Institute for Reference Materials and Measurements

ERM
European Reference Materials

CERTIFICATION REPORT

The Certification of the Mass Concentration of C-Reactive Protein in Human Serum

Certified Reference Material ERM®-DA474/IFCC

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European Commission
Joint Research Centre
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Contact information
Reference materials sales
Retieseweg 111
B-2440 Geel, Belgium
E-mail: jrc-irmm-rm-sales@ec.europa.eu
Tel.: +32 (0)14 571 705
Fax: +32 (0)14 590 406

http://irmm.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

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The Certification of the Mass Concentration of C-Reactive Protein in Human Serum

Certified Reference Material ERM®-DA474/IFCC

G. Auclair, I. Zegers, J. Charoud-Got, A. Muňoz Piňeiro, K. Hanisch, S. Boulo, S. Trapmann, H. Schimmel, H. Emons

European Commission, Joint Research Centre
Institute for Reference Materials and Measurements (IRMM),
Geel (Belgium)

W. Schreiber

Siemens Healthcare Diagnostics Products GmbH, Marburg (Germany)

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Summary

This report describes the production of ERM-DA474/IFCC, a human serum material certified for C-reactive protein mass concentration. The material has been produced following ISO Guide 34:2009 [1].

Serum was produced from blood collected in 4 blood collection centres according to a procedure ensuring that it was obtained from healthy donors, and that the lipid content of the serum was low. The serum was processed, spiked with C-reactive protein, ampouled and frozen.

Between unit-heterogeneity has been quantified and stability during dispatch and storage have been assessed in accordance with ISO Guide 35:2006 [2].

The material was characterised by an intercomparison among laboratories of demonstrated competence. Technically invalid results were removed but no outlier was eliminated on statistical grounds only.

Uncertainties of the certified value were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) [3] and include uncertainties related to possible heterogeneity and instability and to characterisation.

The material is intended for the calibration of immunoassays. As any reference material, it can also be used for control charts or validation studies. The CRM is available in glass ampoules containing 1 mL of human serum closed under argon atmosphere. The minimum amount of sample to be used is 20 µL.

The CRM has been 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]
C-reactive protein (CRP)[1]	41.2	2.5

1) CRP as measured by immunonephelometry and immunoturbidimetry using ERM-DA470 as calibrant (Baudner et al., EUR reports 15423 and 16882 European Communities, Luxembourg (1993)), applying the procedures described for the certification of ERM-DA472/IFCC, ERM-DA470 and 1st Int. St. for CRP Code 85/506.

2) The value is the unweighted mean of 6 accepted mean values, independently obtained by 4 laboratories. The certified mass concentration is traceable to the SI, via ERM-DA470.

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, 2009.

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Glossary

ν_{smeas}	Degrees of freedom for the determination of the standard deviation s_{meas}
$\nu_{MSwithin}$	Degrees of freedom of MS_{within}
$\bar{x}$	Arithmetic mean of all results of a study
α	Significance level
ANOVA	Analysis of variance
b	Slope in the equation of linear regression $y = a + bx$
Bit	Unit of the anallog-to-digital converter of the light detector
C	Mass concentration $c = m / V$ (mass / volume)
C3c	Complement 3c
C3	Complement 3
CASO	Casein soy bean
CRM	Certified reference material
CRP	C-reactive protein
EC	European Commission
ERM®	Trademark of European Reference Materials
FPLC	Fast protein liquid chromatography
g	Relative centrifugal force
HBV	Hepatitis B virus
HBsAg	Hepatitis B surface antigen
HCV	Hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
hs	High sensitivity
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IRMM	Institute for Reference Materials and Measurements
ISO	International Organization for Standardization
IU	International Unit
IVD	In Vitro Diagnostics
JRC	Joint Research Centre
k	Coverage factor

KIU	Kallikrein Inhibitor Unit
m	Mass
MS	Mass spectrometry
MS_{bb}	Mean sum of squares between bottles
$MS_{between}$	Mean squares between-unit from an ANOVA
MS_{wb}	Mean sum of squares within bottles
MS_{within}	Mean squares within-unit from an ANOVA
MWCO	Molecular weight cut-off
n	Number of replicates per unit
N	Number of samples (units) analysed
n.a.	Not applicable
ND	Not detectable
p	Number of accepted datasets
PAGE	Polyacrylamide gel electrophoresis
QC	Quality control
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference Material
RM Unit	Reference Materials Unit
RF	Rheumatoid factors
RSD	Relative standard deviation
RSE	Relative standard error (=RSD/$\sqrt{n}$)
r^2	Coefficient of determination of the linear regression
s	Standard deviation
s_{bb}	Between-unit standard deviation; an additional index "rel" is added as appropriate
$s_{between}$	Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate
SDS	Sodium dodecyl sulphate
SI	International System of Units
s_{meas}	Standard deviation of measurement data; an additional index "rel" is added as appropriate
s_{within}	Standard deviation within groups as obtained from ANOVA; an additional index "rel" is added as appropriate
s_{wb}	Within-unit standard deviation
$t_{\alpha, df}$	Critical t-value for a t-test, with a level of confidence of 1-α and df

	degrees of freedom
t_{sl}	Proposed shelf life
TM	Target material
TF	Transfer factor
TRIS	Tris(hydroxymethyl)aminomethane
u	Standard uncertainty
U	Expanded uncertainty
u_c	Combined standard uncertainty; an additional index "rel" is added as appropriate
u^*_{bb}	Standard uncertainty related to a maximum between-unit heterogeneity 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 heterogeneity; 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}	Standard combined uncertainty of a 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_{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
u_m	Measurement uncertainty
U_{meas}	Expanded measurement uncertainty
u_{rec}	Standard uncertainty related to possible between-unit heterogeneity modelled as rectangular distribution; an additional index "rel" is added as appropriate
u_{sts}	Standard uncertainty of the short-term stability
u_Δ	Combined standard uncertainty of measurement result and certified value
t_i	Time point for each replicate

1 Introduction

1.1 Background: need for the CRM

C-reactive protein (CRP) is an important analyte in clinical chemistry. It is a very sensitive marker of inflammation and tissue damage [4]. Routine clinical uses of CRP results include the diagnosis of bacterial and viral infections and the assessment of disease activity in inflammatory conditions like rheumatoid arthritis.

CRP was originally discovered by Tillett and Francis in 1930 as a substance in the serum of patients with acute inflammation that had the capacity to precipitate the C polysaccharide of Pneumococcus [5] . In the blood of healthy young adult blood donors, the median mass concentration of CRP is 0.8 mg/L, the 90th centile is 3.0 mg/L, and the 99th centile is 10 mg/L. Following an acute-phase stimulus, values may increase to more than 500 mg/L [4]. The finding that modest but persistently increased values are associated with a long-term risk for coronary heart disease [6] has led to the development of assays specifically designed to measure relatively low concentrations of CRP. These assays are often called high sensitivity CRP (*hs* CRP) assays.

The immunoassays used for CRP measurements are capable of a high degree of analytical sensitivity and selectivity, and are convenient in a clinical setting because they give fast results. They are based on the fact that when the antigen (CRP) and specific antibodies are brought together they form complexes or aggregates that scatter incident light. The scattering of the light is measured by turbidimetry (measuring the reduction of light passing through a reaction mixture) or nephelometry (measuring the light scattered by a reaction mixture). The signal is dependent on a large number of factors such as antibody specificity, reaction kinetics and equilibria, multimeric state of the proteins, matrix effects, etc. The quantification with immunoassays is therefore entirely dependent on the comparison of the results with those obtained with a calibrant [7].

The EU Directive on In Vitro Diagnostic Medical Devices (IVD-MD) (Directive 98/79/EC) [8] requires traceability of the assigned values of calibrants and control materials to reference measurement procedures and/or reference materials of higher order.

In 1989 the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) began the processing, characterisation, and calibration of a matrix reference material for human serum proteins. In 1993 the Bureau Communautaire de Référence released the resulting CRM 470 (later renamed to ERM-DA470), certified for 15 proteins [9]. The protein concentration measurements done for the value assignment of CRP in ERM-DA470 were calibrated with the 1st International Standard CRP 85/506.

After the release of ERM-DA470 IVD manufacturers began referencing their calibrants and controls to the material, and the between-laboratory variation for assays of serum proteins became substantially lower for most of the proteins certified in ERM-DA470 [10, 11].

In 2008, ERM-DA472/IFCC was produced to replace ERM-DA470 for the CRP mass concentration [12]. The present material has been produced to replace ERM-DA472/IFCC.

1.2 Choice of the material

The requirements for a material to be used as a reference material for serum protein immunoassays are, next to requirements for homogeneity, stability and metrological traceability:

- the material should behave like fresh patient serum samples (commutability)
- the certified mass concentration of the 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 assays

- the material should be optically clear. This property is important, as most clinical immunoassays use optical measurement methods. This means that the material must have a low content of lipids and lipoproteins.

Therefore it was decided to process the new material according to procedures similar to those applied for ERM-DA472/IFCC [12].

1.3 Design of the project

Serum was produced from blood collected in 4 blood collection centres according to a procedure ensuring that it was obtained from healthy donors, and that the lipid content of the serum was low.

Twelve small pilot batches were produced by spiking serum with recombinant CRP or CRP isolated from pleural fluids under different conditions (method for buffer exchange between dialysis and diafiltration, addition of Ca^{2+} before or after spiking the CRP, addition of sucrose in the final material). The pilot batches were used to verify the recovery for CRP depending on the protocol. With the most suitable protocol, two additional pilot batches were produced to check the scaling up of the procedure to larger volumes. On that basis, it was decided to process the main part of the serum and to spike the material with CRP isolated from pleural fluids.

The serum was processed in 2 batches, then pooled and spiked with CRP. The serum was filled in ampoules, which were afterwards stored at -70 °C.

The material was characterised through a value transfer procedure [13]. The characterisation measurements were calibrated using ERM-DA470 [9]. In this procedure, ERM-DA474/IFCC is used to determine the response curve of the instrument, and the 6 dilutions of ERM-DA470 are measured directly against different dilutions of ERM-DA474/IFCC.

The techniques used to measure the protein concentrations were nephelometry and turbidimetry. The measurements were performed with different instruments (Abbott Architect c16000, Beckman Synchron, Siemens BN™ II, Siemens BN ProSpec®, Siemens Dimension Vista®, Hitachi 917, Roche Integra 800 and Roche Cobas c501) and reagents. In total five laboratories participated in the value assignment. The characterisation measurements were done under an ISO 13485 quality system.

2 Participants

2.1 Project management and evaluation

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

2.2 Provision of serum and proteins

Blutspendedienst SRK Bern, Bern, CH

Etablissement Français du Sang, Rhône-Alpes, Valence, FR

Hralec Králové University Hospital, Hralec Králové, CZ

Siemens Healthcare Diagnostics Products GmbH, Marburg, DE

University College London (UCL), Medical School, London, UK

2.3 Processing, homogeneity and stability study

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

Siemens Healthcare Diagnostics Products GmbH , Marburg, DE (measurements under the scope of ISO 13485 certification TÜV Rheinland 60038820)

2.4 Characterisation

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-TEST)

Beckman Coulter, Brea, US (measurements under the scope of ISO 13485 certification NSAI MD 19.2791/2)

DAKO Denmark, Glostrup, DK (measurements under the scope of ISO 13485 certification UL A12312)

Roche Diagnostics GmbH, Penzberg, DE (measurements under the scope of ISO 13485 certification TÜV SÜD 00 07 45096 005)

Sentinel CH. S.p.A., Milano, IT (measurements under the scope of ISO 13485 certification BSI MD 504781)

Siemens Healthcare Diagnostics Products GmbH , Marburg, DE (measurements under the scope of ISO 13485 certification TÜV Rheinland 60038820)

3 Material processing and process control

3.1 Origin of the starting material

The starting material for the reference material was a serum pool prepared from the blood of healthy donors, collected at four different blood collection centres (Table 1) as well as unprocessed serum as used for ERM-DA470k/IFCC [14].

Selection of the donors:

- Sex — both sexes
- Age — 20 – 70 years
- Blood group — known
- RF — < 30 IU/mL
- Monoclonal components — not detectable (checked by zone electrophoresis)
- Bilirubin serum colour — visually normal
- Hemoglobin serum colour — visually normal
- Lipemia — no turbidity observed
- Infectious agents negative for — HBsAg, anti HIV 1, anti HIV 2, syphillis and anti HCV

Serum collection protocol: 400 – 500 mL blood was withdrawn from each donor, expected to give an average serum volume of 180 mL (140 – 220 mL).

The following protocol was applied for preparing the serum:

a) Pre-prandial blood (tourniquet used).

b) Collection into a dry bag or a polymer bottle, not containing any additives or anticoagulant (such as Baxter, Fenwal).

c) Clotting at room temperature for 3-4 hours.

d) Centrifugation of the bag in a centrifuge bucket or a bottle at 2200 *g* for 20 minutes.

e) Separation of the serum from the clot.

f) 40 aliquots of 0.5 mL were taken from each donor for testing.

g) Freezing of the serum between -70 and -80 °C.

h) Discarding of donations positive for infectious agents.

i) Transport of the serum to IRMM on dry ice, storage at -70 °C.

j) Discarding of donations with RF> 30 IU/mL and/or presenting monoclonal components.

Table 1 : Summary information on the collected serum. The number of donations not fulfilling the inclusion criteria is given in parenthesis.

Collection Centre	Donation period	Number of donors	Male	Female	RF > 30 IU/mL	Monoclonal components	Final Number
Blutspendedienst SRK Bern AG (CH)	18/05/2009 to 05/06/2009	60	39 (2)	21 (0)	1	2	58
Etablissement Français du Sang – Rhône-Alpes (FR)	08/01/2007 to 23/01/2007	40	27 (0)	13 (0)	0	0	40
Hradec Králové University Hospital (CZ)	16/06/2009 to 23/06/2009	60	29 (0)	31 (1)	1	0	59
Siemens Healthcare Diagnostics Products GmbH (DE)	24/09/2009	60	50 (1)	10 (0)	0	1	59

Tests of the serum : The blood collection centres tested each donations for HIV 1+2, HBV or HBsAg and HCV. Monoclonal gammopathies were detected by gel electrophoresis. Two donations were discarded because of the presence of monoclonal components, one donation was discarded because the Rheumatoid Factor (RF) concentration was above 30 IU/mL and one donation presenting both the presence of monoclonal components and a RF concentration above 30 IU/mL was discarded. 216 donations, of which 75 originated from female donors, were fulfilling the inclusion criteria, and were released for further processing.

3.2 Processing of the serum

Overview of the procedure

The processing procedure has been adapted from the procedures that were used for ERM-DA470 as described in the certification report [9] and ERM-DA472 with respect to the final aggregate state and filling [12]. As in the original procedure [12] dialysis was used for buffer exchange after delipidation with Aerosil. The dialysis buffer was supplemented with $CaCl_2$ to maintain its physiological concentration.

3.2.1 Preparation of pools

Donations fulfilling the above criteria were processed as described below:

- thawing at 5 ± 3 °C (2 to 3 days)
- determination of the volume of each donation
- Pool 1: pooling of all suitable CH and CZ donations
- Pool 2: pooling of all suitable donations of the DE, FR and 104 50 mL-aliquots taken before processing from the individual batches used to produce the ERM-DA470k/IFCC [14].
- addition of sodium azide (Merck, Darmstadt, DE) to a final concentration of 7.7 mM (approx. 0.5 g/L)

- removal of aliquots for the measurement of cholesterol, triglycerides, apolipoprotein A-I, apolipoprotein B, total protein (Biuret reaction) and for the measurement and physicochemical analysis of selected proteins

The material is now in State A

Conversion of C3 to C3c

- adjustment of pH to 7.2 ± 0.1 with saturated HEPES buffer (Calbiochem, Merck Biosciences, Schwalbach, DE)
- addition of 20 mM magnesium acetate tetrahydrate (Merck, Darmstadt, DE)
- adjustment of the solution to 37 °C
- addition of Inulin (Sigma Aldrich, Milwaukee, USA) to a final concentration of 0.2 %
- stirring for 1.5 to 2 hours at 37 °C

Delipidation with Aerosil

- adjustment of inulin-treated pool to pH 8.5 ± 0.1 with saturated TRIS solution (Sigma-Aldrich, Milwaukee, USA)
- addition of sodium chloride (crystalline; Merck, Darmstadt, DE) to 50 g/L of the pool while stirring constantly
- calculation of the required amount of Aerosil 200 (Degussa, Frankfurt, DE) based on the total protein concentration of the pool before C3 treatment (approx. 430 mg/g total protein)
- gradual addition of Aerosil while stirring and constantly monitoring and if necessary adjusting the pH to 8.5 ± 0.1
- continued slow stirring for another 30 minutes
- removal of Aerosil and protein precipitates by centrifugation for 30 minutes at approximately 10000 *g* using 500 mL-centrifuge bottles
- determination of the total volume of the clear supernatant
- determination of cholesterol, triglycerides and apolipoproteins A-I and B to verifiy the successful lipid removal

Sterile filtration and dialysis

- filtration of the supernatant using a 0.45 µm Sartobran P filter (Sartorius, Göttingen, Germany) to remove small particles interfering with the following dialysis
- before dialysis removal of aliquot for the measurement of CRP, IgG and total protein (Biuret reaction)
- dialysis (Visking, Typ 36/32, *MWCO* 12000-14000) using isotonic saline with 2.5 mM $CaCl_2$ over 2 to 4 days at +2 °C to +8 °C with a 2-fold buffer exchange (sample/buffer ratio 1:15)
- removal of aliquots for the measurement of IgG and total protein (Biuret reaction).
- ultrafiltration (*MWCO* 10000) to adjust the concentration to 8 to 10 g/L IgG and to 55 to 75 g/L for total protein.

Material is now in State B

Preservation and sterile filtration

- adjustment of the pH with saturated HEPES solution to 7.2 ± 0.1
- addition of sodium azide to a final concentration of 14.6 mM (0.95 g/L)
- addition of aprotinin to 80000 KIU/L (8 mL Trasylol 500 000 KIU/L, Bayer, Leverkusen, DE)

- addition of benzamidine chloride monohydrate (Merck, Darmstadt, DE) to a final concentration of 1 mM
- adjustment of the pH with saturated HEPES solution to 7.2 ± 0.1
- sterile filtration using a Sartopore 2 filter (0.2 µm) (Sartorius, Göttingen, DE)
- sterile removal of
    - 600 mL of Pool 1 (used to produce pilot batches)
    - 300 mL of Pool 2 (used to produce pilot batches)
- sterile removal of aliquots for the measurement of total protein (Biuret reaction), CRP, and physicochemical analysis

Material is now in State C.

Until processing of the final pool the material was stored below -70 °C.

Protein concentration measurements: Throughout the processing, the mass concentrations of CRP, IgG, apolipoprotein A-I and B were determined by immunonephelometry on a BN ProSpec® or BN™ II System using reagents, standards and controls of Siemens Healthcare Diagnostics Products GmbH.

Total protein determination : Determination of total protein was done according to the Biuret method using pure human serum albumin (internal Siemens product) for the calibration and chemicals from Merck (Darmstadt, DE) or Sigma-Aldrich (Milwaukee, USA).

Lipids : Cholesterol was measured using the cholesterol CHOD-PAP Kit and triglycerides with the Triglycerides GPO Kit (Roche Diagnostics, Mannheim, DE).

Blank signal : According to the specifications for the instrument concerned an empty cuvette is considered adequately inserted and optically clear if the scattered light gives a signal between 20 and 600 Bit (unit of the anallog-to-digital converter of the light detector).

3.2.2 Preparation of the combined pool

Preparation of CRP

A purified (as described in [15]) human CRP solution (CRP mass fraction 97 % of total protein; CRP mass concentration 3.88 g/L) was used. The material was stored below -70 °C and thawed in a water bath at 37 °C with occasional turning. Before spiking the pool, the CRP solution was gently mixed while avoiding turbulence.

Preparation of the final material

- thawing of both pools (6 days) at 5 ± 3 °C
- pooling
- ultrafiltration
- measurement of the CRP mass concentration
- preparation of the CRP solution as described
- slow addition of the CRP solution while constantly stirring
- pH adjustment with saturated HEPES solution (pH 7.2 ± 0.1)
- sterile filtration (0.2 µm)
- sterile removal of aliquots for further analysis, including sterility testing

3.2.3 Processing control

Processing of the two pools

Total protein concentration: In line with the protocol for the preparation of the original ERM-DA470 only pools with a total protein concentration between 60 and 80 g/L were considered to qualify as starting material for the processing, and after Aerosil treatments, the pools should be adjusted to protein concentrations between 55 and 75 g/L. As shown in Table 2, the total protein mass concentration of the pools was 77.3 and 81.1 g/L in State A and 54.4 and 57.6 g/L in State C respectively, i.e. within the defined tolerance limits (taking into account the uncertainties for the measurement, the slightly higher value for pool 1 in State A was considered acceptable).

Table 2: Total protein mass concentration (Biuret method) of pools at the different processing stages

	State A	State B	State C
Pool	Total protein [g/L]	Total protein [g/L]	Total protein [g/L]
1	81.1	44.9	57.6
2	77.3	42.5	54.4

Delipidation: The delipidation by the Aerosil treatment is considered successful when the relative mass concentrations of cholesterol and apolipoprotein B are reduced to 1 % of the concentration in the starting material. The relative mass concentrations for triglycerides and apolipoprotein A-I should be below 15 % and 20 % of the starting material, respectively. As shown in Table 3, these criteria were fulfilled for both pools.

Table 3: Lipoprotein mass concentrations before processing and after Aerosil treatment[1]

	State A				State B			
Pool	Chole-sterol [mg/L]	Trigly-cerides [mg/L]	Apo A-I [mg/L]	Apo B [mg/L]	Chole-sterol [mg/L]	Trigly-cerides [mg/L]	Apo A-I [mg/L]	Apo B [mg/L]
1	1910	1122	1500	714	8.4	73.4	ND	ND
2	1254	803	1495	852	2.8	60.4	ND	ND

[1]when the concentration is below the limit of detection this is marked ND (not detectable)

The reduction of the blank value obtained by measuring the signal of undiluted sample using a Siemens BNA System also demonstrated the effective removal of lipoproteins (Table 4).

Table 4: Signal measured with a nephelometer before and after Aerosil treatment

	Measurement value [Bit]	
Pool	State A	State C
1	3626	49
2	3883	55

Preparation of the combined pool

Mixing of the two pools: Pool 1 and 2 were mixed to give the final material pool for the further material processing.

Sterility test: The sterility of the material was verified by inoculating Casein Soy Bean (CASO) Digest Broth with the sterile filtrated pool, and streaking it out on CASO agar, Blood agar and McConkey agar (Institut für Medizinische Mikrobiologie und Krankenhaushygiene, University of Giessen and Marburg, Marburg, DE). No growth was observed on the selected media after five days, and the pool was released for filling.

Blank values: The blank value for the final pool before sterile filtration was 93 Bit (detector values), after sterile filtration 84 Bit, and the material could thus be considered as optically clear.

3.3 Filling and labelling

The serum was ampouled at IRMM in a single batch. Approximately 1 mL (at least) of serum was filled into 3 mL Duran glass ampoules using a Rota ampouling machine R 910 PA (Rota, Wehr, DE). The ampouling machine and the cooled serum were placed in a movable class 1000 cleanroom (Terra Universal Inc., Fullerton, California, USA). The ampoules were first opened, flushed with argon, filled with 1 mL of serum, flushed with argon and flame sealed. During the filling, the bottle containing the serum was kept at low temperature in a tray filled with ice/water under continuous stirring. The transfer lines were made of Teflon®. The speed of the ampouling machine was adapted to minimise the formation of foam in the ampoules. Care was taken that no serum was heated up during the closing of the ampoules. Some ampoules had a black spot on top linked to the presence of droplets of serum falling on the ampoule after filling. The ampoules were scrutinised and the ones presenting black spots of burnt serum were discarded. After filling, the ampoules were kept at +4 °C and labelled according to the filling order. Thereafter, the material was frozen at -70 °C.

After processing, selected samples were analysed with semi-native gel electrophoresis (1/20 SDS-PAGE) followed by Western Blotting in order to check the absence of monomeric CRP in ERM-DA474/IFCC. No evidence of the presence of monomeric CRP was found thus proving that no degradation of the pentameric CRP occurred during the processing.

4 Assessment of homogeneity

A key requirement for any reference material is the equivalence between the various units. In this respect, it is not relevant whether the variation between units is significant compared to the analytical variation, but whether this variation is significant to the certified uncertainty. 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 values of the CRM are valid for all units of the material, within the stated uncertainty.

For the between-unit homogeneity test, 30 units were selected using a random stratified sampling scheme covering the whole batch. For this, the batch was divided into 30 groups (with similar number of units) and one unit was randomly selected from each group. The number of selected units corresponds to approximately the cubic root of the total number of the produced units. From each unit, six independent samples were taken and analysed by immunonephelometry. The analyses were performed in a randomised manner to be able to separate a potential analytical drift from a trend in the filling sequence.

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

sequence were corrected as the trend was significant on at least a 99 % confidence level as shown in formula (1).

corrected result = measured result – b x i (1)

b = slope of the linear regression

i = position of the result in the analytical sequence

A high dispersion of the results was noted for the first two runs. A Cochran test for variance presented the variance from the first run as an outlier thus confirming that the variation within the run was not only due to the lack of homogeneity of the samples. As a consequence, this first run was not retained for the calculations. The second run was not presenting any outlying variance and was retained for further calculations.

The trend-corrected dataset was tested for consistency using Grubbs outlier tests on a confidence level of 99 % on the individual results and the unit means. Some outlying individual results and outlying unit means have been detected. Since no technical reason for the outliers could be found, all the data were retained for statistical analysis with the exception of the first run.

Quantification of between-unit heterogeneity is most easily done by analysis of variance (ANOVA), which can separate 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 unit means which follow at least a unimodal distribution and results for each unit that follow unimodal distributions with approximately the same standard deviations. Distribution of the unit means was tested using histograms and normal probability plots. Too few data are available for each unit to make a clear statement of the distribution of individual results. Therefore, it was checked whether all individual data follow a unimodal distribution using histograms and normal probability plots. Minor deviations from unimodality of the individual values do not grossly affect the estimate of between-unit standard deviations.

One has to bear in mind that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and therefore subject to random fluctuations. Therefore, the mean squares 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 heterogeneity that could be hidden by method repeatability, was calculated as described by Linsinger *et al.* [16]. u^*_{bb} is comparable to the limit of detection of an analytical method, yielding the maximum heterogeneity 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

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}}$$

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

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

MS_{within} mean squares within a unit from an ANOVA

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

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

n average number of replicates per unit

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

The results of the measurements are shown in Annex A. The results of the evaluation of the between-unit variation are summarised in Table 5.

Table 5: Results of the homogeneity study

Measurand	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]	$u_{bb,rel}$ [%]
CRP mass concentration	1.38	0.69	0.22	0.69

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

4.2 Within-unit homogeneity and minimum sample intake

The material is in solution and is not expected to have any relevant heterogeneity. This assumption was confirmed by the homogeneity/stability/characterisation study, where sample intakes as low as 20 µL were found to give acceptable repeatabilities, demonstrating that there is no intrinsic heterogeneity or contamination at a sample intake of 20 µL. Therefore the minimum sample intake is 20 µL.

5 Stability

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

Stability testing is necessary to establish conditions for storage (long-term stability) as well as conditions for dispatch to the customers (short-term stability). During transport, especially in summer time, temperatures up to 60 °C can be reached and stability against these conditions must be demonstrated if transport at ambient temperature will be applied.

The stability studies have been carried out using an isochronous design [17]. In that approach, samples are stored for a certain time at different temperature conditions. Afterwards, the samples are moved to conditions where further degradation can be assumed to be negligible ("reference conditions"), effectively "freezing" the degradation status of the materials. At the end of the isochronous storage, the samples are analysed simultaneously under repeatability conditions. Analysis of the material (after various exposure times and temperatures) under repeatability conditions greatly improves the sensitivity of the stability tests.

5.1 Short-term stability study

For the short-term stability study, samples have been stored at -20 °C, 4 °C, 18 °C and 60 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set to -70 °C. Two units per storage time were selected using a random stratified sampling scheme. From each unit, three samples were measured by immunonephelometry. The measurements were

performed under repeatability conditions, and in a randomised manner to be able to separate a potential analytical drift from a trend over storage time.

Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends according to the time and the temperature. Some significant (99 % confidence level) trends in the analytical sequence were observed (Annex B - Figure B-1). As the analytical sequence and the unit numbers were not correlated, correction for these trends can improve the sensitivity of the subsequent statistical analysis through a reduction in analytical variation without masking potential between-unit heterogeneities. Therefore, as a significant trend in the analytical sequence was noted, the results were corrected using the formula (1) explained in paragraph 4.1.

The obtained data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test. Some outlying individual results were found but as no technical reason for the outliers could be found all data were retained for statistical analysis.

Furthermore, the data were plotted against storage time and regression lines of mass concentration versus time were calculated. The slope of the regression lines was then tested for statistical significance (loss/increase due to shipping conditions). For CRP, the slopes of the regression lines were not significantly different from 0 (at 95 and 99 % confidence level) for -20 °C, 4 °C and 18 °C. The samples stored at 60 °C were clotted and, therefore, no analysis of the CRP mass fraction was possible.

The results of the measurements are shown in Annex B.

No technically unexplained outliers were observed and none of the trends with storage time was statistically significant on a 99 % confidence level for any of the temperatures.

The material was found stable at -20 °C, 4 °C and 18°C. Nevertheless, the material is stored frozen and therefore freeze/thaw cycles should be kept at minimum. With this consideration, the material shall be shipped frozen on dry ice.

5.2 Long-term stability study

For the long-term stability study, samples have been stored at -20 °C and -70 °C for 0, 4, 8 and 12 months (at each temperature). The reference temperature was below -150 °C. Two units per storage time were selected using a random stratified sampling scheme. From each unit, three samples were measured by immunonephelometry. The measurements were performed under repeatability conditions, and in a randomised manner to be able to separate a potential analytical drift from a trend over storage time.

Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. Some significant (95 % confidence level) trends in the analytical sequence were observed (Annex C - Figure C-1). As the analytical sequence and the unit numbers were not correlated, correction for these trends can improve the sensitivity of the subsequent statistical analysis through a reduction in analytical variation without masking potential between-unit heterogeneities. Therefore, as a significant trend in the analytical sequence was noted, the results were corrected using the formula (1) explained in paragraph 4.1.

The obtained data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test. Some outlying individual results were found but as no technical reason for the outliers could be found all data were retained for statistical analysis.

Furthermore, the data were plotted against storage time and regression lines of mass concentration versus time were calculated. The slope of the regression lines was then tested for statistical significance (loss/increase due to storage conditions). For all elements, the slopes of the regression lines were not significantly different from 0 (on 95 and 99 % confidence level) for both -70 °C and -20 °C.

The results of the measurements are shown in Annex C.

No technically unexplained outliers were observed and none of the trends was statistically significant on a 99 % confidence level for any of the temperatures. The material will be stored at -70 °C.

5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results no study can rule out degradation of materials completely, 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, even under ideal conditions, the outcome of a stability study can only be "degradation is 0 ± x % per time".

Uncertainties of stability during dispatch and storage were estimated as described in [18]. For this approach, the uncertainty of the linear regression line with a slope of zero is calculated. The uncertainty contribution (u_{lts}) is then calculated as the product of the chosen shelf life and the uncertainty of the regression lines as

$$u_{lts,rel} = \frac{RSD}{\sqrt{\sum (x_i - \bar{x})^2}} \cdot t_{sl}$$

RSD relative standard deviation of all results of the stability study

t_i time point for each replicate

$\bar{x}$ mean results for all time points

t_{sl} proposed shelf life (12 months at -70 ºC in this case)

The following uncertainty was estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the -20 °C study for a time of 1 month (4 week). The uncertainty therefore describes the possible change during a dispatch on dry ice lasting for one week where the temperature in the parcel may reach -20 °C.
- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the -70 °C study. The uncertainty contribution therefore describes the possible degradation for 12 months at -70 °C.

The results of these evaluations are summarised in Table 6.

Table 6: Uncertainties of stability during storage and dispatch. $u_{sts,rel}$ was calculated for a temperature of -20 °C and 1 week; $u_{lts,rel}$ was calculated for a storage temperature of -70 °C and 1 year.

Measurand	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
CRP mass concentration	0.11 %	0.61 %

After the certification campaign, the material will be subjected to IRMM's regular stability monitoring programme to control its further stability.

6 Characterisation

6.1 Selection of participants

Five laboratories were selected based on criteria that comprised both technical competence and quality management aspects. Each participant was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field of element measurements 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 obligatory. The measurements were performed under a ISO 13485 quality management system and the certification number is stated in the list of participants (Section 2). Furthermore all the selected laboratories have successfully participated to past certification projects for the same measurand in the same matrix.

6.2 General principles

The characterisation of CRP in the human serum reference material ERM-DA474/IFCC is performed according to the principles as documented in [13] and [19]. The characterisation is achieved by transferring values from ERM-DA470 to the candidate reference material. ERM-DA470 is certified for its CRP mass concentration. It is diluted at different concentrations that are measured as samples in the measuring system. The dose-response function of the measuring system is determined by using dilutions of the candidate reference material.

The practical transfer protocol is based on duplicate measurements of 6 dilutions of ERM-DA470 and 6 dilutions of the candidate reference material under conditions as repeatable as possible. The transfer protocol requires several measurements a day (three in this study) repeated on several days (four in this study), an important prerequisite being that all reconstitutions and dilutions are controlled by weighing, thus reducing uncertainty in the transfer.

With regard to the transfer procedure, the following definitions apply ([13] and [19]):

The reference preparation (ERM-DA470) is defined as the protein preparation with known concentration value (Table 7).

Table 7: Certified value and expanded uncertainty of CRP in the calibrant ERM-DA470 [9]

Certified value[1)] [mg/L]	U_{CRM}[2)] [mg/L]
39.2	1.9

1) In the reconstituted material. The certified value is the unweighted mean of 3 accepted mean values, independently obtained by 3 laboratories.
2) The certified expanded uncertainty is the half-width of the 95 % confidence interval of the mean defined in footnote [1)]

Target material is defined as the serum protein matrix material with unknown concentration value. In this case it is the new matrix material ERM-DA474/IFCC.

The transfer method should be based on well established and recognised methods used in clinical chemistry. In this characterisation study immuno-turbidimetry, and immuno-nephelometry were used.

Slight variations in the assay conditions, in the programming of the instruments or in the reagents may lead to different results. This has lead to the prerequisite of method standardisation [7]. To minimise all of these factors contributing to variation, an optimised and practical transfer protocol was made with detailed instructions and emphasis on weighing all solutions used to prepare dilutions. To record the obtained data, special registration forms were made also containing the intended dilution schemes. These raw data forms were subsequently sent for data treatment.

When assigning a value to a target material (a serum) using an existing reference material the value transfer procedure is used [13]. In this procedure dilutions of the target material are used to determine the dose-response function for the instrument or the assay. Dilutions of ERM-DA470 are then measured against this calibration curve in the following way:

1. 6 different dilutions are prepared of the target material (TM). The concentration of the specific protein (although still unknown) in each dilution will be:

$$C_{TM}(i) = F_{TM}(i) \times C_{TM} \quad (2)$$

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

2. A series of 6 test samples are prepared by diluting increasing amounts of the reference material to 6 different test tubes. The final volume of all preparations is then adjusted to be the same by adding a dilution solution (usual diluent used for each assay). The dilutions are made in such a way that the signals will fall within the signal interval of the dose-response curve. The concentration of the samples will be:

$$C_S(j) = F_{470}(j) \times C_{470} \quad (3)$$

where F_{470} is the dilution factor of ERM-DA470 and (j) denotes the different dilutions (e.g., j =1-6).

3. A dose-response function is determined by using the dilutions of TM, and by plotting the signals against the different dilutions of TM.

4. In a sample run the different dilutions of ERM-DA470 are measured. The signals of these test samples $S_S(j)$ are interpolated on the dose-response curve. The resulting concentrations $C_S(j)$ are now obtained as relative concentrations of TM:

$$C_S(j) = F_{TM}(j) \times C_{TM} \quad (4)$$

5. Since the unknown concentrations in $C_S(j)$ can be either calculated using equation (3) or found by interpolation on the dose-response curve (4), a combination of (3) and (4) will give:

$$C_S(j) = F_{TM}(j) \times C_{TM} = F_{470}(j) \times C_{470}$$

$$F_{TM}(j) = C_{470} / C_{TM} \times F_{470}(j) \quad (5)$$

which is the equation of a straight line ($y = \alpha x$).

6. The different dilution factors $F_{TM}(j)$ obtained by the interpolation are now plotted against the different dilution factors $F_{470}(j)$ used for the initial dilution of ERM-DA470. Since all dilutions are controlled by weighing the uncertainty of $F_{470}(j)$ is negligible.

The slope (α) is equal to the ratio of the concentrations of the specific protein in the two materials:

$$\alpha = C_{470} / C_{TM} \quad (6)$$

Since the slope can easily be calculated, the unknown concentration C_{TM} is now found to be:

$$C_{TM} = C_{470} / \alpha \quad (7)$$

The final characterisation study was conducted using ERM-DA470 to assign values to ERM-DA474/IFCC. The methods including instruments, dilution buffers, antibodies and reagents and laboratories are listed in Annex D.

Each of the 4 days a new ampoule of the reference preparation and the target material is used and a new set of dilutions is prepared. Each day 3 calibrations are performed together with determinations of samples and controls (i.e., 3 runs).

Additionally, on each run, duplicate measurements of a specific dilution of the same target material used for the calibration were assessed as control values (measured concentration of

CRP in a control dilution of ERM-DA474/IFCC relative to the concentration in the control sample expected on the basis of the dilution, calculated from the mass values obtained by weighing).

The transfer procedure was optimised separately for each measuring system participating in the value assignment. The dilution scheme was optimised taking into account that dilutions automatically carried out by the instruments should be avoided as much as possible (as these dilutions cannot be corrected by more accurate weighings). Finally it had to be stressed that for each material all 6 dilutions should be prepared from a single vial and that all reconstitutions and dilutions are controlled by weighing.

6.3 Calculation of the dilutions

The liquids used for the reconstitution of the materials and for the preparation of the dilutions were weighed to a standard deviation of maximum 0.0001 g.

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

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 water added to the vial.

In case of predilutions:

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

Where $m_{PR,i}$ and $m_{PD,i}$ are the masses of the reconstituted material and of the diluent for the predilution and $\rho_{PR,i}$ and $\rho_{PD,i}$ are the density of the reconstituted material and of the diluent for the predilution.

For the dilutions:

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

For the dilution *j* of the material *i* with $m_{DM,i}$ and $\rho_{DM,i}$ respectively the mass and the density of the reconstituted material (prediluted or not), and $m_{DD,i}$ and $\rho_{DD,i}$ respectively the mass and the density of the diluent for dilution *j*.

The concentration c_{ij} (with c = m/V (mass / volume) and the unit mg/L) of CRP 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} being the concentration of CRP in material *i* and c_{ij} the concentration of CRP in dilution *j* of the material *i*.

6.4 Data analysis

A variety of measurement instruments existing in the field (8) with different quantification principles (nephelometry and turbidimetry), different dilution buffers, antibodies and reagents were used to characterise the material.

All methods used during the characterisation study are summarised in Annex D. The laboratory code (e.g., L1) is a random number and does not correspond to the order of laboratories in Section 2.4. The lab-method code consists of a number assigned to each laboratory (e.g., L1) and the dataset number, (e.g., Dataset 1).

Datasets were defined as the combination of the triplicate measurements performed on four days by each participant (i.e., up to 12 measurements).

6.4.1 Technical evaluation

For the value transfer procedure, the laboratories used the dilutions to construct the calibration curve directly. The values entered into their instrument for the concentration of these dilutions were the values of the concentration relative to the concentration in ERM-DA474/IFCC, expressed in percent. These relative concentrations were calculated from the masses of the solutions (and predilution if relevant). Then for each run, the laboratories measured the dilutions of ERM-DA470 in duplicate.

The measured values S_{ijk} (signal of the *k*th measurement of material *i* within dilution *j*) were plotted in scatter plots $S_{ijk} = f(c_{ij})$ so as to evaluate outliers. Outliers were only rejected if there was a technical reason for doing so (a transcription error, wrong dilution, etc.). A linear regression with intercept was performed on the means of the S_{ijk} in function of the concentration c_{ij}.

The following specific acceptance criteria were applied for the open procedure:

The mean control value is within 1.00 ± 0.05

The following analyses were performed on all data:

- testing for normality (visual inspection and normal probability plot)
- testing for linearity (visual inspection and evaluation of r^2)
- verification that the intercept ± 4 times the standard deviation covers the origin.

The following general acceptance criteria were applied to the datasets:

- r^2 of the regression must be above 0.98 for all measurement systems
- Data from at least four dilutions must be available
- The completeness of data must be at least 50 % for the data of a particular day. Otherwise the data from that day are declared non-valid
- At least two daily value assignments must be valid
- The day-to-day variation (RSD) of valid datasets for a particular laboratory must be equal to or below 5 %

Rationale for the acceptance criteria (see also reference [13]):

- Non-overlapping confidence intervals of the intercepts of regressions are either an indication of differing matrix effects or of quality problems of measurements resulting in scattering, both of which would result in non-valid results.
- Overall $r^2 < 0.98$ of regression indicates quality problems in the measurements (scattering, outliers, run-to-run variation).

Based on the above some datasets were rejected as not technically valid (see Table 8).

Among all the determinations, four runs were rejected on basis of the control value falling outside of the above-mentioned specifications.

The results for two laboratories (L3 and L5) presented an intercept significantly different from 0 for all the 12 individual determinations and the whole datasets were therefore not included in the calculation.

Nine individual results presented an intercept significantly different from 0 and were not retained. As a result, only one valid dataset remained for one day and was discarded because less than two results were obtained for that specific day.

Table 8: Datasets that showed non-compliances with the analysis protocol and technical specifications, and action taken.

Property measured	Lab-method code	Description of problem	Action taken
C-reactive protein mass concentration	L1-Dataset 1 day 1 – run 3	QC sample out of specification	not used for evaluation
	L5 – Dataset 8 Day 2 – run 1,2 and 3	QC sample out of specification	not used for evaluation
	L3 – Dataset 5 all days – all runs	Intercept significantly different from 0	not used for evaluation
	L5 – Dataset 8 all days – all runs	Intercept significantly different from 0	not used for evaluation
	L3 – Dataset 6 Day 1 – run 2 and 3	Intercept significantly different from 0	not used for evaluation
	L3 – Dataset 6 Day 1 – run 1	Only one daily value accepted	not used for evaluation
	L3 – Dataset 6 Day 2 – run 2	Intercept significantly different from 0	not used for evaluation
	L3 – Dataset 6 Day 3 – run 2	Intercept significantly different from 0	not used for evaluation
	L3 – Dataset 6 Day 4 – run 3	Intercept significantly different from 0	not used for evaluation
	L4 – Dataset 7 Day 2 – run 2	Intercept significantly different from 0	not used for evaluation
	L4 – Dataset 7 Day 3 – run 1,2 and 3	Intercept significantly different from 0	not used for evaluation
	L3 – Dataset 6	statistical outlier, but agrees within uncertainty with certified values	retained

Finally, the characterisation study resulted in six datasets covering twenty-two days and sixty-one runs. All individual accepted results of the participants are displayed in tabular form in Annex E.

6.4.2 Statistical evaluation

The number of accepted datasets were not enough to perform a normality test on the datasets means. The datasets means were tested for outlying means using the Grubbs test and using the Cochran test for outlying standard deviations, (both at a 99 % confidence level). Standard deviation within (s_{within}) and between ($s_{between}$) laboratories were calculated using one-way ANOVA. The results of these evaluations are shown in Table 9.

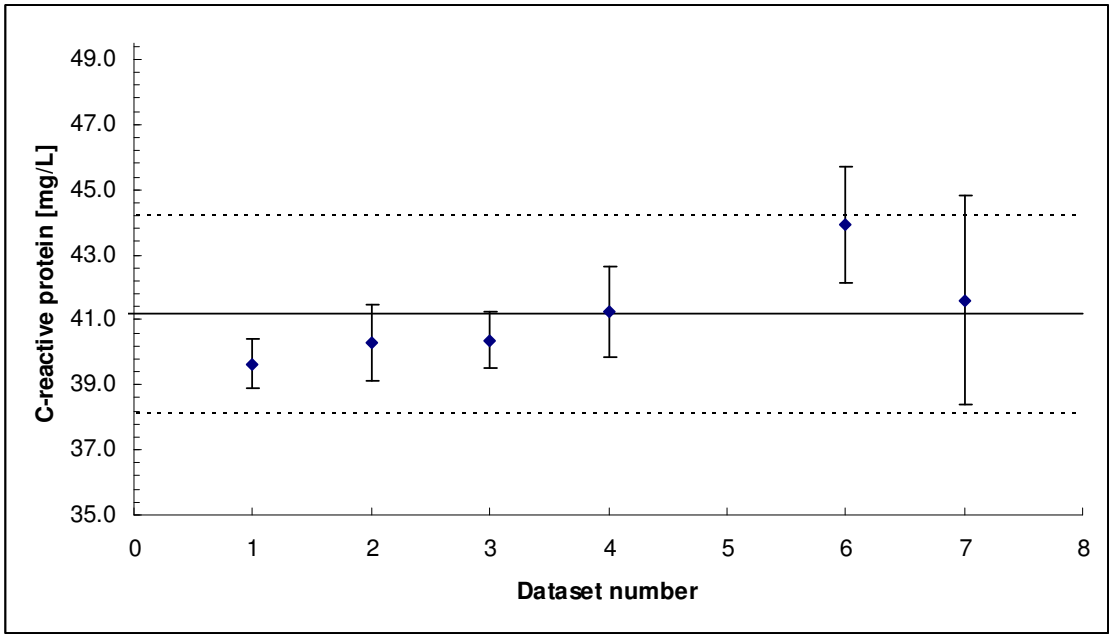
Table 9: Statistical evaluation of the technically accepted datasets for CRP. p: number of technically valid datasets

Measurand	p	Outliers		Normally distributed	Statistical parameters			
		Means	Variances		Average [mg/L]	s [mg/L]	$s_{between}$ [mg/L]	s_{within} [mg/L]
CRP mass concentration	6	1	0	Not enough datasets for the evaluation	41.2	1.5	2.1	1.3

The statistical evaluation flags laboratory L3 – Dataset 6 as outlier for CRP. However, it must be borne in mind that outlier tests do not take uncertainty information into consideration. A closer investigation reveals that the difference between the mean value of laboratory L3 - Dataset 6 and the other results is covered by the measurement uncertainty of laboratory L3 - Dataset 6. There is therefore no evidence that the results of laboratory L3 – Dataset 6 deviate from the other results and this dataset was retained.

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

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



The uncertainty for the characterisation exercise was estimated as the relative standard uncertainty of the mean of laboratory means, i.e., $\frac{s}{\sqrt{p}}$ with s the relative standard deviation of the mean of laboratory means and p the number of datasets, converted into a relative value. The relative uncertainty for the characterisation is presented in Table 10.

Table 10: Uncertainty for the characterisation exercise.

Measurand	$u_{char,rel}$ [%]
CRP mass concentration	1.51 %

7 Value Assignment

For this material, a certified value has been assigned.

Certified values are values that fulfil the highest standards of accuracy. Procedures at IRMM require generally pooling of not less than 6 datasets to assign certified values. Full uncertainty budgets in accordance with the Guide to the expression of uncertainty in measurement [3] must be established.

7.1 Certified value and its uncertainties

The unweighted mean of the means of the accepted datasets as shown in Table 9 was assigned as certified value for the measurand.

The assigned uncertainty consists of uncertainties related to characterisation, u_{char} (see Section 6), potential between-unit heterogeneity, u_{bb} (see Section 4) and potential degradation during transport (u_{sts}) and long-term storage, u_{lts} (see Section 5). These different contributions were combined to estimate the expanded, relative uncertainty of the certified value ($U_{CRM, rel}$) with a coverage factor k as

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

- $u_{char, rel}$ was estimated as described in Section 6.
- $u_{cal, rel}$ is the relative standard uncertainty from ERM-DA470 [9].
- $u_{bb, rel}$ was estimated as described in Section 4.
- $u_{sts, rel}$ was estimated as described in section 5. As can be seen in Table 11, the uncertainty of degradation during dispatch is negligible compared to the other uncertainty contributions and is therefore not included in the final uncertainty budget.
- $u_{lts, rel}$ was estimated as described in Section 5.

Because of the sufficient numbers of the degrees of freedom of the different uncertainty contributions, a coverage factor k of 2 was applied, to obtain the expanded uncertainties.

The certified value and its uncertainty are summarised in Table 11.

Table 11: Certified value and its uncertainties for ERM-DA474/IFCC

Measurand	Certified value [mg/L]	$u_{char, rel}$ [%]	$u_{cal, rel}$ [%]	$u_{bb, rel}$ [%]	$u_{lts, rel}$ [%]	$u_{CRM, rel}$ [%]	U_{CRM} [mg/L]
CRP mass concentration	41.2	1.51	2.42	0.69	0.61	3.00	2.5

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

C-reactive protein in the initial solution used to spike the matrix material is a clearly defined analyte (identity was confirmed by Size Exclusion Chromatography, semi-native gel electrophoresis, 2-D gel electrophoresis and immunoassay response). The measurand in the spiking material is therefore structurally defined and independent of the measurement method.

The participants used different methods for the final determination, different dilution buffers, antibodies and reagents, demonstrating absence of measurement bias. However, the characterisation of the matrix material ERM-DA474/IFCC was performed using only immunoassay-based methods and therefore the measurand is defined as C-reactive protein as measured by the immunoassays used in the presented procedures.

Quantity value

Only validated methods were used for the determination of the assigned value. The value assigned to the common calibrant (ERM-DA470) is traceable to the SI through calibration with the 1st International Standard for CRP WHO 85/506 as described in the certification report [9]. All relevant input parameters were calibrated with this material. The individual results are therefore traceable to the SI, as it is also confirmed by the agreement among the technically accepted datasets. As the assigned values are combinations of agreeing results individually traceable to the SI, the assigned quantity values themselves are traceable to the SI as well.

8.2 Commutability

Many measurement procedures include one or more steps, which are selecting specific (or specific groups of) analytes from the sample for the subsequent steps of the whole measurement process. Often the complete identity of these 'intermediate analytes' is not fully known or taken into account. Therefore, it is difficult to mimic all the analytically relevant properties of real samples within a CRM. The degree of equivalence in the analytical behaviour of real samples and a CRM with respect to various measurement procedures (methods) is summarised in a concept called 'commutability of a reference material'. There are various definitions expressing this concept. For instance, the CLSI Guideline C-53A [20] 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 is essential for its fitness for use and, thus, is a crucial characteristic in case of 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 calibrator. 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.

Commutability was assessed on the pilot batches and their dilutions up to 1/10 on the following platforms (Abbott Architect c8000, Abbott Architect ci8200, Hitachi 917, Olympus AU640, Roche Cobas 600, Roche Integra 800, Siemens BN ProSpec®) using different dilution buffers, antibodies and reagents. As the pilot batches prepared in the same way as the reference material and their dilutions were found to be commutable for the assays tested, it can be expected that ERM-DA474/IFCC and its subsequent dilutions (up to 1/10) is

commutable for the major immunoassays. However, when the material is used as a calibrant in a particular assay the commutability should be verified for the assay concerned.

9 Instructions for use

9.1 Storage conditions

The materials shall be stored at -70 ± 10 °C.

Unopened ampoules should be stored at or below -70 °C. After the opening of the ampoule it is advisable to store the material at 2 to 8 °C in a sealed container. Under the condition that any microbial contamination has been excluded, the solution of ERM-DA474/IFCC can be used for one week.

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

9.2 Safety and protection for the environment

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

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

9.3 Preparation and use of the material

To make it ready for use, the content of the ampoule has to be thawed in a water-bath at room temperature, while gently rotating the ampoule every 5-10 minutes, until the serum is thawed and the content mixed.

9.4 Minimum sample intake

The minimum sample intake is 20 µL.

9.5 Use of the certified value

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

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 [21].

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_m).
- Combine measurement uncertainty (u_m) with the uncertainty of the certified value (u_{CRM}): $u_\Delta = \sqrt{u_m^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty (U_Δ) from the combined uncertainty (u_Δ) using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %

- If $\Delta_m \leq U_\Delta$ then there is no significant difference between the measurement result and the certified value, at a confidence level of about 95 %.

Use as a calibrant

If used as calibrant, the uncertainty of the certified value shall be taken into account in the estimation of the measurement uncertainty.

Use in quality control charts

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

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Annexes

Annex A: Results of the homogeneity measurements

Figure A-1: Regression of the results in the analytical sequence order

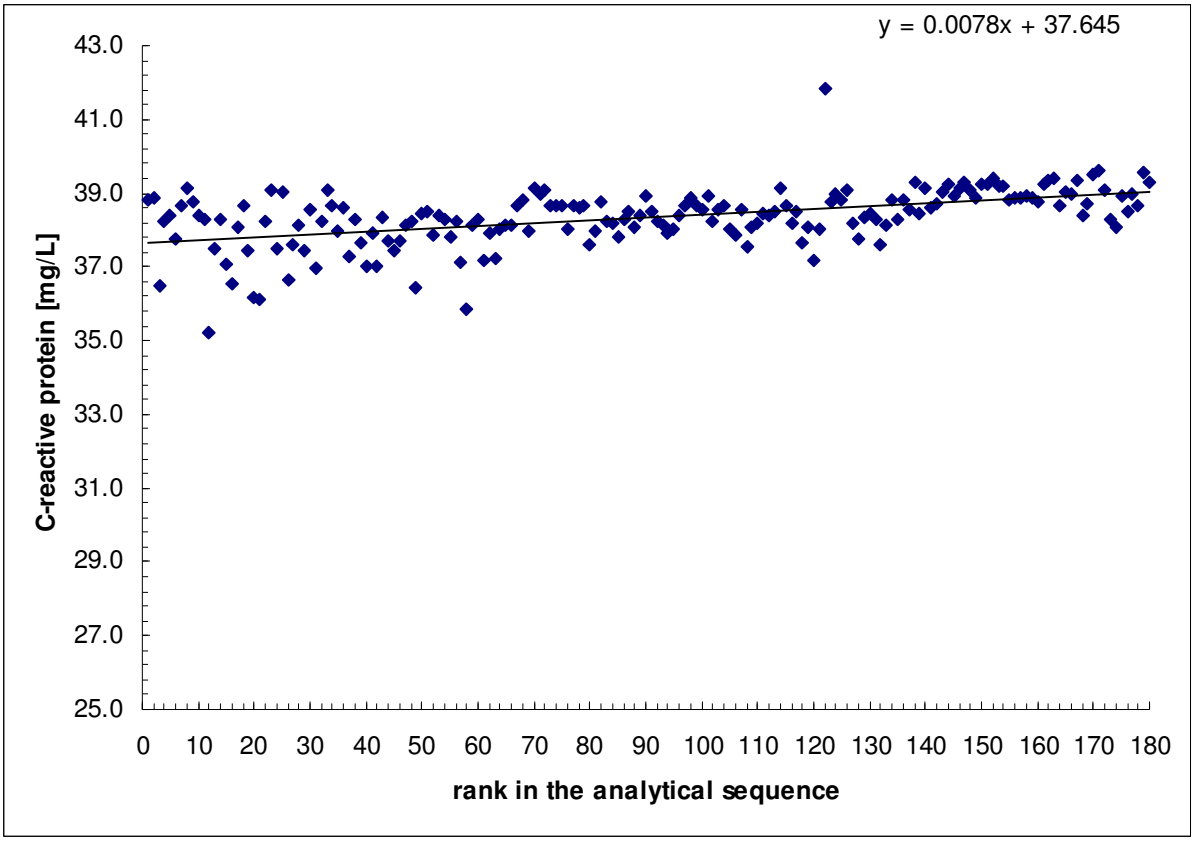
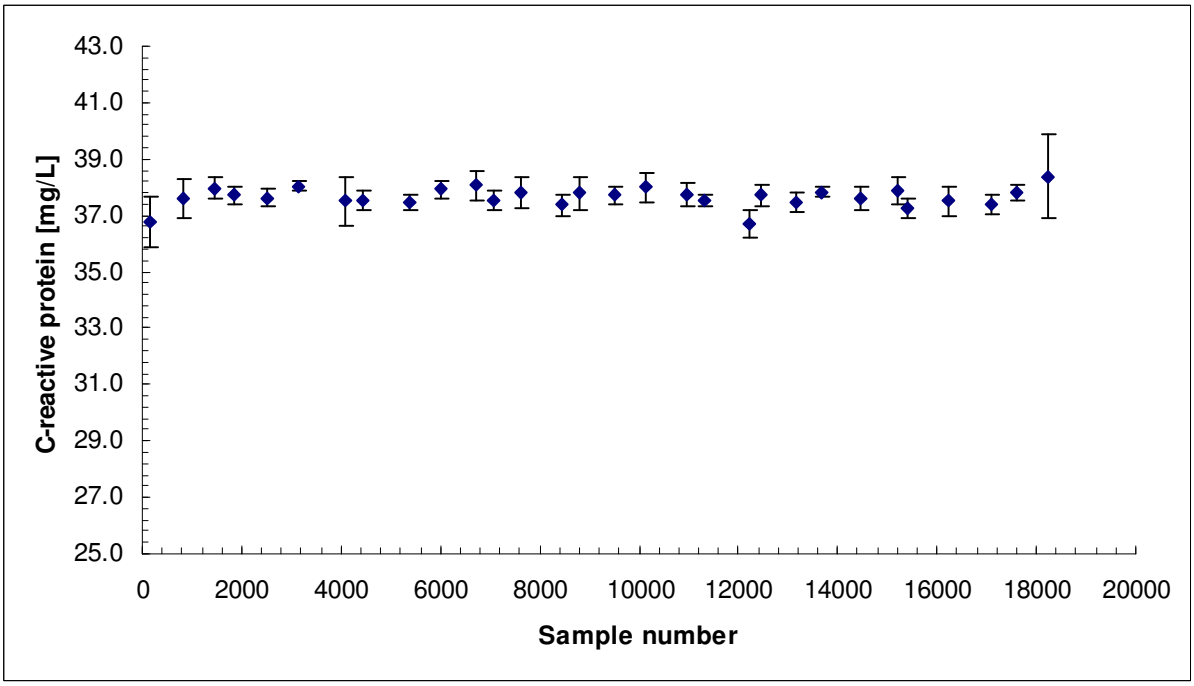


Figure A-2: Homogeneity for CRP in ERM-DA474/IFCC. The bars are the standard deviation for the five replicates.



Annex B: Results of the short-term stability measurements

Figure B-1: Regression of the results in the analytical sequence order

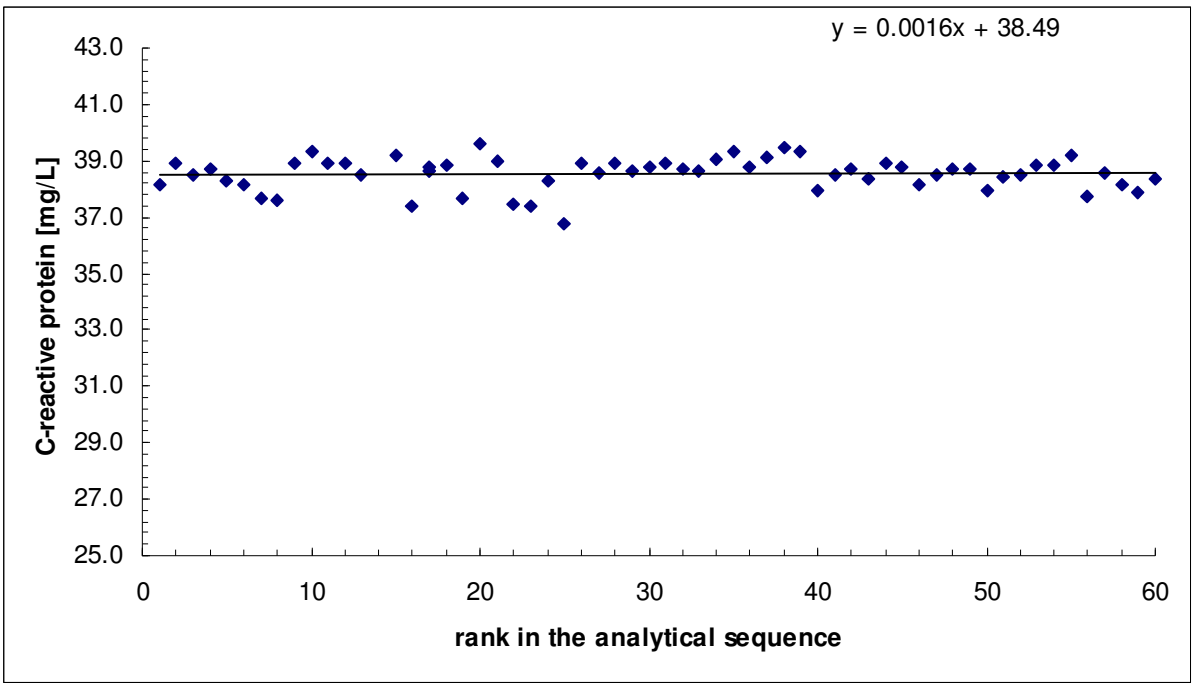


Figure B-2: Short-term stability for CRP in ERM-DA474/IFCC at 18 °C

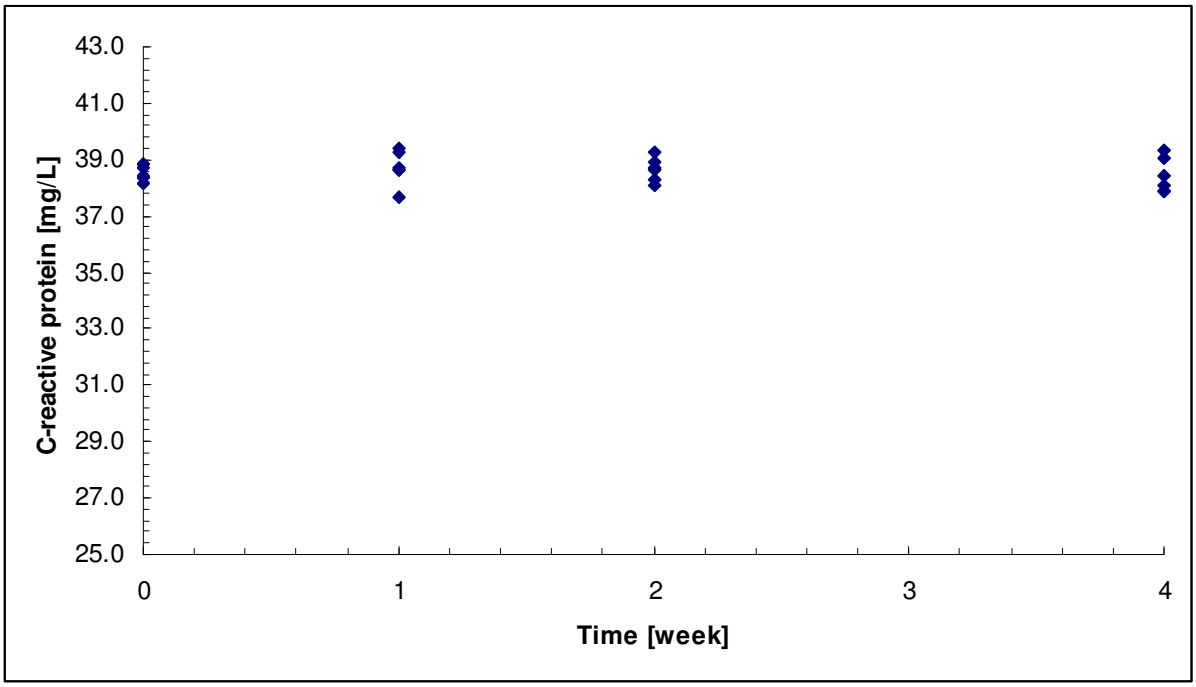


Figure B-3: Short-term stability for CRP in ERM-DA474/IFCC at 4 °C

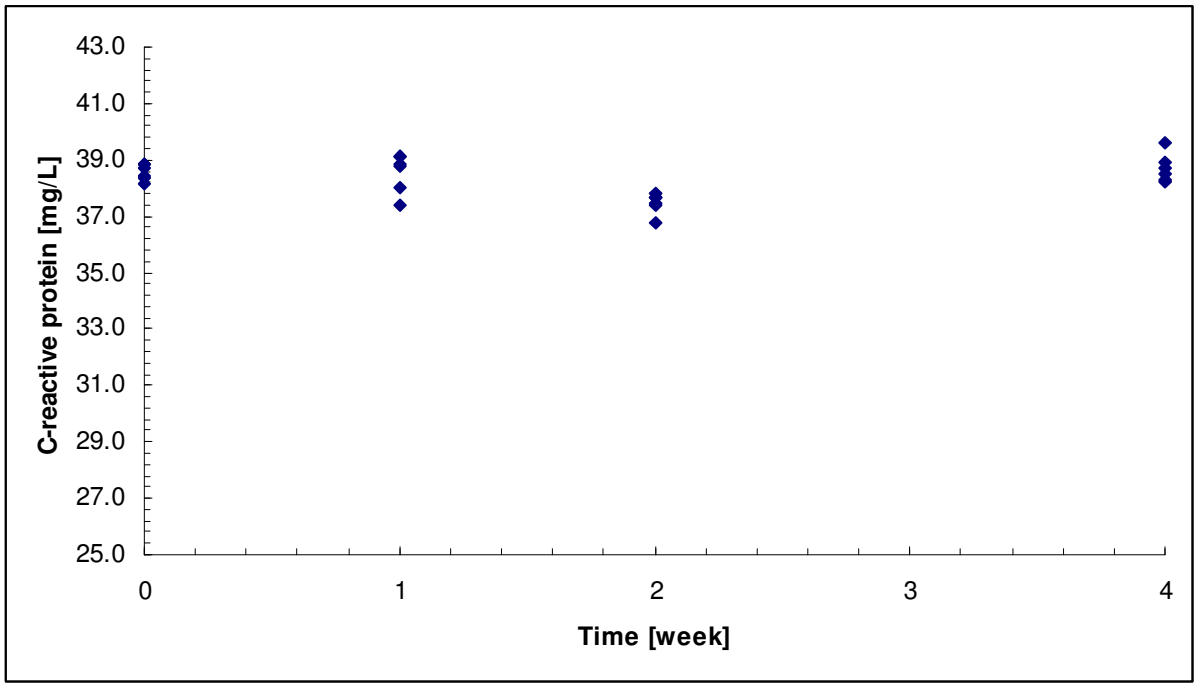
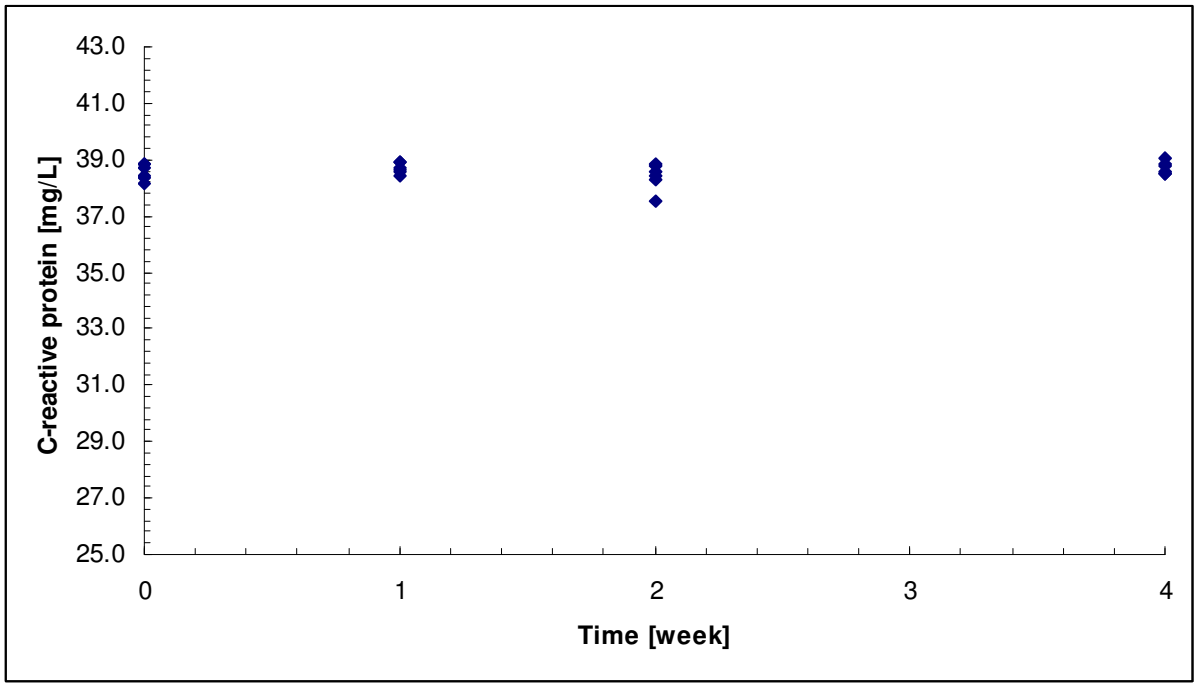


Figure B-4: Short-term stability for CRP in ERM-DA474/IFCC at -20 °C



Annex C: Results of the long-term stability measurements

Figure C-1: Regression of the results in the analytical sequence order

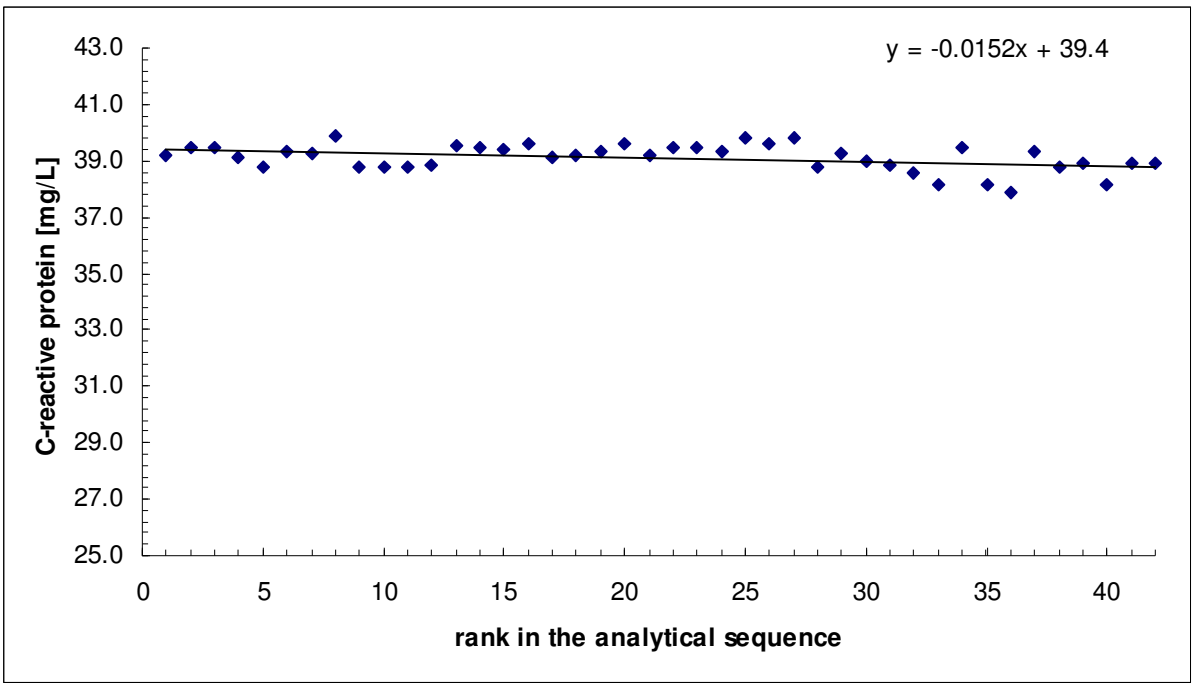


Figure C-2: Long-term stability for CRP in ERM-DA474/IFCC at -20 °C

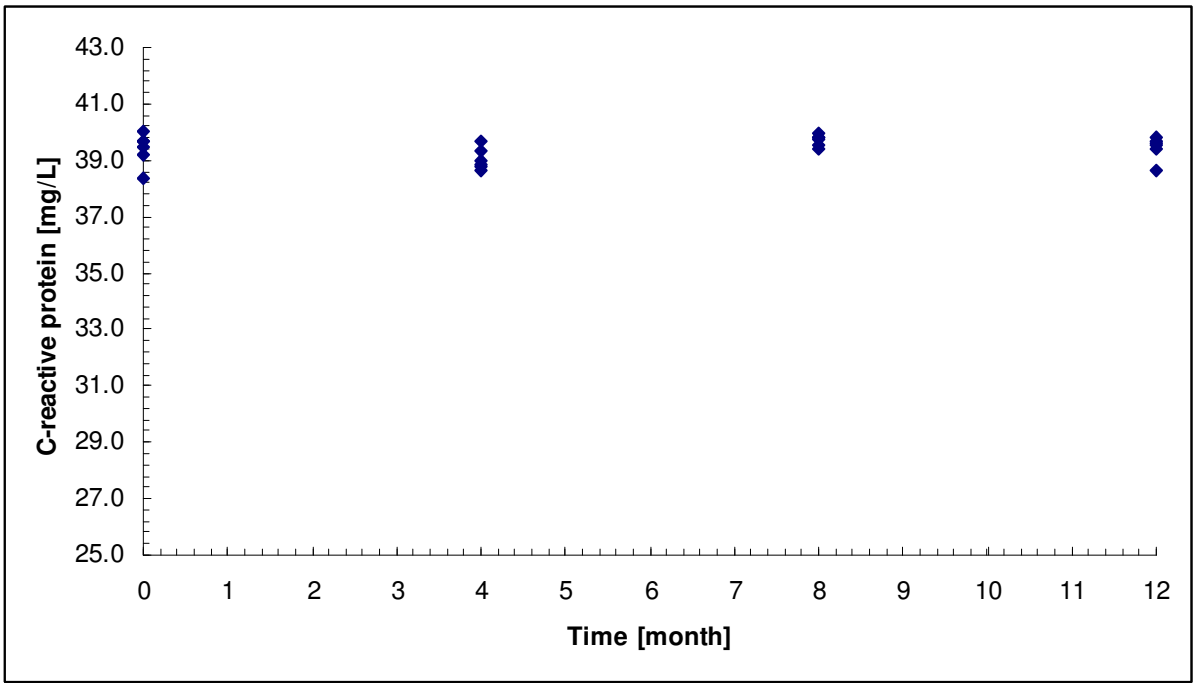
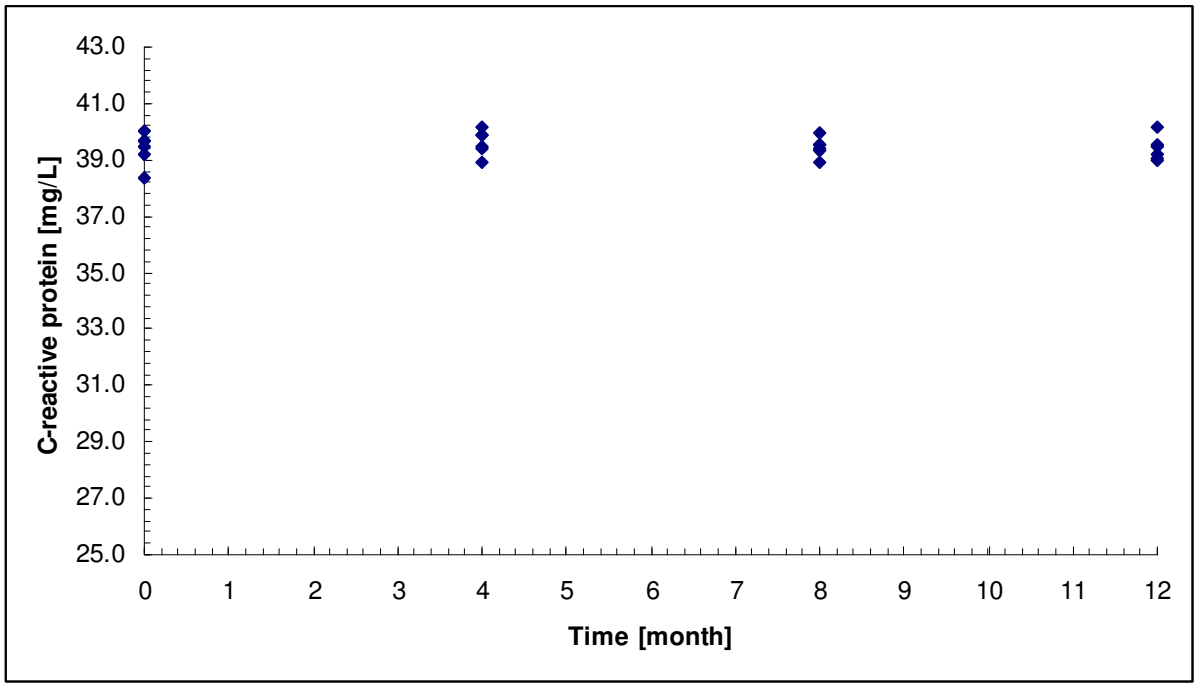


Figure C-3: Long-term stability for CRP in ERM-DA474/IFCC at -70 °C



Annex D: Summary of methods used in the characterisation

Table D-1: Summary of the measurement parameters used by the participating laboratories

Dataset	Laboratory	Method	Instrument	Dilution Buffer	Antibody	Reagent
1	L1	immuno-turbidimetry	Hitachi 917	DAKO S2005	Rabbit anti-human CRP	DAKO S2011
2	L2	immuno-nephelometry	Siemens BN II™	N-Diluent	mouse monoclonal anti-human CRP	*Cardio* Phase *hs* CRP
3	L2	immuno-nephelometry	Siemens BN Prospec	N-Diluent	mouse monoclonal anti-human CRP	*Cardio* Phase *hs* CRP
4	L2	immuno-nephelometry	Siemens Dimension Vista®	N-Diluent	mouse monoclonal anti-human CRP	Flex® reagent hs CRP
5	L3	immuno-turbidimetry	Roche Integra 800	Saline	"Test-kit" 20764930-322	"Test-kit" 20764930-322
6	L3	immuno-turbidimetry	Roche Cobas c501	Saline	"Test-kit" 04956842-190	"Test-kit" 04956842-190
7	L4	immuno-turbidimetry	Abbott Architect c16000	Saline	CRP ultra reagent 2	CRP ultra reagent 1
8	L5	immuno-turbidimetry	Beckman Synchron	Saline	BCI 378020	BCI 378020

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

Dataset	Laboratory	Day	Run 1	Run 2	Run 3
1	L1	1	40.3	40.1	
		2	39.7	39.6	39.2
		3	38.9	40.1	39.5
		4	39.1	39.5	39.7
2	L2	1	40.4	38.8	40.4
		2	39.8	41.0	39.0
		3	41.1	41.7	40.6
		4	39.0	39.5	42.0
3	L2	1	40.1	39.6	39.4
		2	41.0	40.6	40.0
		3	40.5	40.5	40.7
		4	40.7	40.4	40.9
4	L2	1	41.2	41.6	41.5
		2	39.3	39.9	41.4
		3	41.2	42.2	41.5
		4	41.9	41.9	41.4
6	L3	1			
		2	44.6		41.6
		3	43.2		44.3
		4	44.1	45.7	
7	L4	1	43.7	43.1	43.5
		2	40.7		40.7
		3			
		4	40.2	40.8	40.9

European Commission

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Abstract

This report describes the production of ERM-DA474/IFCC, a human serum material certified for C-reactive protein mass concentration. The material has been produced following ISO Guide 34:2009 [1].
Serum was produced from blood collected in 4 blood collection centres according to a procedure ensuring that it was obtained from healthy donors, and that the lipid content of the
serum was low. The serum was processed, spiked with C-reactive protein, ampouled and frozen.
Between unit-heterogeneity has been quantified and stability during dispatch and storage have been assessed in accordance with ISO Guide 35:2006 [2].
The material was characterised by an intercomparison among laboratories of demonstrated competence. Technically invalid results were removed but no outlier was eliminated on statistical grounds only.
Uncertainties of the certified value were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) [3] and include uncertainties related to possible heterogeneity and instability and to characterisation.
The material is intended for the calibration of immunoassays. As any reference material, it can also be used for control charts or validation studies. The CRM is available in glass ampoules containing 1 mL of human serum closed under argon atmosphere. The minimum amount of sample to be used is 20 µL.
The CRM has been accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium

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