CERTIFICATION REPORT

Certification of C-reactive protein in reference material ERM®-DA472/IFCC

Certified Reference Material ERM®-DA472/IFCC

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ERM
European Reference Materials

CERTIFICATION REPORT

Certification of C-reactive protein in reference material ERM®-DA472/IFCC

Certified Reference Materials ERM®-DA472/IFCC

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Abstract

The production and certification of ERM-DA472/IFCC, a new reference material certified for C-reactive protein (CRP), is described.

Serum was produced from blood collected in 6 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 in 5 batches, and then pooled, spiked with β_2 microglobulin (B2M) and CRP and filled into ampoules (approximately 1 mL serum per ampoule). The serum was stored at -70 °C. It was verified that the material was homogenous and stable.

ERM-DA472/IFCC was characterised using the reference material ERM-DA470 as calibrant. This was achieved using a value transfer protocol that can be considered as a reference procedure. The principles used to measure the CRP concentration were immunonephelometry and immunoturbidimetry. The measurements were performed with different platform/reagent combinations (Abbott Architect, Beckman Immage, BN II, different Hitachi instruments, and Olympus AU640). In total 8 laboratories participated in the value assignment. The certified CRP mass concentration is 41.8 mg/L, the expanded uncertainty (k = 2) 2.5 mg/L.

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GLOSSARY

ANOVA	analysis of variance
b	slope in the equation of linear regression $y = a + bx$
Bit	unit of the analog-to-digital converter of the light detector
c	mass concentration c = m / V (mass / volume), the unit is g/L
B2M	β_2 microglobulin
C3c	complement 3c
C3	complement 3
CASO	Casein Soy
CRM	Certified Reference Material
CRP	C-reactive protein
ERM	European Reference Material
HBV	hepatitis B virus
HBsAg	hepatitis B surface antigen
HCV	hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IRMM	Institute for Reference Materials and Measurements
IU	International Unit
IVD	In Vitro Diagnostics
k	coverage factor
KIU	Kallikrein Inhibitor Unit
m	mass
MS_{bb}	mean sum of squares between bottles
MS_{wb}	mean sum of squares within bottles
n	number of replicates
ND	not detectable
PAGE	polyacrylamide gel electrophoresis
RM	reference material
RF	rheumatoid factor
R^2	correlation coefficient of the linear regression
s	standard deviation
s_{bb}	standard deviation between bottles
$s_{bb, rel}$	relative s_{bb}
SDS	sodium dodecyl sulfate
s_{wb}	standard deviation within bottles
T	target material
TF	transfer factor
TRIS	tris(hydroxymethyl)aminomethane
U	expanded uncertainty
u_b	standard uncertainty of the slope b
$u_{bb, rel}$	relative standard uncertainty related to the between-bottle heterogeneity
u^*_{bb}	standard uncertainty related to the between-bottle heterogeneity that can be hidden by the method repeatability
$u^*_{bb, rel}$	relative u^*_{bb}
$u_{c, rel}$	combined relative standard uncertainty
$u_{cal, rel}$	relative standard uncertainty of the calibrant
$u_{char, rel}$	relative standard uncertainty related to the characterisation
$u_{lts, rel}$	relative standard uncertainty related to the long-term stability of the material
$u_{sts, rel}$	relative standard uncertainty related to the short-term stability of the material

1 Introduction and design of the project

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 [1]. 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 [2]. In the blood of healthy young adult volunteer 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 [1]. The finding that modest but persistently increased values are associated with a long-term risk for coronary heart disease [3] 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 antigens (serum proteins) 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, complex matrix effects, etc. The quantification with immunoassays is therefore entirely dependent on the comparison of the results with those obtained with a calibrant [4].

The EU Directive on In Vitro Diagnostic Medical Devices (IVD-MD) (Directive 98/79/EC) 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 re-evaluated and renamed to ERM-DA470), certified for 15 proteins [5]. 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 [6], [7].

The present material has been produced to replace ERM-DA470 as far as CRP is concerned. The material ERM-DA470k/IFCC (available from IRMM) replaces ERM-DA470 for 12 other proteins, but it was not suited for the certification of CRP because of a bias in the CRP values introduced by the lyophilisation process.

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 samples (commutability)

- the concentration of the proteins 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
- there must be continuity of the measurement results from assays calibrated against consecutive reference materials. This is an important issue in clinical chemistry, as the use of reference ranges and decision limits requires that measurement results are comparable over longer time scales

Therefore it was decided to produce the new material according to procedures similar to those applied for ERM-DA470. However, the ERM-DA472/IFCC material for CRP was stored liquid frozen instead of lyophilised, ensuring that the CRP is present in its native pentameric state.

1.3 Design of the project

The material used for this reference material is the same as that used for producing ERM-DA470k/IFCC [5]. The processing is the same up to the point of either filling in vials (ERM-DA470k/IFCC) or ampouling (this material).

Serum was produced from blood collected in 6 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 in 5 batches, and then pooled, spiked with B2M and CRP. The serum was filled in ampoules, which were afterwards stored at -70 °C.

The material was characterised by using the reference material (ERM-DA470) as calibrant. This was achieved using a value transfer protocol that can be considered as a reference procedure [8]. In the so called closed value transfer procedure 6 dilutions of the calibrant (ERM-DA470) and 6 dilutions of ERM-DA472/IFCC are measured against the usual instrument calibrant, and the concentration of the target material is determined from the ratio of the slopes of the regression lines (concentration versus dilution). In the open value transfer procedure, ERM-DA470 is used as a calibrant in the instrument, and the 6 dilutions of the target material are measured directly against different dilutions of that calibrant.

The techniques used to measure the protein concentrations were nephelometry and turbidimetry. The measurements were performed with different platforms (i.e. instruments; Abbott Architect, Beckman Immage, BN II, different Hitachi instruments, Olympus AU640, Roche Integra) and reagents. In total 8 laboratories participated in the value assignment, 6 using open value transfer procedures and 2 using closed value transfer. The majority of the characterisation measurements were either done under an ISO/IEC 17025 accreditation or within the scope of an ISO 13485 quality system. Documented evidence of the technical competence and applied document control was obtained from the remaining laboratories.

2 List of participants

Provision of serum and proteins

- Asahikawa Medical College, Asahikawa (JP)
- Azienda Ospidaliera Policlinico Modena, Modena (IT)
- Blutspendedienst SRK Bern, Bern (CH)
- Centre de Transfusion Sanguine Liège, Service du Sang de la Croix-Rouge de Belgique, Liege (BE) (accred. ISO 15189 BELAC 331-MED)
- Centre for Amyloidosis and Acute Phase Protein, UCL Division of Medicine, London (GB)
- Dade Behring Marburg GmbH - A Siemens Company, Marburg (DE)
- Hralec Králové University Hospital, Hralec Králové (CZ)
- Universita degli Studi di Pavia, Pavia (IT)

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- Institute for Reference Materials and Measurements (IRMM), Joint Research Centre, European Commission, Geel (BE) (Accred. ISO Guide 34 BELAC 268-TEST)

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- Nitto Boseki, Fukushima (JP) (certified ISO 13485 SGS GB06/68575)
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- Protein Reference Unit, St. Georges Hospital, London (GB)
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Data analysis

- Acomed Statistik, Leipzig (DE)
- Institute for Reference Materials and Measurements (IRMM), Joint Research Centre, European Commission, Geel (BE) (Accred. ISO Guide 34 BELAC 268-TEST)

3 Processing

3.1 Preparation of the serum

The starting material for the reference material was the serum prepared from the blood of healthy donors, collected at 6 different blood collection centres (Table 1).

Selection of the donors: Donors with clinical diagnoses of diabetes mellitus, jaundice, high blood pressure, heart, lung or kidney disease, pregnant women and top performance athletes were excluded. Donations with high cholesterol or triglyceride concentrations were rejected. Donations from people with hepatitis and human immunodeficiency virus (HIV) infections were excluded, as well as donors with clinical evidence of bacterial infections.

Serum collection protocol: 400 – 500 mL whole blood was withdrawn from each donor, expected to give an average serum volume of 180 mL (140 – 220 mL). For donors from Japan the volume of blood withdrawn was between 200 and 300 mL.

The following protocol was applied for preparing the serum:

a) Pre-prandial blood (tourniquet used) (the blood collected in Germany was not strictly pre-prandial, but a very stringent selection was made on the transparency of the serum).
b) Collection into a dry bag or a polymer bottle, not containing any additives or anticoagulants (such as Baxter, Fenwal).
c) Clotting at room temperature for 3 - 4 hours.
d) Centrifugation of the bag in a centrifuge bucket or the bottle at 2200 *g* for 20 min
e) Separation of the serum from the clot
f) Freezing of the serum between -70 and -80 °C.
g) Transport of the serum to the IRMM on dry ice, storage at -70 °C. The serum produced in the Czech Republic was stored for a few days at -30 °C before being shipped on dry ice.

Table 1: Summary information on the collected serum. The parenthesised numbers indicate how many male and female donations are fulfilling the inclusion criteria.

Collection centre	Donation period	No.	Male	Female	RF > 30 IU/mL	Monoclonal components	Final No.
Blutspendedienst SRK Bern AG (CH)	12/2006	80	78 (73)	2 (1)	3	3	74
Hradec Králové University Hospital (CZ)	11-12/2006	80	64 (62)*	16 (16)	0	1	78
Croix Rouge de Belgique, Liège (BE)	10/2006 to 01/2007	101	58 (57)	43 (42)	0	2	99
Dade Behring Marburg GmbH (DE)	10/2006	60	46 (44)	14 (13)	3	0	57
Asahikawa Medical College (JP)	09/2006 to 01/2007	26	14 (13)	12 (11)	2	0	24
Policlinico Modena (IT)	12/2006 to 02/2007	60	46 (45)	14 (13)	2	0	58

* one donation lost due to a leaking container

Tests of the serum: The blood collection centres tested each donation for HIV 1+2, HBV or HBsAg, and HCV. Monoclonal gammopathies were detected by gel electrophoresis. Six donations were discarded because of the presence of monoclonal components, and ten donations were discarded because their Rheumatoid Factor (RF) concentration was above 30 IU/mL (Table 1). 390 donations, of which 96 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 is summarised in Figure 1. It followed largely that used for CRM 470 (ERM-DA470) [5]. The major differences were that after the delipidation step a diafiltration with a membrane with a cut-off of 10 kDa was used instead of a dialysis with membranes with a cut-off of 12-14 kDa, that no sodium azide was added to the serum before the pooling of the individual donations, and that the final sodium azide mass concentration was kept below 1 g/L.

The serum donations from each collection centre were pooled and processed as collection centre pools. Particulate matter and lipids were removed, and a uniform degree of 'maturation' of the serum achieved. As the batch from Japan was small it was processed together with the serum from Belgium. The serum from different collection centres was then pooled, spiked with B2M and CRP and sterile filtrated after addition of preservatives (sodium azide, aprotinin and benzamidine hydrochloride), before the filling.

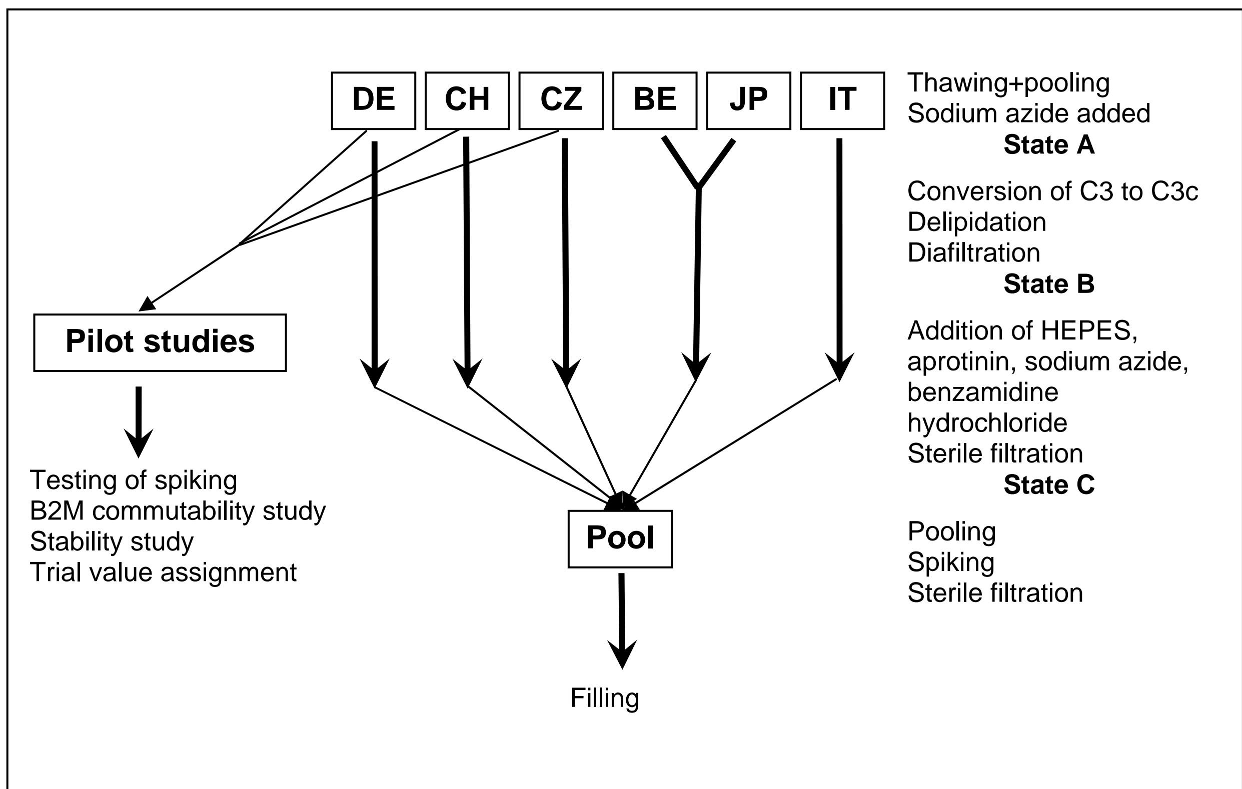

Figure 1: Overview of the processing of the serum.

3.2.1 Preparation of pools per collection centre

Donations fulfilling the criteria were processed as follows:

- thawing overnight at 2 - 8 °C
- determination of the volume of each donation
- pooling of all accepted donations of the same collection centre
- addition of sodium azide (Merck, Darmstadt, DE) to a final amount of substance concentration of 7.7 mmol/L (approximately 0.5 g/L)
- removal of $1/8^{th}$ of the volume of each pool for verification and testing purposes and storage in 50 mL plastic tubes at a temperature below -70 °C
- removal of aliquots for the measurement of cholesterol, triglycerides, apolipoprotein A-I, apolipoprotein B, total protein (Biuret reaction) and for the concentration 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 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Calbiochem, Merck Biosciences, Schwalbach, DE)
- addition of magnesium acetate tetrahydrate, final amount of substance concentration 20 mmol/L (Merck, Darmstadt, DE)
- adjustment of the solution to 37 °C
- addition of Inulin (Sigma Aldrich, Milwaukee, US) to a final mass concentration of 0.2 %
- stirring for 2 hours at 37 °C.

Delipidation with Aerosil

- adjustment of the inulin-treated pool to pH 8.5 ± 0.1 with a saturated tris(hydroxymethyl)aminomethane (TRIS) solution (Sigma-Aldrich, Milwaukee, USA)
- addition of sodium chloride (crystalline; Merck, Darmstadt, DE) to a final concentration of 50 g/L while stirring constantly
- calculation of the required amount of Aerosil 200 (Degussa, Frankfurt, DE) based on the total protein concentration of the pool before the C3 conversion (430 mg aerosil / 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 min
- removal of Aerosil (Degussa, DE) and protein precipitates by centrifugation for 30 min at approximately 10000 *g* using 500 mL centrifugation bottles
- determination of the total volume of the clear supernatant
- determination of the concentrations of cholesterol, triglycerides and apolipoproteins A-I and B to verify the successful lipid removal.

Sterile filtration and diafiltration

- filtration of the supernatant using a 0.45 µm Sartobran P filter (Sartorius, Göttingen, DE) to remove small particles interfering with the following diafiltration
- diafiltration (Centrasette with Omega membrane with a molecular weight cut-off of 10000; Pall, Hauppauge, USA) using an isotonic sodium chloride solution to remove TRIS and Aerosil-derived free silica; this step combines buffer exchange and adjustment to the desired volume
- removal of aliquots for the measurement of the total protein mass concentration and for the measurement and physicochemical analysis of selected proteins.

Material is now in State B

Preservation and sterile filtration

- adjustment of the pH with a saturated HEPES solution to 7.2 ± 0.1
- addition of sodium azide to achieve a final amount of substance concentration of 14.6 mmol/L (0.95 g/L)
- addition of aprotinin (Trasylol 500000 KIU/L, Bayer, Leverkusen, DE) to achieve a final concentration of 80000 KIU/L
- addition of benzamidine hydrochloride monohydrate (Merck, Darmstadt, DE) to a final amount of substance concentration of 1 mmol/L
- sterile filtration using a Sartopore 2 filter (0.2 µm) (Sartorius, Göttingen, DE)
- sterile removal of aliquots for the measurement of the total protein content and for the measurement and physicochemical analysis of selected proteins
- sterile removal of 150 mL of the DE, CZ and CH pools; these aliquots were stored below -70 °C until processing of the pilot batches.

The material is now in State C.

Until their combination the collection centre pools were stored at temperatures below -70 °C.

Protein concentration measurements: Throughout the processing, the mass concentrations of the proteins certified in ERM-DA470k/IFCC and CRP, as well as of apolipoprotein A-I and B were determined by immunonephelometry on a BN ProSpec or BN II System using reagents, standards and controls of Dade Behring Marburg GmbH.

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

Lipids: The cholesterol concentration 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 platform concerned an empty cuvette is considered adequately inserted and optically clear if the scattered light gives a signal between 20 and 600 Bit.

Physicochemical structure of CRP: The integrity (oligomeric state, behaviour in terms of charge/size in electrophoretic mobility) of the main serum proteins during processing was verified using several methods:

- 2-dimensional immunoelectrophoresis according to Clarke and Freeman [9]
- native PAGE followed by western blotting (CRP only)
- size exclusion chromatography followed by SDS PAGE and Western blotting (CRP only)
- 2-dimensional electrophoresis.

3.2.2 Preparation of the combined pool

Preparation of CRP and B2M

463 mL of a purified 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 any turbulence. The starting material for B2M was a lyophilised purified recombinant protein.

Preparation of the final material

- thawing of all 5 collection centre pools (2 to 3 days)
- pooling

- sterile removal of aliquots for the measurement of B2M and CRP concentrations, and for the physicochemical analysis
- preparation of the CRP solution as described
- slow addition of the total amount of the CRP solution (463 mL) while constantly stirring
- reconstitution of recombinant B2M in sterile water
- addition of B2M to the pool to a final mass concentration of approximately 2.6 mg/L (total added 65 mg)
- 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

The same combined pool was used for preparing the lyophilised ERM-DA470k/IFCC and the liquid frozen ERM-DA472/IFCC.

3.2.3 Processing control

Pools per collection centre

Total protein concentration: In line with the protocol of 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 processing, and the Aerosil treated pools should be adjusted to between 55 and 75 g/L. As shown in Table 2, the total protein mass concentration of the pools was between 69 and 77 g/L in State A and between 64 and 69 g/L in State C, i.e. within the defined tolerance limits.

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

<table>
<tr><th></th><th>State A</th><th>State B</th><th>State C</th></tr>
<tr><th>Collection centre pool</th><th>Total protein [g/L)]</th><th>Total protein [g/L]</th><th>Total protein [g/L]</th></tr>
<tr><td>DE</td><td>69.0</td><td>68.4</td><td>65.8</td></tr>
<tr><td>CZ</td><td>72.0</td><td>68.1</td><td>69.2</td></tr>
<tr><td>CH</td><td>77.3</td><td>67.7</td><td>68.8</td></tr>
<tr><td>BE</td><td>69.9</td><td rowspan="2">66.9</td><td rowspan="2">63.8</td></tr>
<tr><td>JP</td><td>73.7</td></tr>
<tr><td>IT</td><td>71.1</td><td>70.9</td><td>69.4</td></tr>
</table>

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 all collection centre pools. The reduction of the blank value obtained by measuring the signal of undiluted sample using a BN100 System also demonstrated the effective removal of lipoproteins (Table 4).

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

<table>
<tr><th></th><th colspan="4">State A</th><th colspan="4">State B</th></tr>
<tr><th>Pool</th><th>Chole-sterol [mg/L]</th><th>Trigly-cerides [mg/L]</th><th>Apo A-I [mg/L]</th><th>Apo B [mg/L]</th><th>Chole-sterol [mg/L]</th><th>Trigly-cerides [mg/L]</th><th>Apo A-I [mg/L]</th><th>Apo B [mg/L]</th></tr>
<tr><td>DE</td><td>1627</td><td>1189</td><td>1555</td><td>806</td><td>ND</td><td>95.5</td><td>ND</td><td>ND</td></tr>
<tr><td>CZ</td><td>1788</td><td>2266</td><td>1565</td><td>931</td><td>ND</td><td>56.4</td><td>ND</td><td>ND</td></tr>
<tr><td>CH</td><td>1760</td><td>920</td><td>1715</td><td>874</td><td>11.5</td><td>95.5</td><td>ND</td><td>ND</td></tr>
<tr><td>BE</td><td>1903</td><td>767</td><td>1620</td><td>870</td><td rowspan="2">ND</td><td rowspan="2">8.3</td><td rowspan="2">ND</td><td rowspan="2">ND</td></tr>
<tr><td>JP</td><td>1875</td><td>1158</td><td>1595</td><td>783</td></tr>
<tr><td>IT</td><td>1846</td><td>725</td><td>1645</td><td>842</td><td>5.8</td><td>8.3</td><td>ND</td><td>ND</td></tr>
</table>

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

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

<table>
<tr><th></th><th colspan="2">Blank value [Bit]</th></tr>
<tr><th>Pool</th><th>State A</th><th>State C</th></tr>
<tr><td>DE</td><td>3497</td><td>55</td></tr>
<tr><td>CZ</td><td>1992</td><td>75</td></tr>
<tr><td>CH</td><td>3082</td><td>103</td></tr>
<tr><td>BE</td><td>1553</td><td rowspan="2">99</td></tr>
<tr><td>JP</td><td>>[2]</td></tr>
<tr><td>IT</td><td>2498</td><td>101</td></tr>
</table>

[1] exceeds measuring interval (0-4096 Bit)

Preparation of the combined pool

Sterility test: The sterility of the material was verified by inoculating Casein Soy (CASO) Bean 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.

Mass density measurements: The mass density of ERM-DA472/IFCC and reconstituted ERM-DA470 were measured with an Anton Paar densitometer (Graz, AT), and found to be 1.0236 and 1.0234 g/mL, respectively, at 20 °C. As the difference between the densities of the two materials is negligible compared to the uncertainty of the certified value no systematic density correction was applied to the transfer factors.

3.3 Filling and labelling

The serum was ampouled at the IRMM. Approximately 1 mL serum was filled into 3 mL Duran glass ampoules using a Rota ampouling machine R 910 PA (Rota, Wehr, DE). The ampoules were first opened, filled, 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. Teflon tubing was used. The ampouling machine was adapted to minimise the formation of foam in the ampoules. After filling, the ampoules were kept at + 4 °C and labelled according to the filling order. Thereafter the material was frozen at -70 °C.

4 Homogeneity

4.1 Between and within vial homogeneity

The homogeneity of ERM-DA472/IFCC was verified by measuring CRP in 15 ampoules randomly selected from the whole batch. The CRP mass concentration was measured 6 times in each ampoule using a BN ProSpec platform and Dade Behring reagents.

Grubbs tests were performed to detect outlying individual values as well as averages measured for each vial. No outliers were detected at the 95 % confidence level.

Regression analyses were used to evaluate potential drifts in values related to the analysis sequence or to the filling sequence. It was furthermore checked whether the data followed a normal or at least unimodal distribution using normal probability plots and histograms respectively. The individual data and the averages were normally distributed. A small but significant (at the 95 % confidence level) drift in function of the analytical sequence was detected, but not corrected for, as it was small compared to the overall uncertainty.

One-way ANOVA statistics showed that there was no significant heterogeneity at the 95 % confidence level (F-test). The between bottle standard deviation (s_{bb}) and the maximum standard uncertainty related to the inhomogeneity that can be hidden by the method repeatability (u^*_{bb}), were calculated using the formulas:

$$s_{\text{bb}} = \sqrt{\frac{MS_{\text{bb}} - MS_{\text{wb}}}{n}} \qquad u^*_{\text{bb}} = \sqrt{\frac{MS_{\text{wb}}}{n}} \cdot \sqrt[4]{\frac{2}{df_{wb}}}$$

(MS_{bb} = mean sum of squares between bottles; MS_{wb} = mean sum of squares within bottles; n = number of replicates; df_{wb} = degrees of freedom within bottles). The values were converted into relative uncertainties.

The $s_{bb, rel}$, which is the highest of the two values $s_{bb, rel}$ = 0.290 % and $u_{bb, rel}^*$ = 0.274 %, was used as an estimate of the uncertainty related to inhomogeneity, and was included into the calculation of the overall uncertainty of the certified values (Section 7.1).

4.2 Minimum sample intake for analysis

The material forms a clear solution. Therefore it is not expected to have any relevant heterogeneity in the CRP concentration at sample intakes even below nL volumes. The sample intake of the homogeneity study on the material measured with a BN ProSpec platform was 20 µL. The standard deviation within a bottle (s_{wb}) is lower or equal to the expected method variability (data not given), so there is no indication of intrinsic heterogeneity at a sample intake of 20 µL.

5 Stability

Short and long-term stability studies were carried out using an isochronous set-up [10] that consists of the simultaneous analysis of samples from reference and test ampoules. For each study a defined set of ampoules was exposed for different periods of time to elevated temperatures and then brought back to the reference temperature. At the end of the study all ampoules were analysed for the concentrations of CRP within one analytical run, in triplicate. The data were analysed by determining the regression line for the CRP concentration in function of time [10].

5.1 Short-term stability

A short-term stability study was performed in order to assess the possible effect of transport at different temperatures on the stability of the material. Test ampoules were kept for 0, 1, 2, and 4 weeks at -20, 4, 18 and 60 °C before being brought back to the reference temperature (-70 °C in the short term stability study). For each combination of time and temperature 2 ampoules were analysed in triplicate. The CRP concentration was measured by turbidimetry using a Hitachi 917 measuring system and DAKO reagents.

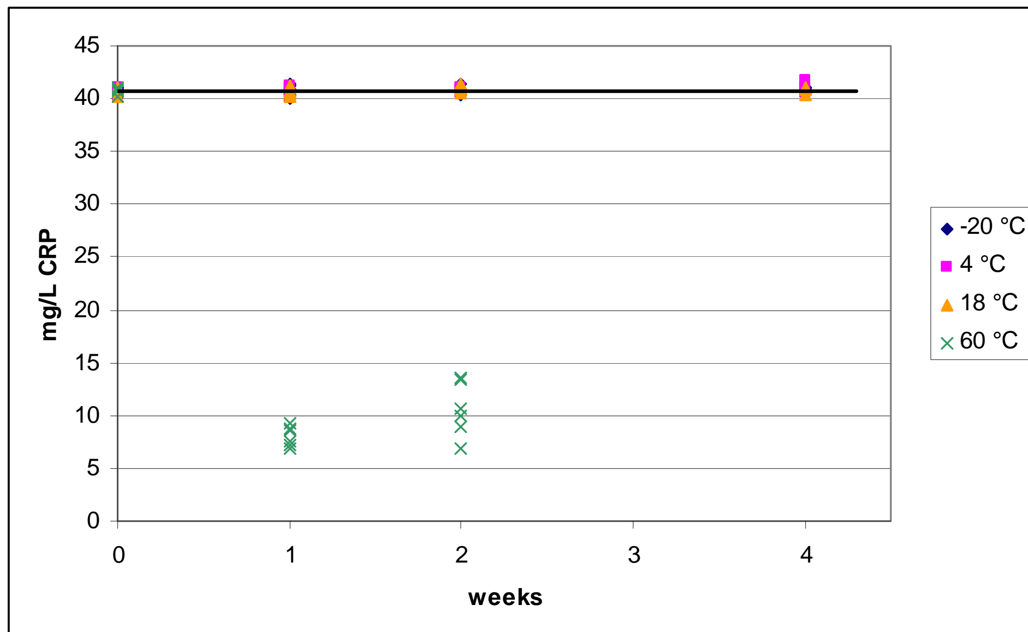

Figure 2: Results of the short-term stability study. The trendline for the CRP concentration in function of storage time is shown for samples stored at -20 °C.

Table 5: Short-term stability study: slope (*b*), test for significance of the slope ($|b/u_b|$), and relative standard uncertainty after two weeks storage at the specified temperature ($u_{sts, rel}$)

Temperature [°C]	b [(mg/L)/week]	$\|b/u_b\|$	$u_{sts, rel}$ [%]
-20	0.011	0.22	0.24
4	0.061	0.13	0.24
18	0.029	0.74	0.19
60	-15.1	2.6	92

For ampoules stored at -20, 4 and 18 °C there were 24 measurements, or 22 degrees of freedom for the linear regression. The slope of the CRP concentration in function of time is significantly different from 0 when the absolute value of slope b divided by its uncertainty u_b ($|b/u_b|$) is larger than $t_{0.05, 22}$ = 2.07. When ampoules were kept at -20, 4, and 18 °C none of

the slopes was significantly different from 0. At 60 °C the solution became turbid with a precipitate within one week, and the CRP concentration dropped significantly (at a 95 % confidence level).

From this study it was concluded that for the shipment (for maximum two weeks) of ampoules at temperatures at or below 4 °C the possible contribution of the transport to the uncertainty of the certified values is negligible compared to the overall uncertainty. For practical reasons the material will be shipped on dry ice.

5.2 Long-term stability

A 6 month stability study was performed in order to confirm the stability of the material upon storage at -70 and -20 °C. The reference temperature was at or below -150 °C (stored above liquid nitrogen). The test ampoules were kept for 0, 2, 4, and 6 months at -70 and -20 °C. For each combination of time and temperature 2 ampoules were analysed 6 times. The CRP concentration was measured by turbidimetry using a Hitachi 917 measuring system and DAKO reagents.

For both test temperatures there were 48 measurements, or 46 degrees of freedom for the linear regression. The slope of the CRP concentration as a function of time is significantly different from 0 when the absolute value of slope b divided by its uncertainty u_b ($|b/u_b|$) is larger than $t_{0.05,\,22} = 2.01$. None of the slopes (CRP mass concentration versus time) was significantly different from 0 (at a 95 % confidence level). The uncertainty given for the long-term stability corresponds to the maximum uncertainty due to instability that could be hidden by the measurement variation after a period of 6 months. The results indicate that the material is stable when stored at either -70 or -20 °C. It was decided to store the material at -70 °C, and the corresponding value of $u_{lts,\,rel}$ was used for the calculation of the uncertainty.

Table 6: Long-term stability study: slope (b), test for significance of the slope ($|b/u_b|$), and relative standard uncertainty contribution $u_{lts,\,rel}$ due to storage for 6 months at the specified temperature

Temperature [°C]	b [(mg/L)/month]	$|b/u_b|$	$u_{lts,\,rel}$ [%]
-70	0.023	0.07	0.468
-20	-0.038	1.20	0.461

6 Characterisation

6.1 General principles

The measurements were calibrated with the matrix certified reference material ERM-DA470 (see Table 7) by value transfer procedures that minimise the effects of calibration curve fitting and matrix differences [8]. The methods used for transferring values are validated, well established routine methods based on turbidimetry and nephelometry. The laboratories used either of two different approaches, called the open and closed value transfer procedures. For both procedures, measurements were performed over 4 days, with independent reconstitution of the reference material and preparation of dilutions on each of the 4 days. All reconstitutions and dilutions were obtained by weighing.

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

Certified value[1)] [mg/L]	U_{CRM}[2)] [mg/L]	Calibrant
39.2	1.9	1st Intl. Std. CRP 85/506

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).

In the open value transfer procedure 6 dilutions of ERM-DA470 were used for calibrating the instrument, and 6 dilutions of ERM-DA472/IFCC were assayed against this calibration. This procedure makes it possible to check for equivalent analytical responses of the materials (in the assays for which they are intended to be used) by assessing the linearity and the intercept of the regression line, as these are indicators for different matrix effects in the two materials.

The open transfer procedure was optimised separately for each platform participating in the value assignment. The dilution scheme was optimised taking into account that dilutions done by the platforms should be avoided as much as possible (as these dilutions can not be corrected by more accurate weighings). On most measurement systems the option exists to provide 'pre-diluted' samples to the instrument, and to switch off the dilutions normally done by the platforms. Further it was required that the interval of concentrations for the dilutions of the calibrant was at least 10 % broader than the expected concentration interval of the dilutions of the candidate reference material at the high and low end of this expected concentration interval. Finally it had also to be taken into account that for each material all 6 dilutions should be prepared from a single vial or ampoule.

In the closed procedure 6 dilutions of ERM-DA470 and 6 dilutions of ERM-DA472/IFCC were assayed against the calibrants normally used by the laboratories. These calibrants mostly have values traceable to ERM-DA470. This procedure has in principle the disadvantage that the precision is lower, and that the proportionality of the materials can not be assessed directly. The presence of a matrix effect in either material may lead to non-linearity of the regression line, and/or to an intercept different from 0. Here again the dilution scheme was optimised for the two platforms, taking into account the working interval of the assays, the requirement that all dilutions should be prepared from a single vial, and that the volumes of the dilutions need to be large enough to allow for all the measurements.

6.2 Performance of the characterisation measurements

The characterisation measurements were done in parallel with those for the certification of 12 proteins in ERM-DA470k/IFCC.

The laboratories were provided with detailed protocols and reporting sheets, as well as with vials of ERM-DA470 and ERM-DA472/IFCC. Laboratories were asked to specify the platform and reagents used, and the order in which the measurements were performed. Both procedures required that the ERM-DA470 was reconstituted the day before the measurements, according to the procedure described in the certificate of ERM-DA470 [5].

For each of the 4 measurement days a new vial of the ERM-DA470 was reconstituted and a new set of 6 dilutions of ERM-DA470 and ERM-DA472/IFCC prepared.

Open value transfer procedure

On each measurement day, 3 runs were performed. Each run was done with new calibrations using 6 dilutions of ERM-DA470. The 6 dilutions of ERM-DA472/IFCC and the control material (which consisted of a separate dilution of ERM-DA470) were measured as samples. Each calibrant, target and control material dilution was measured in duplicate at each run. Laboratory 1 used a different validated procedure. According to its procedure all the dilutions are measured in triplicate in each run, over 3 days, resulting in a slightly higher number of measurements.

Closed value transfer procedure

On each measurement day each of the 6 dilutions of ERM-DA470 and 6 dilutions of ERM-DA472/IFCC were measured in triplicate. The laboratories used the dilution scheme given below. The intended volume fractions of the candidate reference material and the calibrant in the dilution schemes are given as percent of the reconstituted material present in the dilutions.

Dilutions: 40, 50, 60, 70, 80 % of the reconstituted material, as well as the undiluted material.

6.3 Data analysis

Principle of the analysis

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

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

where C_T and C_R are the mass concentration of CRP in the target material (T, ERM-DA472/IFCC) and in the calibrant (RM, ERM-DA470), respectively.

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

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

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

The concentrations of CRP in the dilutions of the target material will be:

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

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

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

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

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

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

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

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

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

For the closed value transfer procedure the dilutions of the RM can not be used directly to calibrate the platform. When the measured signals for the target material

$C_{S,T}(j) = F_T(j) \times C_T$

and for the RM

$C_{S,R}(i) = F_R(i) \times C_R$

are plotted against the dilutions $F_T(j)$ and $F_R(i)$ respectively, the TF = C_T / C_R can be derived as the ratio of the slopes of the respective linear regressions.

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 following mass corrections were applied:

For the reconstitution:

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

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

In the case of predilutions:

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

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

For the dilutions:

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

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

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

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

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

Determination of the transfer factors (TFs)

For the open value transfer procedure the laboratories used the dilutions to construct the calibration curve directly. The values entered into their instrument for the concentrations of these dilutions were the values of the concentrations relative to the concentrations in ERM-DA470, 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 the target material in duplicate. The measured values S_{ijk} (signal of the kth 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 (measured concentration of CRP in a control dilution of ERM-DA470 relative to the concentration in the control sample expected on the basis of the dilution, calculated from the mass values obtained by weighing) is within 1.00 ± 0.05

For the closed value transfer procedure the laboratories measured the 6 dilutions of the target (Y1-Y6) and the reference material (X1-X6) in triplicate on each day. For both the reference and the target materials, single measurement results S_{ijk} (signal of the kth measurement of material i within dilution j, in g/L) were plotted in scatter plots $S_{ijk} = f(c_{ij})$ so as to evaluate outliers. For both the reference and the target material, 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
- testing for the homoscedasticity of the residuals (whether the variances are homogeneous) via a *t*-test
- testing for normality (visual inspection and normal probability plot)
- testing for linearity (visual inspection and evaluation of R^2)
- testing for outliers of the regression model according to the method of Lund [11]
- verification that the intercept ± 4 times the standard deviation covers the origin.

The TFs were calculated as the slope of the linear regression line for the open value transfer procedure, and as the ratio of the slopes of the linear regression lines for the candidate reference material and for the calibrant for the closed value transfer procedure.

The following general acceptance criteria were applied to the datasets:

1. The 95 % confidence intervals of the intercepts of the regressions of the calibrant and the target materials must be overlapping (closed procedure only).
2. R^2 of the regression must be above 0.97 for Immage data and above 0.98 for all other measurement systems
3. Data from at least 4 dilutions must be available
4. 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
5. At least two daily value assignments must be valid
6. 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 [8]):

- Non-overlapping confidence intervals of the intercepts of closed protocol 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 TFs.

- Overall R^2 < 0.98 of regression indicates quality problems in the measurements (scattering, outliers, run-to-run variation). Laboratory 5 worked with an instrument with higher scattering of the data. Therefore the threshold for this laboratory was reduced to 0.97.

- On the basis of criteria 3, 4, 5, and 6, datasets with general quality problems were excluded.

6.4 Results of the characterisation measurements

6.4.1 General considerations

The measurements were performed by 8 laboratories. Of these laboratories 6 used an open value transfer procedure and 2 a closed value transfer procedure (Table 8). They are coded from laboratory 1 to 8.

The requirement that all the dilutions are prepared from a single vial made the volumes of the dilutions available small, and required the optimisation of the dilution scheme for the different platforms, depending on their sample intake. One of the possible problems was the evaporation of samples, which can become significant when small sample volumes are used, and when the samples are stored on the platform for a long time before they are measured (as is the case with slower platforms and when many proteins are measured). This problem was avoided in different ways, for example by measuring fewer proteins per series of dilutions, or capping the samples between measurements. The dilutions have been measured in different orders by different laboratories, and some laboratories have measured in different orders on different days. Overall no significant differences were detected between laboratories running the samples in different orders.

6.4.2 Scrutiny of the data

For all 8 laboratories valid datasets were obtained. Data measured on day 1 by laboratory 6 were not valid because for technical reasons less than 50 % of the measurements had been done (criterion 4). Laboratory 5 only performed three measurement sets, and data measured on day 2 by laboratory 5 was not valid because the R^2 of the regression was below 0.97 (criterion 2). Data from laboratory 7 measured on day 1 were excluded because of non-overlapping confidence intervals of the intercepts (criterion 1).

Table 8 gives the average transfer factors for each laboratory, and Figure 3 shows the results of the characterisation study.

Table 8: Summary of the measurements performed by the participating laboratories

Laboratory/ procedure used	Platform	TF
L1/ open	AU640	1.055
L2/ open	BN II	1.044
L3/ open	Hitachi 917	0.9939
L4/ open	Hitachi 917	1.076
L5/ closed	Immage	1.159
L6/ open	Architect	1.073
L7/ closed	Hitachi 919	1.053
L8/ open	Hitachi 917	1.073

The individual daily measured values are normally distributed at $\alpha = 0.05$ (error probability upon rejection of the zero hypothesis). The lab means distribution is not normally distributed

at an α = 0.05 (K-crit = 3.7, K=3.74) according to a Kurtosis test, but is at α = 0.01. The skewness test indicates normality at α = 0.05. Laboratory 5 is an outlier at α = 0.05 when the data are evaluated either with the Dixon's test or Nalimov t-test. However, the estimated expanded uncertainty of the result of laboratory 5 covers the mean of laboratory means. Therefore Laboratory 5 was retained in the analysis.

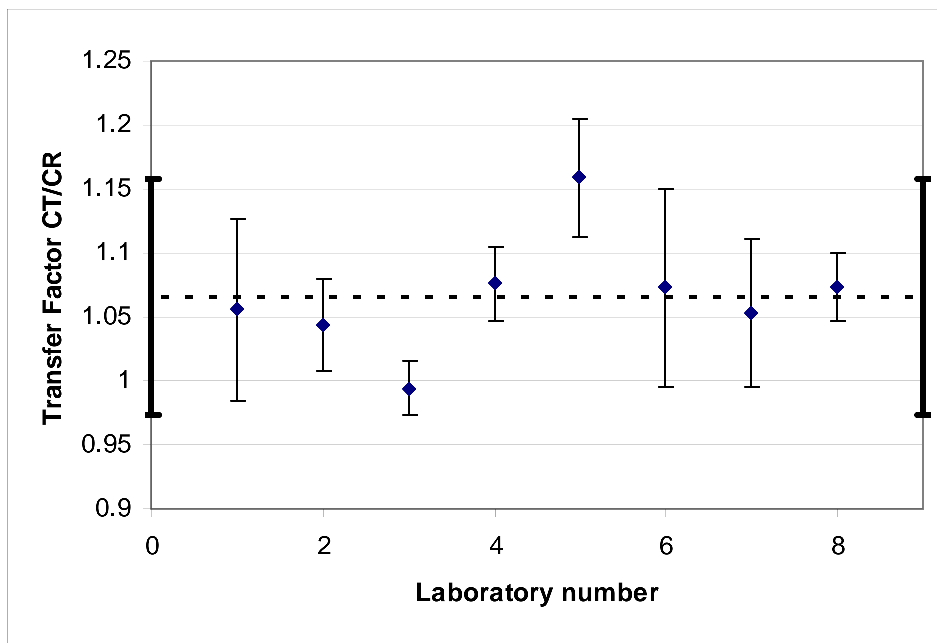

Figure 3: Results of the characterisation measurements for CRP. The bars represent the laboratory means ± 2*s*. The dotted line represents the mean of means, and the bars at the beginning and the end of the dotted line represents the mean of means ± 2*s*.

Table 9: Mean of means and relative standard deviation of the TFs, calculated using all valid results

TF	
Mean of means	s_{rel} [%]
1.066	4.32

7 Uncertainty budget and certified value

7.1 Estimation of the uncertainty

The certified uncertainties consist of the relative standard uncertainty related to characterisation ($u_{char, rel}$), the relative standard uncertainty of the calibrant ($u_{cal, rel}$), and the uncertainties due to the between-bottle heterogeneity ($u_{bb, rel}$), and possible degradation during long-term storage ($u_{lts, rel}$) [12].

- $u_{char, rel}$ was estimated as the relative standard uncertainty of the mean of laboratory means, i.e. $s/(p)^{0.5}$ with s the relative standard deviation of the mean of laboratory means and p the number of datasets, converted into a relative value
- $u_{cal, rel}$ was estimated as half of the half-width of the 95 % confidence interval of the certified value of CRP in the calibrant, ERM-DA470, relative to the certified value.
- $u_{bb, rel}$ was taken as the $s_{bb, rel}$ as defined in Section 4.1.
- $u_{lts, rel}$ was estimated from stability tests and were taken from Table 6 (6 months stability study).

The relative combined standard uncertainty was calculated as the square root of the sum of squares of the individual contributions, according to:

$$u_{c, rel} = \sqrt{u_{char, rel}^2 + u_{cal, rel}^2 + u_{bb, rel}^2 + u_{lts, rel}^2}$$

The various uncertainty contributions and the relative combined standard uncertainty ($u_{c, rel}$) are shown in Table 10.

Table 10: Uncertainty budget for ERM-DA472/IFCC

$u_{char, rel}$ [%]	$u_{cal, rel}$ [%]	$u_{bb, rel}$ [%]	$u_{lts, rel}$ [%]	$u_{c, rel}$ [%]
1.53	2.42	0.29	0.47	2.92

7.2 Certified value

The certified value (Table 11) was calculated from the TF (Table 9) and the certified value in ERM-DA470 (Table 6) according to:

$$c_{ERM-DA472} = TF \cdot c_{ERM-DA470}$$

The relative expanded uncertainty was calculated from the relative combined standard uncertainty $u_{c, rel}$ (Table 10) by multiplication with a coverage factor k. This coverage factor was taken as 2. The relative expanded uncertainty was multiplied with the mean of dataset means to obtain the expanded uncertainty U_{CRM}.

Table 11: Certified value and expanded uncertainty for the mass concentration of CRP in ERM-DA472/IFCC

Certified value [mg/L]	U_{CRM} (k = 2) [mg/L]
41.8	2.5

8 Metrological traceability

The measurements of CRP were calibrated with ERM-DA470 applying the two value transfer procedures as described in the report. The value transfer measurements were strictly controlled with respect to the adherence to the procedure and the adequate functioning of equipment and reagents verified. The methods used were all immunochemical. Different combinations of reagents and platforms were used, and gave consistent results. Therefore the results are not dependent on the individual measurement procedures.

The value assignment measurements of CRP in ERM-DA470 had been calibrated with the 1st International Standard CRP 85/506, to which values had been assigned by a using a pure protein as calibrant, also using immunochemical methods and a collaborative study [5].

Therefore ERM-DA472 is traceable to the pure protein calibrant, and thus to the SI, applying the procedures described in the present report and in the report for ERM-DA470 [5], using immunochemical methods.

9 Commutability

During the value assignment process it was found that, as was the case for ERM-DA470, there were no significant matrix effects (closed protocol) or differences in matrix effect between the CRM and ERM-DA470 (open protocol). This was done by verifying that the regression line of the signal measured for the CRP concentrations in the dilutions in function of the volume fraction of the certified reference material in the dilutions was linear, and passed through the origin. Also, the following combinations of platforms and reagents produced consistent results:

- Abbott Architect, Abbott reagents
- Beckman Immage, Beckman reagents
- BN II, Dade Behring reagents
- Hitachi 917, DAKO reagents
- Hitachi 917, Roche reagents
- Hitachi 919, Nitto Boseki reagents
- AU640, Olympus reagents

The commutability of the material has to be demonstrated for each particular assay. However, the results of external quality assessment schemes show that the inter-assay variation of CRP certified has generally been significantly reduced during the long-term use of ERM-DA470 [6], [7]. As this is a strong indication of commutability, ERM-DA472/IFCC was produced in a similar manner up until the point where the materials were either ampouled (this material), or filled in vials and lyophilised (ERM-DA470).

10 Intended use and instructions for use

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

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

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 so as to mix the contents every 5-10 minutes, until the serum is thawed.

The minimum sample intake for which the material was shown to be homogenous was 20 μL, but it can be expected to be homogenous at nL levels, as it is a true solution

Storage

Unopened ampoules should be stored at temperatures at or below -20 °C. Under the condition that any microbial contamination during the reconstitution procedure has been excluded, the solution of ERM-DA472/IFCC can be used for one week, as it was verified that changes to the certified concentration observed during that period are not significant. After the opening of the ampoule it is advisable to store the material at 2 to 8 °C in a sealed container.

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

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Abstract

The production and certification of ERM-DA472/IFCC, a new reference material certified for C-reactive protein (CRP), is described.

Serum was produced from blood collected in 6 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 in 5 batches, and then pooled, spiked with β_2 microglobulin (B2M) and CRP and filled into ampoules (approximately 1 mL serum per ampoule). The serum was stored at -70 °C. It was verified that the material was homogenous and stable.

ERM-DA472/IFCC was characterised using the reference material ERM-DA470 as calibrant. This was achieved using a value transfer protocol that can be considered as a reference procedure. The principles used to measure the CRP concentration were immunonephelometry and immunoturbidimetry. The measurements were performed with different platform/reagent combinations (Abbott Architect, Beckman Immage, BN II, different Hitachi instruments, and Olympus AU640). In total 8 laboratories participated in the value assignment. The certified CRP mass concentration is 41.8 mg/L, the expanded uncertainty (k = 2) 2.5 mg/L.

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