of reference materials by isochronous measurements, Fres. J Anal Chem, 1998, 360:359-361
14. T.P.J. Linsinger, J. Pauwels, A. Lamberty, H. Schimmel, A.M.H. van der Veen, L. Siekmann, Estimating the Uncertainty of Stability for Matrix CRMs, Fres J Anal Chem, 2001, 370:183-188

15. ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories, International Organization for Standardization, Geneva, Switzerland, 2005
16. ISO 15189:2012, Medical laboratories — Requirements for quality and competence, International Organization for Standardization, Geneva, Switzerland, 2012
17. M. Schönenberger, Streulichtmessungen an Plasmaproteinen. Z. Naturforsch, 1955, 10b:474
18. C.B. Reimer, S.J. Smith, T.W. Wells, R.M. Nakamura, P.W. Keitges, R.F. Ritchie, G.W. Williams, D.J. Hanson, D.B. Dorsey, Collaborative calibration of the U.S. national and the College of American Pathologists reference preparations for specific serum proteins. Am J Clin Pathol, 1982, 77:12–19
19. I. Zegers, W. Schreiber, J. Sheldon,S. Blirup-Jensen, A. Muñoz, G. Merlini, Y. Itoh, A.M. Johnson, S. Trapmann, H. Emons, H. Schimmel, The certification of proteins in the human serum, Certified Reference Material ERM® - DA470k/IFCC, EUR 23431 EN, European Commission, Luxembourg, 2008, ISBN 978-92-79-09490-3
20. T.P.J. Linsinger, ERM Application Note 1: Comparison of a measurement result with the certified value, www.erm-crm.org

Annexes

Annex A: Results of the homogeneity measurements

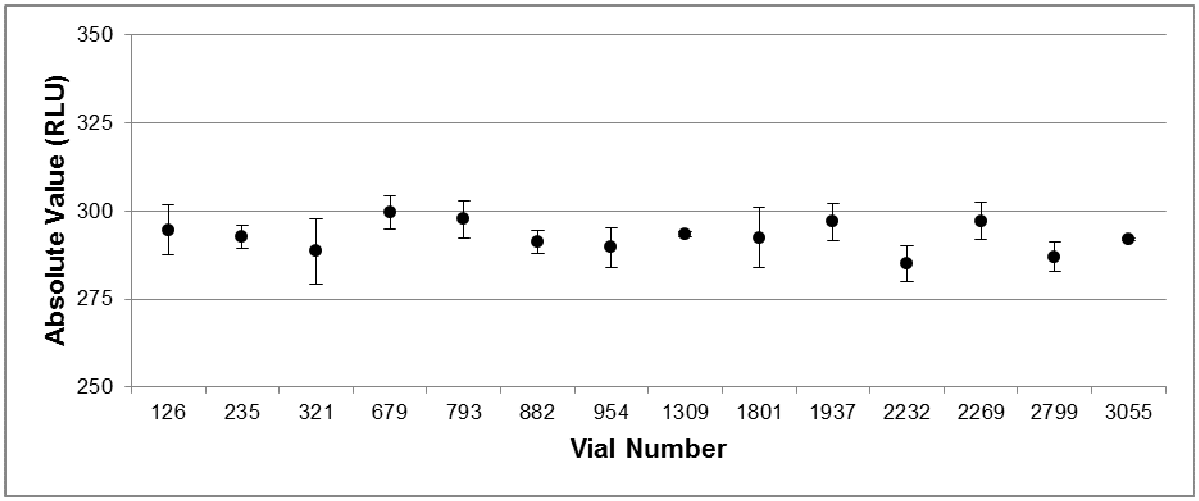

Figure A1: Measured values (in Relative Light Units) as a function of the filling sequence. The error bars indicate the relative standard deviation between the triplicate measurements

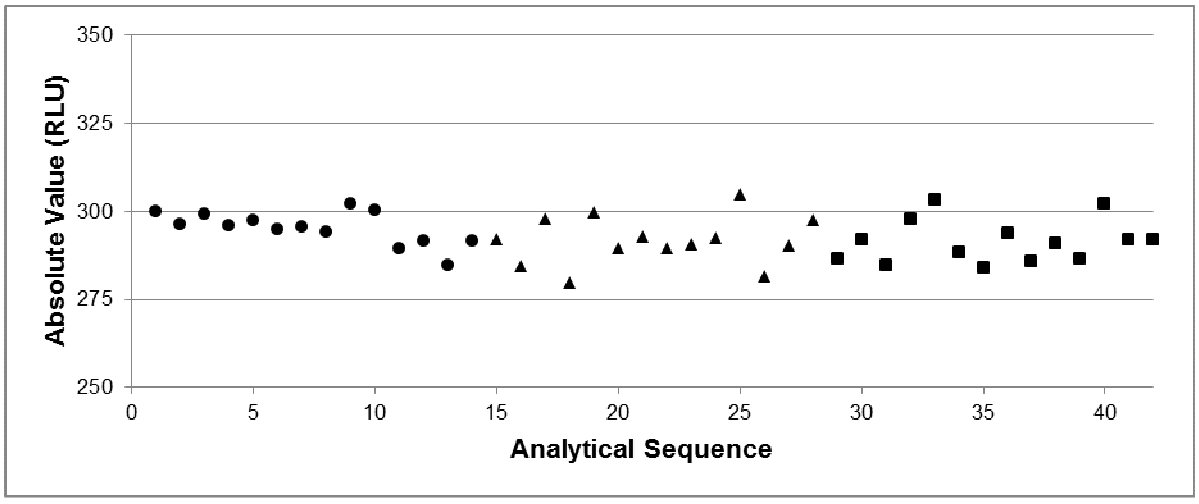

Figure A2: Measured values (in Relative Light Units) as a function of the analytical sequence. In the graph values of all replicates are shown (● replicate 1, ▲ replicate 2 and ■ replicate 3)

Annex B: Results of the long-term stability measurements

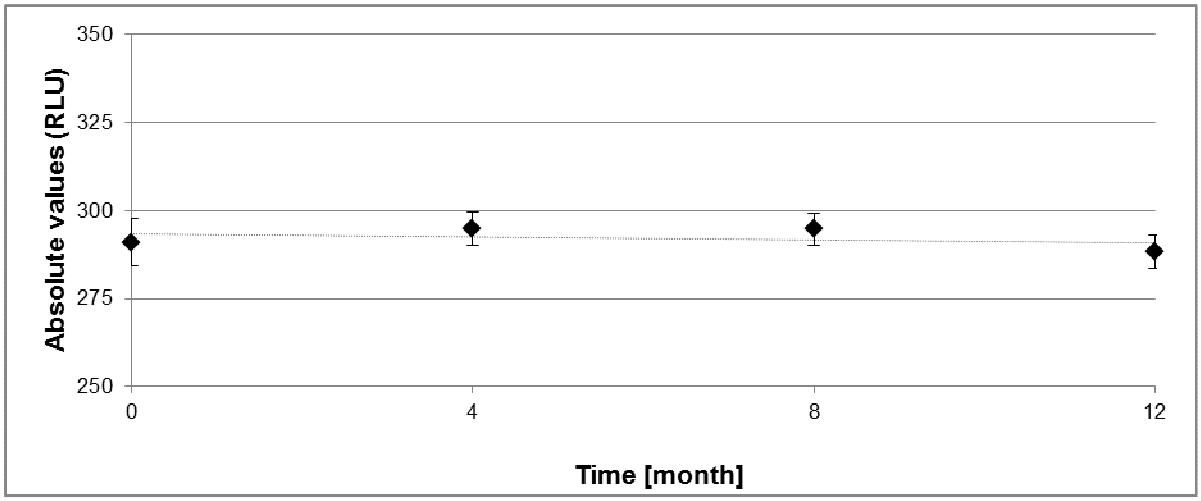

Figure B1: Long-term stability data (mean values) of ERM-DA483/IFCC; Measured values by a chemiluminescent immunoassay (Relative Light Units), when stored up to one year at -20 °C. Results at time point 0 months correspond to units that were stored at the reference temperature at most of -150 °C.

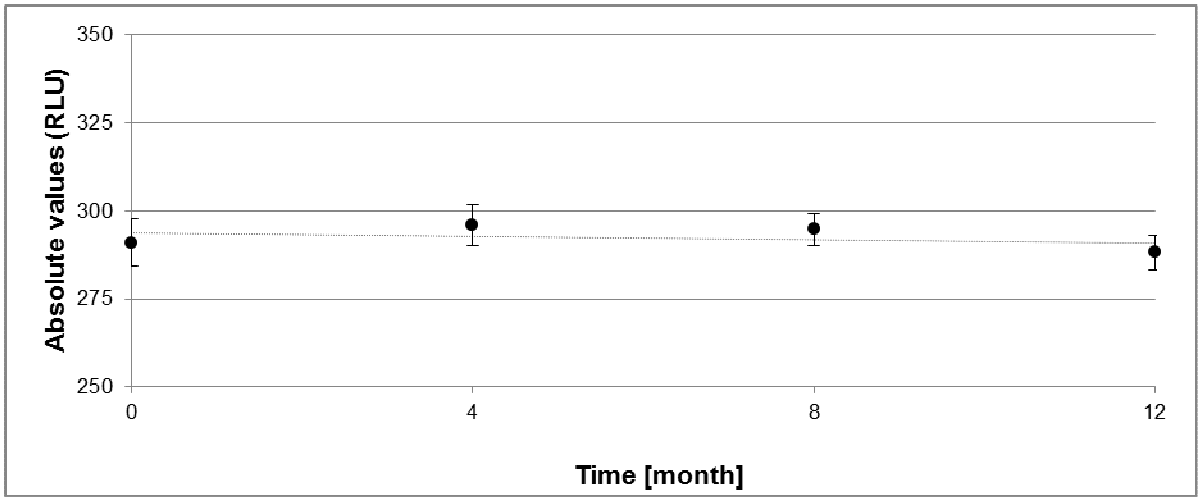

Figure B2: Long-term stability data (mean values) of ERM-DA483/IFCC; Measured values by a chemiluminescent immunoassay (Relative Light Units), when stored up to one year at -70 °C. Results at time point 0 months correspond to units that were stored at the reference temperature at most of -150 °C.

Annex C: Characterisation

IgG PR3 ANCA

Laboratory	Platform	Method Principle	Mass Concentration [mg/L]
L1	Cobas 6000/8000 c 501	Turbidimetry	294.85
L2	Cobas c 501	Turbidimetry	295.26
L3	Behring Nephelometer (BN) II	Nephelometry	305.42

Table C1: All laboratories giving acceptable results, together with the platforms used and the principles of the methods that these platforms employ are listed. The concentration given represents the average values of tree replicates over two days under intermediate precision conditions.

ERM-DA483/IFCC

Laboratory	Method Number	Method Name	Method Principle	Mass Concentration [mg/L]
L1	1	ImmuLisa™ Proteinase 3 (PR3) antibody Enhanced	ELISA	333.63
L2	2	ORG 618 PR3 hs	ELISA	319.75
L3	3	BioPlex 2200 Vasculitis	Multiplex Flow Immunoassay	252.95
	4	Anti-PR3 EIA	ELISA	247.58
L4	5	QUANTA Lite PR 3	ELISA	219.43
	6	QUANTA Flash	Chemiluminescent immunoassay	255.01
L5	7	PR3 ANCA Wieslab®	ELISA	258.59
L6	8	AESKULISA PR3 sensitive	ELISA	238.77
L7	9	EliA PR3^{S}	Fluorescence immunoassay	275.33
L8	10	Anti-PR3-hn-hr-ELISA (IgG)	ELISA	301.77

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. The last column gives the average concentrations of the ERM-DA483/IFCC.

ERM-DA483/IFCC

Laboratory	Method Number	Slope ratio Day 1	Slope ratio Day 2	Slope ratio Day 3	Slope ratio Day 4	TF [mean slope ratio]	Day-to-Day variation [%]
L1	1	1.254	n.i.	1.024	1.186	1.155	0.102
L2	2	1.126	1.068	1.081	1.151	1.107	0.035
L3	3	0.782	0.891	0.988	0.840	0.875	0.100
	4	0.695	0.891	0.992	0.849	0.857	0.144
L4	5	n.i.	0.782	0.780	0.717	0.759	0.049
	6	0.864	0.835	0.991	0.840	0.882	0.083
L5	7	0.820	1.034	0.894	0.832	0.895	0.110
L6	8	n.i.	0.897	0.755	n.i.	0.826	0.122
L7	9	1.104	0.916	0.974	0.818	0.953	0.125
L8	10	1.023	1.067	1.028	1.059	1.044	0.021

n.i.: results not included due to technical reasons

Table C3: Individual results for the value assignment for the characterisation of the target material, ERM-DA483/IFCC. The day-to-day variation per laboratory is presented.

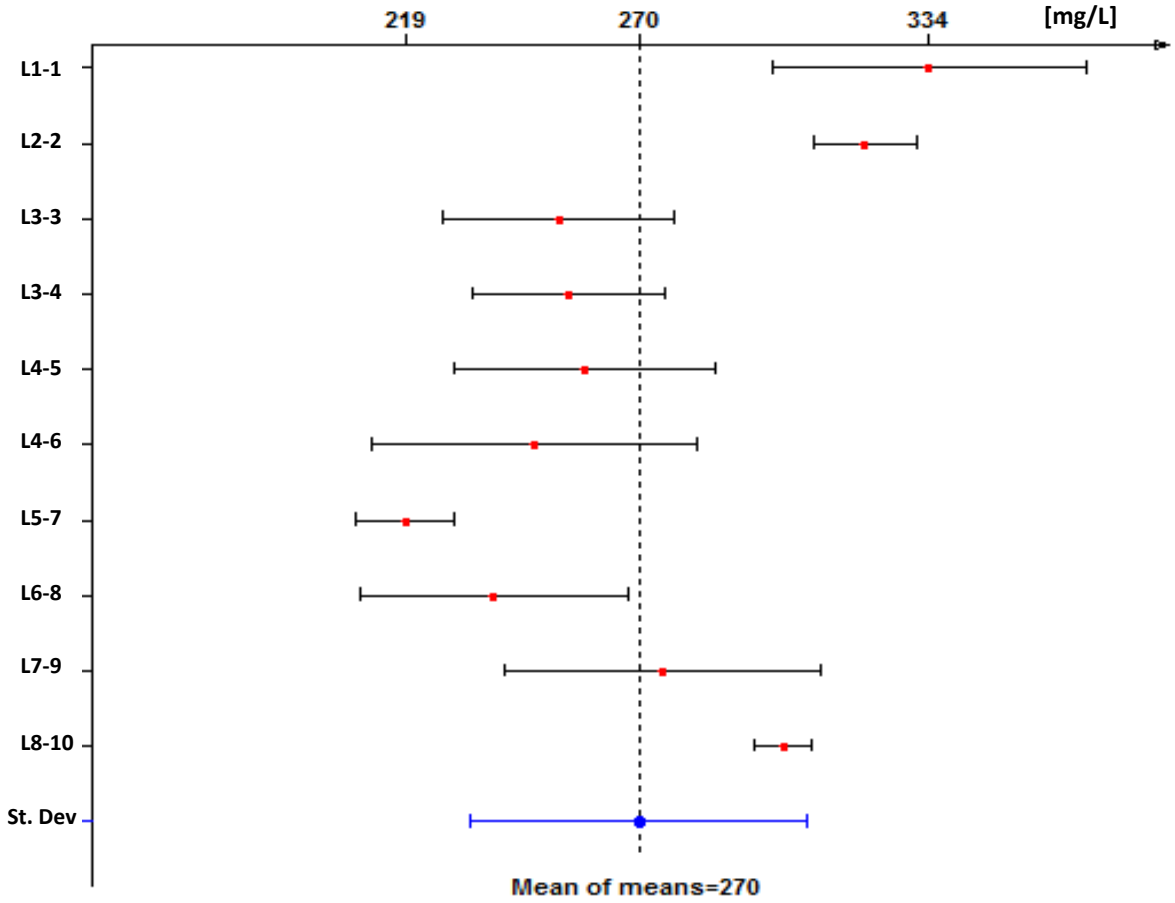

Figure C1: Mean values of the concentration (mg/L) of IgG PR3 ANCA for all participating laboratories and their corresponding standard deviations for the value transfer of ERM-DA483/IFCC

Annex D: Commutability

Laboratory	Method Name	Method Principle
L1	Varelisa™ PR3 ANCA	ELISA
L2	Wieslab® Anti-PR3 ELISA	ELISA
L3	anti-PR3-hr-hn ELISA (IgG)	ELISA
L4	QUANTA Lite PR3 IgG	ELISA
L5	ORG 518 Anti-PR3 (cANCA)	ELISA
L6	DIASTAT anti-PR3 (cANCA)	ELISA

Table D1: Laboratories that participated in the commutability studies and the methods for which pilot batches processed in the same way as ERM-DA483/IFCC were found to be commutable.

European Commission

EUR 28537 EN – Joint Research Centre – Directorate F – Health, Consumers and Reference Materials
Title: **CERTIFICATION REPORT The certification of the mass concentration of immunoglobulin G proteinase 3 anti-neutrophil cytoplasmic autoantibodies (IgG PR3 ANCA) in human serum: ERM® - DA483/IFCC**
Author(s): Evanthia Monogioudi, Dana Petronela Hutu, Jean Charoud-Got, Joanna Sheldon, Heinz Schimmel, Stefanie Trapmann, Pier Luigi Meroni, Hendrik Emons and Ingrid Zegers

Luxembourg: Publications Office of the European Union
2017 – 34 pp. – 21.0 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1831-9424
ISBN 978-92-79-66974-3
doi: 10.2760/373057

As the Commission's in-house science service, the Joint Research Centre's mission is to provide EU policies with independent, evidence-based scientific and technical support throughout the whole policy cycle.

Working in close cooperation with policy Directorates-General, the JRC addresses key societal challenges while stimulating innovation through developing new methods, tools and standards, and sharing its know-how with the Member States, the scientific community and international partners.

Key policy areas include: environment and climate change; energy and transport; agriculture and food security; health and consumer protection; information society and digital agenda; safety and security, including nuclear; all supported through a cross-cutting and multi-disciplinary approach.

KJ-NA-28537-EN-N

Publications Office

doi:10.2760/373057

ISBN: 978-92-79-66974-3