Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

Certification of proteins in the human serum

Certified Reference Material ERM®- DA470k/IFCC

EUR 23431 EN - 2008

JRC EUROPEAN COMMISSION

The mission of the IRMM is to promote a common and reliable European measurement system in support of EU policies.

European Commission
Joint Research Centre
Institute for Reference Materials and Measurements

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/

EUR 23431 EN
ISBN 978-92-79-09490-3

ISSN 1018-5593
DOI 10.2787/63869

Luxembourg: Office for Official Publications of the European Communities

Printed in Belgium

CERTIFICATION REPORT

Certification of proteins in the human serum

Certified Reference Material ERM®- DA470k/IFCC

I. Zegers[1], W. Schreiber[2], J. Sheldon[3],S. Blirup-Jensen[4], A. Muñoz[1], G. Merlini[5], Y. Itoh[6], A.M. Johnson[7], S. Trapmann[1], H. Emons[1], H. Schimmel[1]

[1]European Commission, Joint Research Centre
Institute for Reference Materials and Measurements (IRMM),
B-2240 Geel (Belgium)

[2] Dade Behring Marburg GmbH
A Siemens Company
Marburg (Germany)

[3] Protein Reference Unit
St. Georges Hospital
London (United Kingdom)

[4] Universitetsshukhuset I Lund (Sweden)

[5] Universita degli Studi di Pavia
Pavia (Italy)

[6] Asahikawa Medical College
Asahikawa (Japan)

[7] Greensboro (United States)

Summary

The production and certification of ERM-DA470k/IFCC, a new serum protein reference material intended to replace ERM-DA470, 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 B2M and CRP and filled into vials (1 mL serum per vial). The serum was lyophilised in the vials, afterwards closed with rubber stoppers and screw caps and stored at -70 °C. The stability and homogeneity of the material were assessed for 14 proteins, including CER and B2M.

ERM-DA470k/IFCC was characterised for 12 proteins using the reference material ERM-DA470 as calibrant. This was achieved using a value transfer protocol that can be considered as reference procedure.

The techniques used to measure the protein concentrations were immunonephelometry, immunoturbidimetry, and for ALB also visible spectrometry. The measurements were performed with different platform/reagent combinations (Abbott Architect, Beckman Immage, BN II and BN ProSpec, different Hitachi instruments, Roche Integra, LX 2200, and Olympus AU640). In total 18 laboratories participated in the value assignment, 4 using open value transfer procedures, 12 using closed value transfer procedures, and 2 using both open and closed procedures.

The results show that open and closed value transfer procedures give very similar results, and lead to robust values for A2M, AAG, AAT, ALB, C3c, C4, HPT, IgA, IgG, IgM, TRF, and TTR.

GLOSSARY

AAG	α_1 acid glycoprotein (orosomucoid)
AAT	α_1 antitrypsin (α_1-protease inhibitor)
A2M	α_2 macroglobulin
ACT	α_1 antichymotrypsin
ALB	albumin
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
C4	complement 4
CASO	Casein Soy
CER	ceruloplasmin
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
HPT	haptoglobin
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IRMM	Institute for Reference Materials and Measurements
IU	International Units
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 samples analysed

n	number of subsamples analysed
NC	not calculable
ND	not detectable
PAGE	polyacrylamide gel electrophoresis
RM	reference material
RF	Rheumatoid Factor
R^2	correlation coefficient of the linear regression
RSD	relative standard deviation
s_{bb}	standard deviation between bottles
SD	standard deviation
SDS	sodium dodecyl sulfate
s_{wb}	standard deviation within bottles
SI	International System of Units
TM	target material
TF	transfer factor
TRIS	tris(hydroxymethyl)aminomethane
TRF	transferrin
TTR	transthyretin (also called prealbumin)
U	expanded uncertainty
u_{bb}	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_c	combined relative standard uncertainty
u_{cal}	relative standard uncertainty of the calibrant
u_{char}	relative standard uncertainty related to the characterisation
u_{lts}	relative standard uncertainty related to the long-term stability of the material
u_{sts}	relative standard uncertainty related to the short-term stability of the material
USNRP	United States National Reference Preparation

1 Introduction and design of the project

1.1 Background: need for the CRM

Serum proteins levels are important measurands in clinical chemistry. The measurement of their concentrations is used in routine medical evaluations and in a large number of specific indications, and can be performed repeatedly without (major) injury to the patient.

The immunoassays used for serum protein measurements are capable of a high degree of sensitivity and specificity, 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.

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

The requirements for a calibrant are that the assigned values are metrologically traceable, and accompanied by an uncertainty statement. The stability and homogeneity with respect to all the certified properties must be verified, and the calibrant must be commutable, i.e. resemble the patient samples [1]. These issues are particularly challenging for serum proteins, as they form a mixture of interacting proteins with different isoforms and complexes.

In 1989 the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) began the processing, characterisation, and calibration of a secondary matrix reference material for human serum proteins. In 1993 the Bureau Communautaire de Référence released the resulting CRM 470 (later transformed to ERM-DA470), certified for 15 proteins [2]. The protein concentration measurements were calibrated with pure proteins for transthyretin (TTR), α_1 acid glycoprotein (orosomucoid, AAG), α_1 antitrypsin (α_1-protease inhibitor, AAT), transferrin (TRF), and α_1 antichymotrypsin (ACT), with the matrix material USNRP 12-0575C [3] for albumin (ALB), ceruloplasmin (CER), α_2 macroglobulin (A2M), haptoglobin (HPT), complement 3c (C3c), complement 4 (C4), immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) and with the 1st International Standard CRP 85/506 for C-reactive protein (CRP).

After the release of ERM-DA470 IVD manufacturers began referencing their calibrants and controls to the material, and the among-laboratory variances for assays of serum proteins became substantially lower for most of the proteins certified in ERM-DA470 [4, 5].
The present material has been produced to replace ERM-DA470 when that will be exhausted.

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, traceability and 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 detection 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

For most proteins certified in ERM-DA470, the material is sufficiently commutable to lead to a considerable reduction of inter-assay variances. Therefore it was decided to produce the new material according to procedures similar to those applied for ERM-DA470.

1.3 Design of the project

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.

Two pilot batches were produced, one spiked with only CRP and one spiked with CRP and β_2 microglobulin (B2M). The pilot batches were used to verify that the processing procedures resulted in material with the required properties (homogeneity, stability, commutability of B2M, optical clarity of the material). On that basis it was decided to further process the main part of the serum, and to spike the material with both CRP (from pleural fluids) and recombinant B2M.

The serum was processed in 5 batches, and then pooled, spiked with B2M and CRP and filled into vials (1 mL serum per vial). The serum was lyophilised in the vials and afterwards stored at -70 °C.

The homogeneity and stability of the material were assessed for 14 proteins: A2M, AAG, AAT, ALB, B2M, C3c, C4, CER, HPT, IgA, IgG, IgM, TRF, and TTR. Compared to ERM-DA470, ACT was not certified in the new material. B2M was added to the list of proteins in order to allow for its certification later on. For CER and CRP no values were assigned to the material during this certification campaign.

The material was characterised for 12 proteins by using the reference material (ERM-DA470) as calibrant. This was achieved using a value transfer protocol that can be considered as reference procedure [6]. In the so called closed value transfer procedure 6 dilutions of the calibrant (ERM-DA470) and 6 dilutions of ERM-DA470k/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 (measured 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 concentration of the target material is determined using the slope of the regression line (interpolated concentration versus dilution).

The techniques used to measure the protein concentrations were nephelometry, turbidimetry, and for ALB also visible spectrometry. The measurements were performed with different platforms (i.e. instruments; Abbott Architect, Beckman Immage, BN II and BN ProSpec, different Hitachi instruments, Eiken LX 2200, Olympus AU640, Roche Integra) and reagents. In total 18 laboratories participated in the value assignment, 4 using open value transfer procedures, 12 using closed value transfer procedures, and 2 using both open and closed procedures (see Section 6.1). 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 (JP)

Azienda Ospidaliera Policlinico Modena (IT)

Blutspendedienst SRK Bern (CH)

Centre de Transfusion Sanguine Liège, Service du Sang de la Croix-Rouge de Belgique (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 Kralove University Hospital (CZ)

Università degli Studi di Pavia (IT)

Characterisation and processing of the serum

Dade Behring Marburg GmbH - A Siemens Company, Marburg (DE) (certified ISO 13485 TÜV Rheinland Product Safety GmbH SX 60014517 0001)

Universitetssjukhuset I Lund (SE) (accred. ISO/IEC 17025 SWEDAC 1424)

Stability and homogeneity studies

DAKO Denmark, Glostrup (DK) (certified ISO 13485 UL A12312)

Dade Behring Marburg GmbH - A Siemens Company, Marburg (DE) (certified ISO 13485 TÜV Rheinland Product Safety GmbH SX 60014517 0001)

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

Olympus Life and Materials Science, Clare (IE) (certified ISO 13485 TÜV Rheinland Product Safety GmbH SX 60021010 0001)

Characterisation

Abbott Diagnostics, Irving (US) (certified ISO 13485 UQA 0103128/B)

Centro Interdipartimentale sulla Riferibilità Metrologica in Medicina di Laboratorio (CIRME), Università Degli Studi di Milano (IT)

Beckman Coulter, Brea (US) (certified ISO 13485 NSAI MD 19.0779)

Dade Behring Marburg GmbH - A Siemens Company, Marburg (DE) (certified ISO 13485 TÜV Rheinland Product Safety GmbH SX 60014517 0001)

DAKO Denmark, Glostrup (DK) (certified ISO 13485 UL A12312)

Denka Seiken, Tokyo (JP) (certified ISO 13485 TÜV Japan Ltd SD 951 05 3207)

Eiken Chemical Co., Tokyo (JP) (certified ISO 13485 TÜV Management Service GmbH Q1N 05 10 56541 004)
Foundation for Blood Research (FBR), Scarborough (US)
Klinisk Kemi Malmö, Lunds Universitet (SE) (accred. ISO/IEC 17025 SWEDAC 1340)
Kreiskliniken Altötting-Burghausen (DE)
Laboratoire d'Immunologie, Centre Hospitalier Lyon-Sud (FR)
Laboratorio di Biotecnologie e Tecnologie Biomediche, Policlinico San Matteo, Pavia (IT)
Medical Biological Laboratories Co., Nagano-Ken (JP) (certified ISO 13485 SGS GB06/69985)
Nitto Boseki, Fukushima (JP) (certified ISO 13485 SGS GB06/68575)
Odense Universitetshospital (DK)
Olympus Life and Materials Science, Clare (IE) (certified ISO 13485 TÜV Rheinland Product Safety GmbH SX 60021010 0001)
Protein Reference Unit, St. Georges Hospital, London (GB)
Roche Diagnostics GmbH, Penzberg (DE) (certified ISO 13485 TÜV SÜD Q1N 07 08 45096 003)

Data analysis

Acomed Statistik, Leipzig (DE)
Andrew M. Johnson, Greenboro (US)
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.

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. Samples with high cholesterol or triglyceride concentration were rejected. Donations from subjects 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 blood 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 briefly stored at -30 °C before being shipped on dry ice

Tests of the serum: The blood banks tested the serum for HIV 1+2, HBV or HBsAg, and HCV. Monoclonal gammopathies were detected by gel electrophoresis.
6 donations were discarded because of the presence of monoclonal components, and 10 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.

Table 1: Summary information on the collected serum. In case certain donations had to be excluded, 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 sample lost due to a leaking container

3.2 Processing of the serum

Overview of the procedure

The processing procedure is summarised in Fig. 1. It followed largely that used for CRM 470 (ERM-DA470) [2]. The major differences were that after the delipidation step a dialysis membrane with a cut-off of 10 kDa was used instead 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 so as to remove particulate matter and lipids, and to achieve a uniform degree of 'maturation' of the serum. As the batch from Japan was small it was processed together with the serum from Italy. The serum from different collection centres was then pooled, and sterile filtrated after addition of preservatives (sodium azide, aprotinin and benzamidine hydrochloride) and before the filling and lyophilisation.

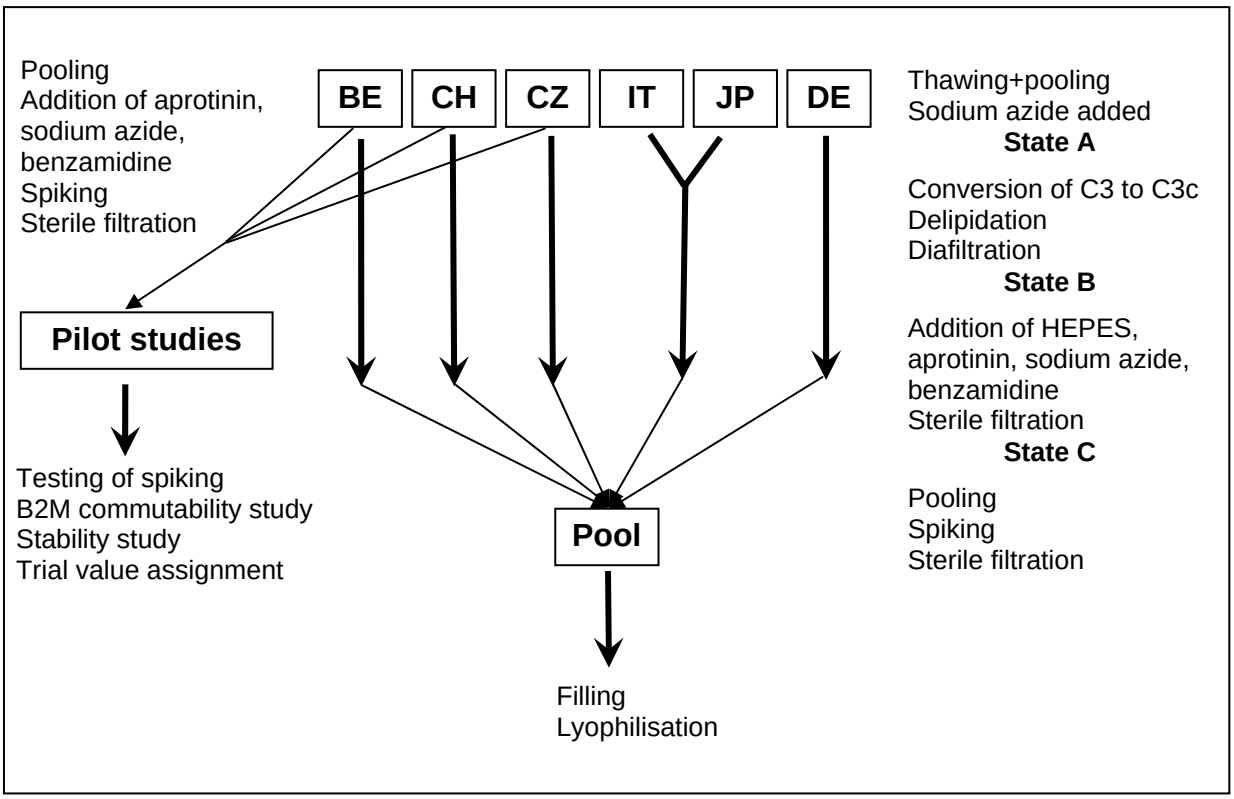

Figure 1: Overview of the processing of the serum.

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 concentration of 7.7 mmol/L (approximately 0.5 g/L)
- removal of 1/8th of the volume of each pool for verification and testing purposes and storage in 50 mL plastic tubes at 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 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 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 approx. 10000 x *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 Dalton; Pall, Hauppauge, US) 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 total protein 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 saturated HEPES solution to 7.2 ± 0.1
- addition of sodium azide to a final concentration of 14.6 mmol/L (0.95 g/L)
- addition of aprotinin to 80000 KIU/L (8 mL Trasylol 500000 KIU/L, Bayer, Leverkusen, DE)
- addition of benzamidine hydrochloride monohydrate (Merck, Darmstadt, DE) to a final 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 total protein and for the measurement and physicochemical analysis of the proteins to be certified
- sterile removal of 150 mL of the DE, CZ and BE pools; these aliquots were stored below -70 °C until processing of the pilot batches

The material is now in State C.

Until processing of the final pool the collection centre pools were stored below -70 °C.

Protein concentration measurements: Throughout the processing, the concentrations of the proteins to be certified and of apolipoprotein A-I and B were determined by immunonephelometry (double determinations) on a BN ProSpec or BN II System using reagents, standards and controls of the 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).

Physicochemical structure of the plasma proteins: The integrity of the proteins during processing was studied using several methods:

- 2-dimensional immunoelectrophoresis according to Clarke and Freeman [7]
- non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent immunoblotting (DE, CZ and BE pools only) for C3/C3c, C4, AAT and CER
- native PAGE
- 2-dimensional electrophoresis.

3.2.2 Preparation of the combined pool

Preparation of CRP and B2M

463 mL of a purified human CRP solution (mass fraction 97 % of total protein; 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 lyophilised purified recombinant protein.

Preparation of the final pool

- thawing of all 5 separate pools (2 to 3 days)
- pooling
- sterile removal of aliquots for the measurement of B2M and CRP, and for the physicochemical analysis
- preparation of 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 concentration of approximately 2.6 mg/L (total amount added 65 mg)
- pH adjustment with saturated HEPES solution (pH 7.2 ± 0.1)
- sterile filtration (0.2 µm)
- sterile removal of 3.5 L serum for storage as liquid frozen material (in ampoules containing 1 mL serum each, stored below -70 °C)
- sterile removal of aliquots for further analysis, including sterility testing
- analysis of liquid and lyophilised material (total protein, measurement of the concentration of and physicochemical analysis of the proteins to be certified)

3.2.3 Processing control

Pools per country of origin

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 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 concentration (Biuret method) and volumes of pools at different processing stages

	State A		State B		State C	
Collection centre pool	Volume [L]	[total protein] [g/L)]	Volume [L]	[total protein] [g/L]	Volume [L]	[total protein] [g/L]
DE	10.5[1]	69.0	8.8	68.4	8.6[3]	65.8
CZ	11.2[1]	72.0	9.3	68.1	9.1[3]	69.2
CH	11.2[1]	77.3	9.4	67.7	9.4[3]	68.8
BE	15.2[2]	69.9	14.0	66.9	14.0[4]	63.8
JP	2.6[2]	73.7				
IT	8.9[1]	71.1	7.0	70.9	7.1	69.4

[1] starting volume for processing (1/8th of the volume of the pool was stored for other purposes)
[2] starting volume for processing (1 L of BE-Pool and 200 mL of JP-Pool stored for other purposes)
[3] final volume after processing and subtraction of 150 mL used for pilot batches
[4] final volume after processing and subtraction of 200 mL for pilot batches

C3 conversion: Conversion of C3 to C3c was verified with 2-dimensional immunoelectrophoresis (see Fig. 2) using aliquots of the pool before starting the processing (State A), and after sterile filtration (State C). In the A State a small amount of C3 was already converted to C3c. Only in the serum from Japan and Belgium the conversion had progressed to a higher extent, probably due to different time schedules in the blood collection process. 2-dimensional immunoelectrophoresis results of the material in State C demonstrate that the major part of C3 was converted to C3c.

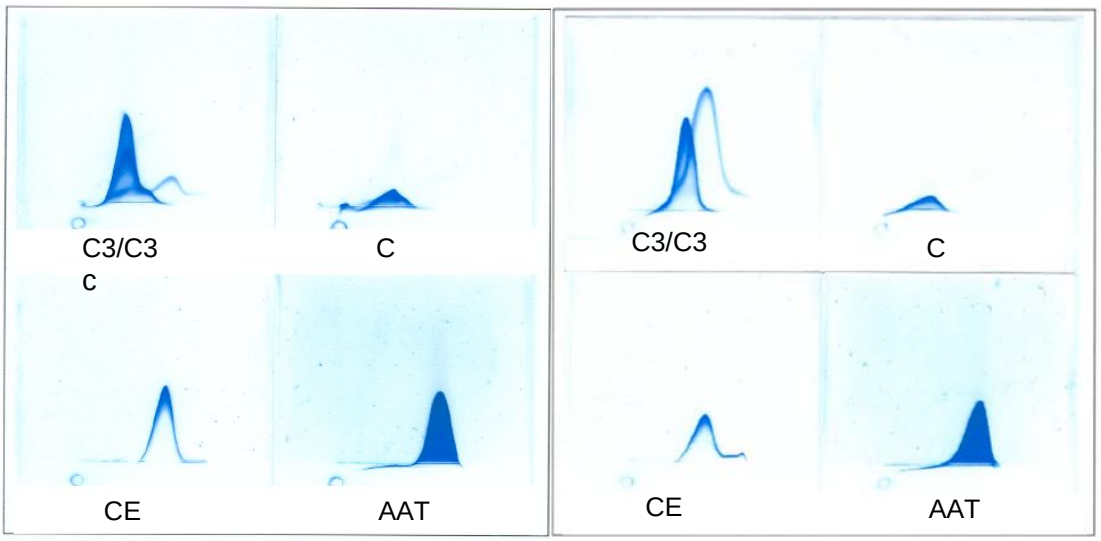

Figure 2: Crossed immunoelectrophoresis measurement profiles of C3/C3c, C4, CER, and AAT in the serum from the Czech Republic, before (State A, left) and after (State C, right) maturation.

Delipidation: For the delipidation by the Aerosil treatment to be considered successful, relative mass concentrations of cholesterol and apolipoprotein B should be 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 individual 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 3: Lipoproteins before processing and after Aerosil treatment[1]

	State A					State B				State C
Pool	Chole-sterol [mg/L]	Trigly-cerides [mg/L]	Apo A-I [mg/L]	Apo B [mg/L]	Blank value [Bit]	Chole-sterol [mg/L]	Trigly-cerides [mg/L]	Apo A-I [mg/L]	Apo B [mg/L]	Blank value [Bit]
DE	1627	1189	1555	806	3497	ND	95.5	ND	ND	55
CZ	1788	2266	1565	931	1992	ND	56.4	ND	ND	75
CH	1760	920	1715	874	3082	11.5	95.5	ND	ND	103
BE	1903	767	1620	870	1553	ND	8.3	ND	ND	99
JP	1875	1158	1595	783	>[2]					
IT	1846	725	1645	842	2498	5.8	8.3	ND	ND	101

[1] when the concentration is below the limit of detection this is marked by ND (not detectable)
[2] exceeds measuring interval (0-4096 Bit)

Concentration of 14 serum proteins: The protein loss caused by the delipidation process was compensated to a certain extent by reducing the volume by 19 % by diafiltration, so that the mass concentrations of most proteins were found within 90 to 100 % of the starting values (Table 4). Higher losses were observed for IgM, C4 and CER due to the known affinity of these proteins to Aerosil. The B2M concentration decreased similarly. Nevertheless, the concentration of B2M was adjusted in the final pool with recombinant material.

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, DE). No growth was observed on the selected media after five days, and the pool was released for filling.

Homogeneity of the filling volume: The consistency of the filling volume was determined during the filling process by taking samples (4 consecutive vials each) at regular intervals (approx. every 15 min). Altogether 24 samples consisting of 4 vials each were taken. The average mass of the liquid filled into these 96 vials was 1004 mg with a standard deviation (SD) of 5 mg.

Residual moisture and dry mass: The dry mass of the vial contents and the residual moisture were determined in parallel. To determine the dry mass, the mass of a vial was determined before and after removal of the vial content. The remaining moisture in the lyophilised material was analysed by Karl Fischer titration [8]. 10 vials each were analysed from the beginning, middle and end of the filling process (N=30). The measurement results and the standard deviation (SD) and relative standard deviation (RSD) are:

average dry mass	76.0 mg	average residual moisture	4.3 mg/g
interval	74.0 – 78.0	interval	3.6 – 6.0 mg/g
SD	0.9 mg	SD	0.6 mg/g
RSD	1.2 %	RSD	14 %

The average dry mass of the new material is similar to that in ERM-DA470 (76.0 mg versus 71.8 mg), whereas the average residual moisture is lower in the new material (4.3 mg/g versus 5.7 mg/g), but similar to the values of the pilot batches (4.3 mg/g).

Blank values: The blank value for the final pool before sterile filtration was 93 Bit (detector values), after sterile filtration 84 Bit. The final preparation had blank values of 204 Bit (liquid) and 183 Bit (lyophilised and reconstituted). 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. Therefore these results show that the material is optically clear.

Mass density measurements: The mass density of the reconstituted ERM-DA470k/IFCC, the liquid frozen processed serum, and reconstituted ERM-DA470 were measured with an Anton Paar densitometer (Graz, AT), and found to be 1.0221, 1.0236 and 1.0234 g/mL, respectively, at 20 °C. As the difference between the density of ERM-DA470 and the density of ERM-DA470k/IFCC is negligible compared to the uncertainties of the certified values no systematic density correction was applied to the transfer factors.

Protein concentration measurements on the final preparation: The concentrations of the selected serum proteins were analysed on BN ProSpec systems. Lyophilised materials were reconstituted according to the procedure described in section 9. Liquid and lyophilised and reconstituted materials were measured in triplicate in the same analytical series. The final pool was measured in duplicate before and after sterile filtration

The results show that there is a relative loss of 4 - 8 % of measured protein concentration upon lyophilisation and reconstitution for most proteins. This is mostly due to a dilution effect, as the volume of water removed by lyophilisation of a 1.0 mL serum sample is less than 1.0 mL, and the material is reconstituted with 1.0 mL water.

Table 4: Approximate protein concentrations and optical clarity at the final processing stages

	Protein mass concentration (n = 3) [g/L][1]			Relative decrease in concentration by lyophilisation [%]
Protein	Sterile filtrated final pool	After filling	Reconstituted lyophilised material	
A2M	1.52	1.52	1.43	5.9
AAG	0.640	0.696	0.649	6.8
AAT	1.16	1.16	1.09	6.0
ALB	39.6	42.0	39.4	6.2
B2M[1]	2.59	2.79	2.63	5.7
C3c	1.08	1.07	1.02	4.7
C4	0.172	0.184	0.167	9.2
CER	0.187	0.186	0.172	7.5
HPT	0.940	0.979	0.921	5.9
IgA	1.98	1.96	1.85	5.6
IgG	10.9	10.6	9.89	6.7
IgM	0.773	0.777	0.739	4.9
TRF	2.50	2.47	2.37	4.0
TTR	0.217	0.236	0.219	7.2
Total protein	66.5	63.3	58.2	8.1
Blank value (Bit)	84	204	183	

[1][mg/L] for B2M

3.3 Filling, lyophilisation, capping and labelling

1 mL serum was filled into each vial under clean-room conditions. Uncoloured threaded and siliconised glass vials were used.

Within one hour after filling the lyophilisation program was started. Closing of the bottles using grey rubber stoppers was performed under a low-pressure nitrogen atmosphere.

Capping and labelling was performed using red screw caps. Due to the large number of vials this step had to be done on two machines in parallel.

4 Homogeneity

4.1 Homogeneity

The homogeneity of ERM-DA470k/IFCC was verified by measuring the proteins in triplicate in 40 vials taken randomly over the whole batch. The measurements were performed with a BN ProSpec and Dade Behring reagents, or with a Hitachi 917 instrument and DAKO reagents, depending on the protein. The protein concentrations were corrected for the mass of the water used to reconstitute each sample.

Grubbs tests were performed to detect outlying individual results as well as averages measured for each vial. For HPT, IgM, and TRF outliers (one each) were found at the 99 % confidence level, but these were retained in the analysis.

Regression analyses were used to evaluate potential drifts in results related to the analysis sequence or to the filling sequence. Small but significant trends (at the 99 % confidence level) were observed for A2M, AAT, C3c, and CER. The homogeneity measurements had been done in such a sequence that trends due to analysis sequence and filling sequence could still be confounded. The 6 months stability data (see Section 5.2) had been measured in such a manner that these trends could be separated, and the analysis of these data showed that there is no trend in function of the filling sequence. Therefore the trend in the results of the homogeneity measurements is due to a drift in function of the analytical sequence.

It was furthermore checked whether the data followed a normal and unimodal distribution using normal probability plots and histograms respectively. The individual data and the bottle averages were unimodal for all proteins, although some deviations from normality were observed.

ANOVA statistics were used to calculate 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}), using the formulas:

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

(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)

Both values were converted into relative uncertainties (Table 5). The larger of both values was included into the calculation of the overall uncertainty of the certified values (Section 7.1).

Table 5: Relative between bottle homogeneity[1]

Protein	Relative between bottle heterogeneity ($s_{bb,rel}$) [%]	Relative maximum hidden heterogeneity ($u^*_{bb,rel}$) [%]
A2M	NC	0.517
AAG	NC	0.484
AAT	0.675	0.527
ALB	NC	0.612
B2M	NC	0.666
C3c	NC	0.557
C4	NC	0.418
CER	NC	0.672
HPT	0.637	0.413
IgA	NC	0.340
IgG	NC	0.425
IgM	0.745	0.573
TRF	NC	0.498
TTR	1.153 (0.880)[2]	0.520 (0.144)[2]

1) When MS_{bb} was smaller than MS_{wb}, s_{bb} could not be calculated. Those cases are marked as NC (non-calculable).
2) Values obtained on a Hitachi 917 using DAKO reagents are given in parentheses.

4.2 Minimum sample intake for analysis

The reconstituted material forms a clear solution, and as a true solution is not expected to have any relevant heterogeneity in protein concentration at sample intakes even below nL volumes. The sample intakes of homogeneity studies on the material measured with a Hitachi 917 platform were between 2 and 24 µL, depending on the protein. For all the proteins 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 or contamination at a sample intake of 2 µL.

5 Stability

Short and long-term stability studies were carried out using an isochronous set-up [9] that consists of the simultaneous analysis of reference and test samples. For each study a defined set of samples was exposed for different periods of time to elevated temperatures and then brought back to the reference temperature (above liquid nitrogen or -70 °C). At the end of the study all samples were analysed for the concentrations of the proteins within one analytical run under repeatability conditions. The data were analysed by determining the regression line for the protein concentration in function of time, and determining whether it is significantly different from zero [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. The reference temperature was below -140 °C, as the reference samples were stored above liquid nitrogen. Test samples were kept for 0, 1, 2, and 4 weeks at -70, -20, 4, and 60 °C before being brought back to the reference temperature. For each combination of time and temperature 2 samples were analysed in triplicate. The samples were analysed for all proteins by turbidimetry using a Hitachi 917 platform (i.e. equipment) and DAKO reagents, except for IgM and B2M, which were analysed on an Olympus AU640. The values were corrected for the variable reconstitution volume. The results are shown in Table 6.

Table 6: Short-term stability study: slope (b), test for significance of the slope ($|b/u_b|$), and relative standard uncertainty after one week storage at the specified temperature u_{sts}

	-70 °C			-20 °C		
Protein	b [(mg/L)/week]	$\|b/u_b\|$	u_{sts} [%]	b [(mg/L)/week]	$\|b/u_b\|$	u_{sts} [%]
A2M	2.4	1.1	0.15	0.8	0.5	0.13
AAG	0.04	0.0	0.11	0.9	1.0	0.15
AAT	2.6	1.5	0.14	-0.3	0.2	0.13
ALB	-61.0	0.9	0.17	-7.1	0.2	0.13
B2M	-0.0069	1.3	0.25	-0.0027	0.6	0.19
C3c	2.3	1.9	0.12	1.3	1.1	0.12
C4	-0.1	0.5	0.16	0.1	0.5	0.15
CER	0.3	1.0	0.21	0.3	0.7	0.27
HPT	-0.1	0.1	0.12	2.0	1.7	0.14
IgA	1.9	0.5	0.21	1.4	0.4	0.18
IgG	-2.6	0.2	0.16	-16.8	1.7	0.12
IgM	-1.7	0.6	0.35	-3.4	1.4	0.35
TRF	5.2	1.7	0.14	1.3	0.4	0.14
TTR	-0.2	1.0	0.12	-0.3	1.0	0.13

Table 6 Continued

	4 °C			60 °C		
Protein	b [(mg/L)/week]	$\|b/u_b\|$	u_{sts} [%]	b [(mg/L)/week]	$\|b/u_b\|$	u_{sts} [%]
A2M	2.4	0.7	0.15	14.6	7.3	0.57
AAG	1.3	1.4	0.15	-2.6	2.2	0.20
AAT	0.2	0.13	0.13	-1.3	0.7	0.15
ALB	-100.0	2.0	0.15	-250.0	5.4	0.18
B2M	-0.0093	1.6	0.27	-0.0054	1.1	0.23
C3c	1.7	1.4	0.12	1.6	1.1	0.15
C4	0.2	1.0	0.14	5.3	13.3	0.58
CER	0.1	0.3	0.24	7.6	15.2	0.67
HPT	1.2	1.2	0.11	-0.5	0.4	0.15
IgA	1.3	0.4	0.19	2.0	0.6	0.18
IgG	-17.0	1.6	0.13	-43.4	5.2	0.19
IgM	-3.0	1.1	0.35	0.9	0.4	0.30
TRF	5.5	1.7	0.15	-5.8	1.4	0.18
TTR	-0.4	1.3	0.13	-0.1	0.3	0.14

For each protein and temperature there were 24 measurements, or 22 degrees of freedom for the linear regression. The slope of the protein 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 samples were kept at -70, -20 and 4 °C none of the slopes was significantly different from 0. However at 60 °C significant (at a 95 % confidence level) negative slopes were found for AAG, ALB, and IgG, and significant positive slopes for CER, C4 and A2M, although even at 60 °C the added uncertainties due to storage at this temperature for one week would be small. It was concluded from this study that the uncertainty due to degradation during dispatch is negligible, if the material is shipped with cooling elements.

5.2 Long-term stability

The ERM-DA470k/IFCC has been produced according to procedures that follow those for ERM-DA470 very closely. One of the reasons for doing so is that ERM-DA470 has proven to be stable for all proteins for which values were certified in the material. As the protein concentrations have been monitored in that material for over 14 years without detecting any instability it can be expected that ERM-DA470k/IFCC will also be stable over longer periods.

A 6-months stability study was performed in order to confirm the stability of the material upon storage at -70 and -20 °C. The reference temperature was below -140 °C, as the reference samples were stored above liquid nitrogen. The test samples were kept for 0, 1, 2, 4, 5, and 6 months at -70 and -20 °C. The samples were analysed by nephelometry, using a BN ProSpec. Each protein in each randomly selected vial was measured in triplicate, in

three separate runs. The values were corrected for the variable reconstitution volumes of the vials, and normalised to the run mean to correct for the evaporation of samples between runs. The measurements were divided by the protein concentration averaged over all the vials of that particular run and multiplied with the protein concentration averaged over all vials and all three runs.

The results are shown in Table 7. For IgA, TRF, A2M, CER, IgM, HPT, and TTR there were 54 measurements, or 52 degrees of freedom for each linear regression of each protein at each temperature. IgG, ALB, B2M, C3c, C4, AAT, and AAG were measured from a different set of vials. One of these vials was reconstituted incorrectly, and thus for these proteins there were 51 measurements per temperature, or 49 degrees of freedom. None of the slopes (protein mass concentration versus time) of any of the proteins was significantly different from 0 at a 99 % confidence level. At a 95 % confidence level the $|b/u_b|$ for C3c at -20 °C was just above the $t_{0.05, 51}$=2.01. 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 it is safe to store the material at either -20 or -70 °C.

Table 7: Long-term stability study: slope (b), test for significance of the slope ($|b/u_b|$), and relative standard uncertainty contribution u_{lts} due to storage for 6 months at the specified temperature

	-70 °C			-20 °C		
Protein	*b* [(mg/L)/month]	$\|b/u_b\|$	u_{lts} [%]	*b* [(mg/L)/month]	$\|b/u_b\|$	u_{lts} [%]
A2M	-2.7	1.6	0.729	-1.6	1.2	0.557
AAG	-0.3	0.8	0.355	-0.2	0.3	0.500
AAT	0.9	0.8	0.627	-0.5	0.4	0.635
ALB	61.7	1.8	0.540	51.6	1.4	0.556
B2M	-1.1	0.3	0.824	-0.5	0.2	0.609
C3c	-0.2	0.3	0.486	1.8	2.4	0.439
C4	-0.04	0.3	0.461	-0.04	0.3	0.482
CER	-0.3	1.5	0.786	0.04	0.0	0.664
HPT	-0.6	0.7	0.722	-1.0	0.9	0.573
IgA	-4.1	1.7	0.788	-1.7	1.1	0.498
IgG	17.2	1.8	0.563	11.9	1.4	0.537
IgM	1.8	1.6	0.883	-0.4	0.3	0.919
TRF	-3.4	1.1	0.800	-2.7	1.4	0.498
TTR	-0.3	0.8	1.043	-0.4	1.3	0.751

In parallel a 12 months stability study was performed on a pilot batch of serum processed in exactly the same manner as the final material, including the spiking with B2M and CRP. The reference temperature was -70 °C. The test samples were kept for 0, 2, 8, and 12 months at -20, 4, and 18 °C. The samples were analysed by turbidimetry, using a Hitachi 917 and DAKO reagents. Each protein in each randomly selected vial was measured in triplicate, in

three separate runs. The values were corrected for the variable reconstitution volumes of the vials.

The results for -20 and 18 °C are shown in Table 8. For each protein and temperature there were 24 measurements, or 22 degrees of freedom for each linear regression. None of the slopes (protein mass concentration versus time) of any of the proteins was significantly different from 0 at a 99 % confidence level at -20 °C. At 18 °C significant slopes (at the 95 % confidence level) are found for AAG, AAT, CER, IgG, and TRF. 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 12 months. The results indicate that it is safe to store the material at -20 °C for 12 months.

Table 8: Long-term stability study: slope (b), test for significance of the slope ($|b/u_b|$), and uncertainty u_{lts} added by the storage for 12 months at the specified temperature

	-20 °C			18 °C		
Protein	*b* [(mg/L)/month]	$\|b/u_b\|$	u_{lts} [%]	*b* [(mg/L)/month]	$\|b/u_b\|$	u_{lts} [%]
A2M	0.8	0.9	0.669	0.0	0.0	0.626
AAG	0.1	0.3	0.632	-0.7	2.3	0.910
AAT	0.4	1.0	0.410	1.5	2.5	1.062
ALB	-13	0.7	0.567	38	1.7	0.734
B2M	0.1	0.0	2.012	-4.0	0.8	2.676
C3c	0.1	0.3	0.445	-0.6	1.2	0.602
C4	0.01	0.0	0.497	-0.2	2.0	1.149
CER	-0.004	0.0	0.880	0.3	3.0	1.640
HPT	0.3	0.8	0.494	0.9	1.8	0.692
IgA	0.0	0.0	0.357	-1.3	1.4	0.573
IgG	-0.3	0.7	0.615	-13.7	3.3	1.206
IgM	0.4	1.3	0.562	-0.4	1.0	0.661
TRF	-1.2	0.9	0.646	-3.5	2.7	1.248
TTR	0.1	1.0	0.592	-0.2	2.0	0.609

6 Characterisation

6.1 General principles

Values were assigned by calibration with the matrix certified reference material ERM-DA470 (see Table 9) by value transfer procedures that minimise the effects of assay methods, calibration curve fitting and matrix differences [6]. The methods used for transferring values are validated, well established routine methods such as turbidimetry, nephelometry, and occasionally visible spectrometry. 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 the independent reconstitution of the reference and target materials and preparation of dilutions on each of the four days. All reconstitutions and dilutions were verified by weighing, and the concentrations calculated from the masses rather than from the intended volumes.

Table 9: Certified values and expanded uncertainties of the calibrant ERM-DA470

Protein	Certified value[1)] [g/L]	U_{CRM}[2)] [g/L]	Calibrant
A2M	1.64	0.05	USNRP 12-0575C
AAG	0.656	0.005	Pure protein
AAT	1.206	0.011	Pure protein
ACT	0.245	0.015	Pure protein
ALB	39.7	0.8	USNRP 12-0575C
C3c	1.091	0.027	USNRP 12-0575C
C4	0.151	0.005	USNRP 12-0575C
CER	0.205	0.011	USNRP 12-0575C
CRP	0.0392	0.0019	1st Intl. Std. CRP 85/506
HPT	0.893	0.009	USNRP 12-0575C
IgA	1.96	0.04	USNRP 12-0575C
IgG	9.68	0.010	USNRP 12-0575C
IgM	0.797	0.023	USNRP 12-0575C
TRF	2.45	0.006	Pure protein
TTR	0.243	0.018	Pure protein

[1)] When the material is reconstituted according to the specified procedure. The values are the unweighted means of 3-14 accepted mean values, independently obtained by 3-14 laboratories.
[2)] The certified uncertainty is the half-width of the 95 % confidence interval of the mean defined in footnote [1)]. *t*-factors were chosen according to the *t*-distribution depending of the number of accepted sets of results.

In the open value transfer procedure 6 dilutions of ERM-DA470 were used for calibrating the instrument, and 6 dilutions of ERM-DA470k/IFCC were assayed against this calibration. This procedure makes it possible to check for proportionality between 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 measures of differences in matrix effect between the two materials.

The open transfer procedure was optimised separately for each platform participating in the value assignment, for each protein measured with that platform. The main parameter optimised was the dilution scheme. It was taken 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 platforms the option exists to provide 'pre-diluted' samples to the instrument, and to switch off the dilutions normally done automatically 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. Proteins for which similar dilution schemes could be used were grouped, and common dilution schemes were developed for these groups of proteins. Finally it had also to be taken into account that for each material all six dilutions should be prepared from a single vial.

In the closed procedure 6 dilutions of ERM-DA470 and 6 dilutions of ERM-DA470k/IFCC were assayed against the calibrants normally used by the laboratories. These calibrants are usually the ones provided by the companies selling the assay reagents, and are most often 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 will lead to non-linearity of the regression line for that material, and to a non-zero intercept. Here again dilution schemes were optimised for the different platforms, taking into account the working range of the assays, the requirement that all dilutions should be prepared from a single vial, and that the volumes of the dilutions be large enough to allow for all the measurements.

20 laboratories participated in a trial run using the closed value transfer procedure. The trial run was aimed at testing that all the procedures linked to the value transfer (reconstitution, reporting of the results, etc.) were well controlled. Potential problems either in the procedures or in their application could be identified, and it was assured that all laboratories had a comparable level of performance. Some problems were detected during the trial run, but overall the results ranged from acceptable to very good for all participating laboratories.

6.2 Performance of the value assignment measurements

The laboratories were provided with detailed protocols and reporting sheets, as well as with vials of ERM-DA470, and ERM-DA470k/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 material was reconstituted the day before the measurements, according to the procedure described in the certificate of ERM-DA470 and ERM-DA470k/IFCC.

For each of the 4 measurement days a new vial of the reference preparation and the target material was reconstituted and a new set of 6 dilutions of each 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-DA470k/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 3 used a different procedure that had already been validated previously. According to their procedure all the dilutions are measured in triplicate, over 3 days only, 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-DA470k/IFCC were measured in triplicate. The laboratories used one of three dilution schemes, depending on the assay intervals of particular proteins and on the volumes required for the measurements. The intended volume fractions of the candidate reference material and the calibrant in the dilution schemes are given in percent of the reconstituted material present in the dilutions.

Scheme A: 40, 50, 60, 70, 80 and 100 % of the reconstituted material

Scheme B1: 33.3, 40, 50, 60, 66.7 and 80 % of the reconstituted material

Scheme B2: 25, 33.3, 40, 50, 66.7, 80 % of the reconstituted material

6.3 Data analysis

Principle of the analysis

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

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

where C_T and C_R are the mass concentration of the protein in the target material (TM, ERM-DA470k/IFCC) and in the calibrant (RM, ERM-DA470), respectively.

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

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

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

The concentrations of the specific proteins in the dilutions of the target material will be:

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

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

For the open value transfer procedure a calibration run is made, and the calibration curve is constructed by plotting the signals against the different dilutions of R. In a measurement run

the different dilutions of TM are assayed. The signals of these unknown test samples $C_S(j)$ are interpolated on the calibration curve, and are measured in relative concentrations of R:

$C_S(j) = F_R(j) \times C_R$ (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 of a straight line through the origin (y = TF x).

The different dilution factors $F_R(j)$ obtained by interpolation are plotted against the different dilution factors $F_T(j)$ used for the initial dilution of 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 as calibrant. 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 precision of the values for the dilutions were considerably improved by weighing all the liquids used for the reconstitution of the materials and for the preparation of the dilutions to a precision of at least 0.0001 g.

The following mass corrections were applied:

For the reconstitution:

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

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

For eventual 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:

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

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

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

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

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

Determination of the transfer factors (TFs)

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. The relative concentrations were corrected for the masses of the dilutions (and predilution if relevant).

Then for each run, the laboratories measured the dilutions of the target material in triplicate. The measurement results S_{ijk} (signal of the k^{th} measurement of material *i* within dilution *j*, expressed in %) were plotted in scatter plots $S_{ijk} = f(c_{ij})$ for the single measurements 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 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 a particular protein in a control dilution of ERM-DA470 relative to the concentration in the control sample expected on the basis of the dilution, corrected for 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 k^{th} measurement of material *i* within dilution *j*, in g/L) were plotted in scatter plots $S_{ijk} = f(c_{ij})$ so as to evaluate outliers. 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] (only in case of homoscedasticity).
- verification that the intercept ± 4 times the SD 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 datasets only).
2. R^2 of the regression must be above 0.97 for Immage data and above 0.98 for all other platforms
3. Data from at least 4 dilutions must be available
4. The completeness of data must be at least 50 % for the data for a particular protein on 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 protein and laboratory must be below 5 % (8 % for A2M)

Rationale for the acceptance criteria:

- 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). Laboratories 2, 9, 12, and 21 work with an instrument with higher scattering of the data. Therefore the threshold for these laboratories was reduced to 0.97.

- During the data analysis, the results obtained when linear regressions were forced through 0 ($y = bx$) were compared to those when linear regressions were performed with an intercept ($y = a+bx$). The mean of means of the TFs were very comparable, but the standard deviations were higher in the second case. However, as in the second case only measured data were used and no model other than the linearity within the actual measurement interval was assumed it was decided to process the data allowing for intercepts in the linear regression, provided that the intercept ± 4 SD covers the origin. As a consequence, the criterion for the acceptance of datasets was adapted, and a (within laboratory) day-to-day RSD of 5 % defined.

- For A2M many methods have a lower precision, which results in higher day-to-day variations for these proteins. Therefore, in these cases day-to-day RSD of 8 % were accepted.

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

6.4 Results of the value transfer measurements

6.4.1 General considerations

The characterisation measurements were performed by 18 laboratories. Of these laboratories 6 used an open value transfer procedure and 14 a closed value transfer procedure (Table 11). They are coded from laboratory 1 to 22, as some laboratories which first intended to participate dropped out. In the plots number 23 was used for laboratories having measured a second dataset for a particular protein, the reader can refer to Table 11 to know which laboratories performed these measurements. Two of the laboratories normally measuring with the open protocol did additional measurements using the closed protocol. The planning of the measurements was governed by the aim to obtain at least twelve datasets per protein, with preferably at least one dataset for all major platform / reagent combinations in an open mode. The Beckman Immage can not be run in the open mode, for this platform only data with a closed value transfer procedure were obtained. The measurement of A2M is only supported by a limited number of platforms and laboratories, and only 6 datasets were retained for this protein. For the other proteins the characterisation is based on between 10 and 14 valid datasets each.

The performance of the measurements was very demanding, particularly for laboratories measuring many proteins. As each platform has its particularities (time taken by the measurements, order of the measurements, intermediate storage of the samples in a closed environment or not, etc.) the planning of the order of the measurements had to be done separately by each laboratory.

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 (as was the case here), 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. Laboratory 2 kindly accepted to measure the dilutions one by one, instead of in one analytical series. There is no significant evaporation between dilutions in their measurements, and their results are mostly in line with those of other laboratories. Hence they confirm that there is no significant impact from evaporation on the measurement results.

6.4.2 Scrutiny of the data

The results are summarised in Tables 10 (summary of data problems) and 11 (results obtained). In Table 11 the grey cases correspond to laboratory / protein combinations for which measurements were reported. If, for these cases, no number is given there were no valid measurements for that combination (according to the acceptance criteria defined under Section 6.3, and the results from the analysis described below). In those cases the main reason for the invalidity of the results is indicated.

The results obtained with closed and open characterisation procedures are very similar (Fig. 3). The only protein for which the difference is significant according to a *t*-test at the 95 % confidence interval is A2M, but for A2M only 2 datasets were measured with the open instrument mode.

As part of the scrutiny, all outlying values were identified, at the level of individual measurements and at the level of TFs. Outliers were not deleted automatically, but possible technical reasons for outlying values were examined with the laboratories involved. As a result the following decisions were taken:

- A number of typographical errors were corrected.

- For laboratory 2, data from day 1 were not used, as the data for this day were incomplete, and some issues concerning sample evaporation were still being tested. As on days 2, 3, and 4 the dilutions were not measured in one analytical sequence, but separately, data giving a correlation coefficient of 0.96 were accepted (instead of 0.97).

- For laboratory 8, data from measurement day 2 on group 1 proteins (AAT, HPT, IgA, IgG, and TRF) were not used. There had been a delay in the measurement of certain dilutions because of technical problems with the platform, and this had led to evaporation of these dilutions.

- For laboratory 5 data on C4 were withdrawn because of technical reasons leading to reproducibility problems.

- For laboratory 4, the data points for the highest concentration were not used for IgG and HPT, because they are at the limit of the measuring interval.

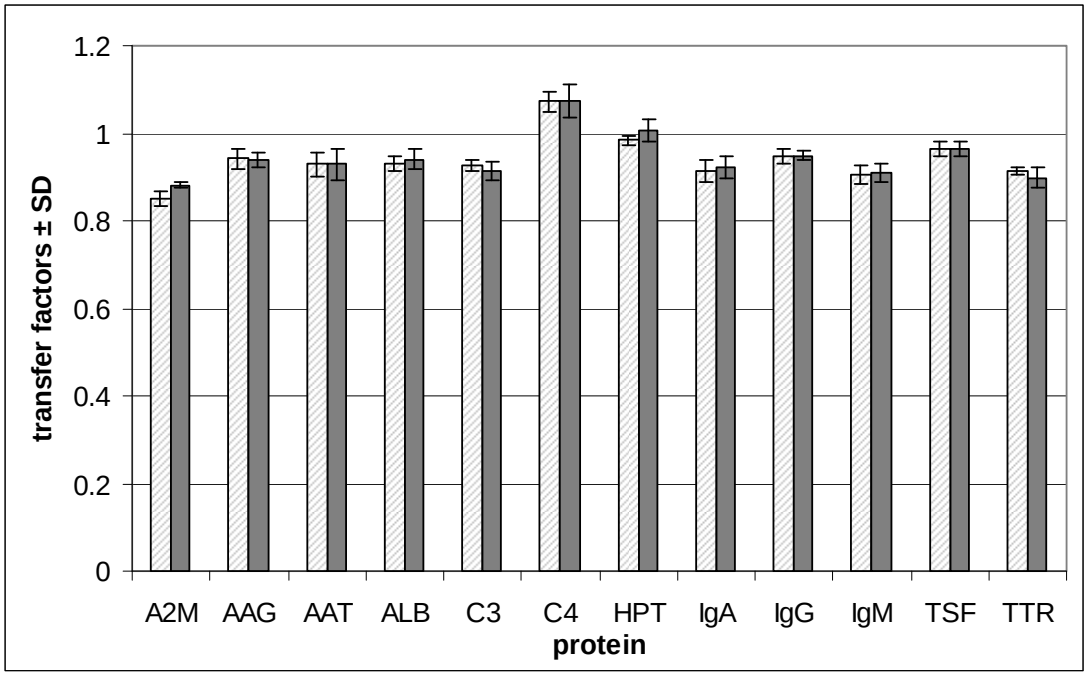

Figure 3: Comparison of TFs obtained with open (light bars) and closed (dark bars) characterisation protocols. The height of the bars correspond to the average values per protein. The error bars represent ± SD.

Table10: Number of measurement days excluded and occurrence of different data problems. Note that the data of one measurement day may have different types of problems

			Number of occurrences of:					
Protein	Number of measurement days		Days not used	> 50 % of the data is missing	Non-overlapping confidence intervals	Correlation coefficient below limit	< 2 valid days	Day-to-day variation above limit
	Analysed	Non-valid						
A2M	32	6	1	1	1	1		4
AAG	63	17	1	2	2	5	1	12
AAT	51	7	2			6	1	
ALB	52	6	1	1	1	3		
C3c	51	1		1				
C4	55	11	5			6		4
HPT	54	9	2		1	4		4
IgA	54	7	2	1	2	2		
IgG	53	5	2	1	1	2		
IgM	51	11	1		1	5	1	4
TRF	55	15	2		1	6		8
TTR	47	7	1		2			4

Table 11: Summary of the measurements performed by the participating laboratories[1]. For each protein / laboratory combination the mean of the valid daily TFs is given

Laboratory/ procedure used	Platform	A2M	AAG	AAT	ALB	C3c	C4	HPT	IgA	IgG	IgM	TRF	TTR
L1/ closed	BNII	0.8892	0.9228	0.9416	0.9243	0.9095	1.0768	0.9726	0.8994	0.9308	0.9294	DTD	
L2/ closed	Immage	CI	DTD	CC	0.9301	0.8936	1.0006	1.0136	0.9223	0.9404	DTD	0.9648	0.9103
L3/ open	AU640		0.9579	0.9437		0.9399	1.0836	0.9907	0.9341	0.9523	0.9286	0.9640	0.9202
L4/ open	BN II	0.8405	0.9016	0.8769	0.9100	0.9166	1.038	0.9668	0.8706	0.9205	0.8686	0.9716	0.9012
L4/ closed	BN ProSpec	0.8820											
L5/ open	Hitachi 917	0.8602	0.9641	0.9394	0.9301	0.9271		0.9838	0.9215	0.9699	0.9125	0.9658	0.9121
L7/ closed	BN ProSpec		0.9408		0.9222								0.8988
L8/ open	Hitachi 917		0.9348	0.9420	0.9488	0.9367	1.0956	0.9788	0.9225	0.9486	0.9113	0.9704	0.9213
L8/ closed	Hitachi 917						1.1002						
L9/ closed	Immage		CC, DTD	0.9516									
L10/ closed	Integra		0.9434	0.9340				1.0104				0.9409	
L11/ closed	BN ProSpec	DTD	DTD	0.9303	0.9740	DTD	1.1248	DTD	0.8972	0.9508	CC	CC	DTD
L12/ closed	Immage	0.8796	DTD	0.8650	0.9797	0.8887	1.0534	1.0208	0.9208	0.9449	0.8853	DTD	0.8655
L13/ open	Architect		0.9437	0.9419	0.9373	0.9148	1.0601	0.9845	0.9115	0.9346	0.9007	0.9359	0.9117
L13/ open	Architect				0.9301								
L14/ closed	LX-2200[2]		0.9654			0.9430	DTD	1.0422	0.9684	0.9584	0.9303	0.9899	
L17/ closed	Hitachi 917					0.9253	1.0844		0.9134	0.9595	0.9033	0.9666	
L18/ closed	Hitachi 717		0.9258			0.9204	1.0735	0.9701	0.9203	0.9437	0.8993	0.9594	
L19/ closed	Hitachi 919		0.9408	0.9516	0.9231								0.9227
L21/ closed	Immage	0.8752			0.9444			1.0080	0.9296	0.9581			0.8941
L22/ open	Hitachi 917		0.9473	0.9336	0.9167	0.9213	1.0533	0.9964	0.9244	0.9546	0.9140	0.9804	0.9078

[1]cases are coloured grey for those laboratory / protein combinations for which data has been analysed. In case the data was valid the mean of the valid daily determinations is given. In case the data for that laboratory/protein combination was not valid the main reason is indicated by one of the following abbreviations: CI non-overlapping confidence intervals; DTD: the day-to-day variation exceeds the acceptance limit; CC: the correlation coefficient exceeds the acceptance limit.

6.4.3 Results per protein

A2M

Only 8 laboratories could measure A2M, and only 2 of those measured it with the open instrument protocol. The 6 valid datasets provided acceptable results, but the uncertainty of the characterisation is higher than for most other proteins (see Table 12) because of the scattering of the values and the limited number of datasets (Fig. 4).

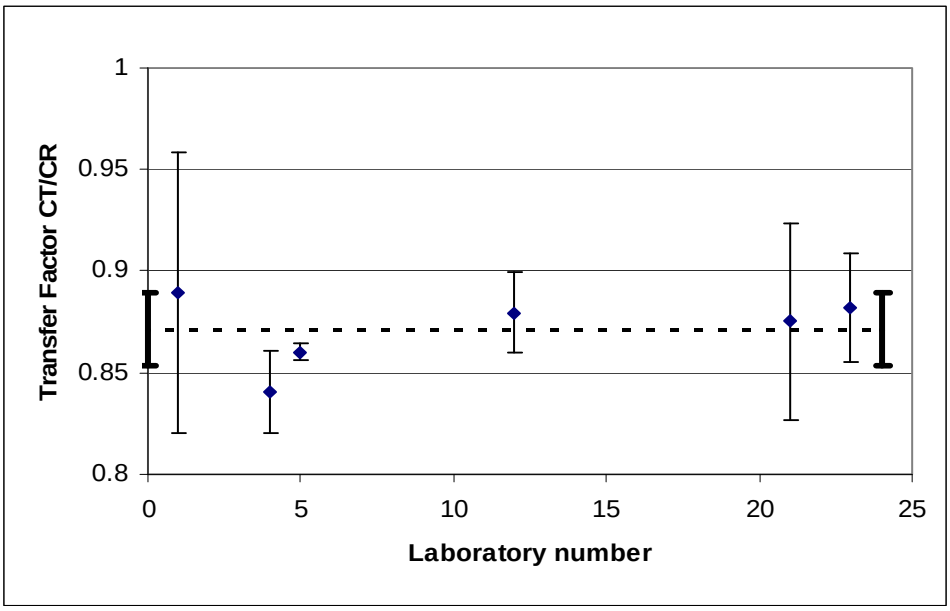

Figure 4: Results of the characterisation measurements for A2M The bars represent the laboratory means ± SD. The dotted line represents the mean of means, and the bars at the beginning and the end of the dotted line represents the mean of means ± SD.

AAG

For AAG the assigned value is the mean of means of 12 datasets. None of these was flagged as an outlier (Fig. 5).

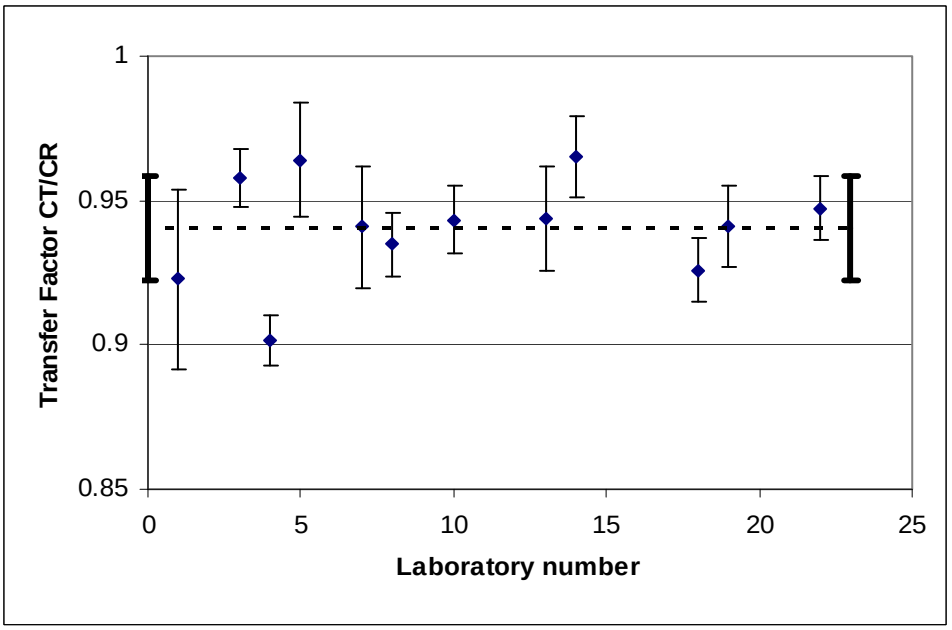

Figure 5: Results of the characterisation measurements for AAG The bars represent the laboratory means ± SD. The dotted line represents the mean of means, and the bars at the beginning and the end of the dotted line represents the mean of means ± SD.

AAT

For the measurement of AAT the mean value of laboratory 12 is an outlier (Fig. 6) at the 95 % confidence level according to Grubbs test results. Two other laboratories were using the same platform/reagent combination, and there is thus also no reason to suspect that the differing results are method dependent. The uncertainty of the value of Laboratory 12, as calculated from the day-to-day variation, is such that the 95 % confidence interval of the value covers the mean of means of all the laboratories. Therefore the value from Laboratory 12 was retained for the calculation of the mean of means.

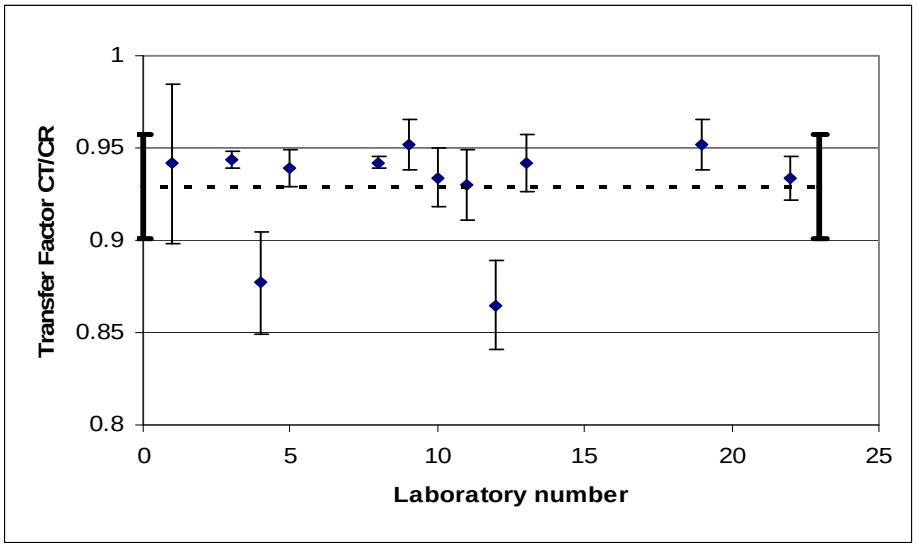

Figure 6: Results of the characterisation measurements for AAT The bars represent the laboratory means ± SD. The dotted line represents the mean of means, and the bars at the beginning and the end of the dotted line represents the mean of means ± SD.

ALB

The mean TFs of Laboratory 11 and 12 are outliers (Fig. 7) at the 95 % confidence level according to Grubbs test results, but they do not represent a single method. They are also within the 95 % tolerance interval, and values were accepted..

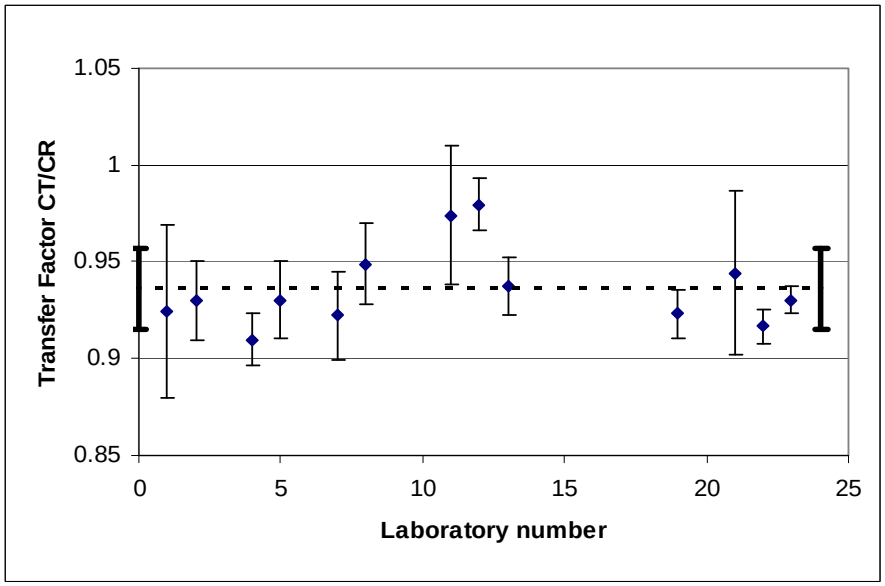

Figure 7: Results of the characterisation measurements for ALB The bars represent the laboratory means ± SD. The dotted line represents the mean of means, and the bars at the beginning and the end of the dotted line represents the mean of means ± SD.

C3c

The value for C3c is the mean of means of 12 valid datasets (Fig. 8). Many laboratories show a very low day-today variation for this protein, but the scatter of the laboratory means is not reduced.

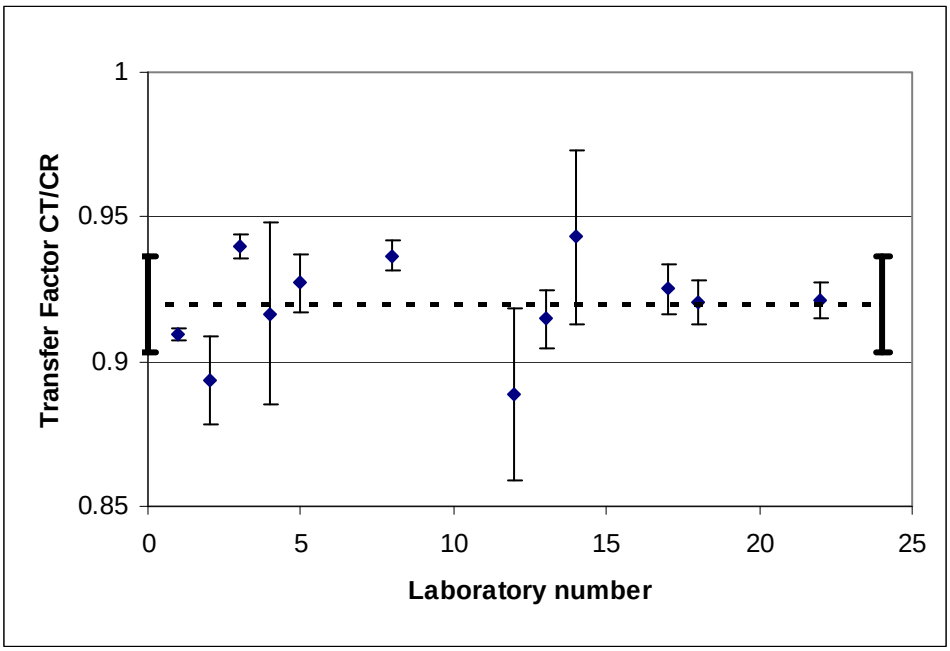

Figure 8: Results of the characterisation measurements for C3c The bars represent the laboratory means ± SD. The dotted line represents the mean of means, and the bars at the beginning and the end of the dotted line represents the mean of means ± SD.

C4

The value for C4 is the mean of means of 12 valid datasets (fig. 9). None of these were flagged as outliers.

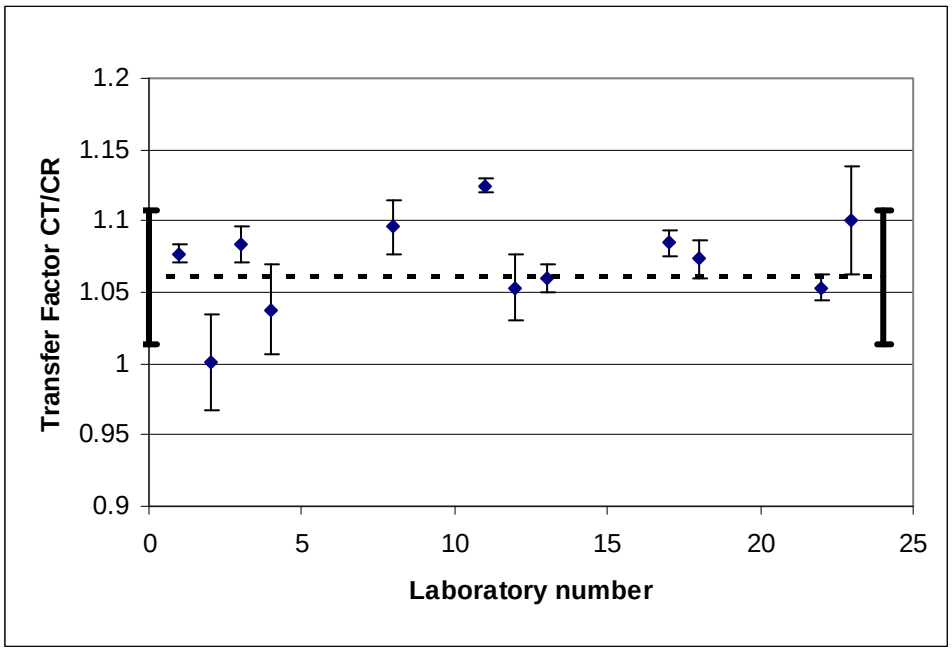

Figure 9: Results of the characterisation measurements for C4 The bars represent the laboratory means ± SD. The dotted line represents the mean of means, and the bars at the beginning and the end of the dotted line represents the mean of means ± SD.

HPT

The value for HPT is the mean of means of the results of 13 laboratories (Fig. 10).

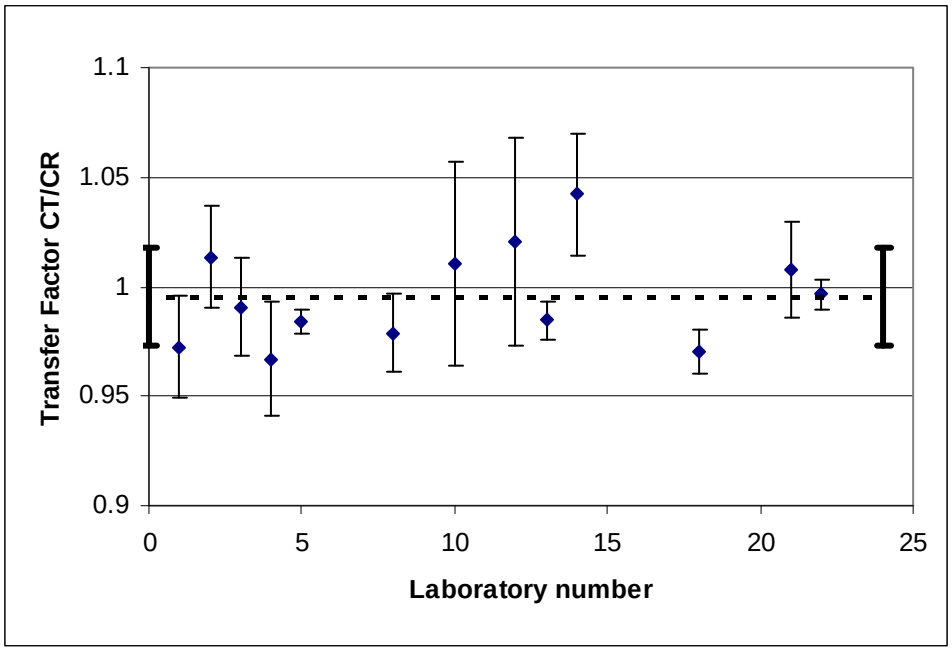

Figure 10: Results of the characterisation measurements for HPT The bars represent the laboratory means ± SD. The dotted line represents the mean of means, and the bars at the beginning and the end of the dotted line represents the mean of means ± SD.

IgA

There are data from 14 laboratories for IgA, and most of them cluster quite closely (Fig. 11). The mean TF of Laboratory 14 is an outlier at a 95 % confidence level according to Grubbs test results, and it is also a unique platform. However, as the day-to-day variation of Laboratory 14 is also high the 95 % confidence interval of this laboratory covers the mean of means, and Laboratory 14 was included for the calculation of the certified value.

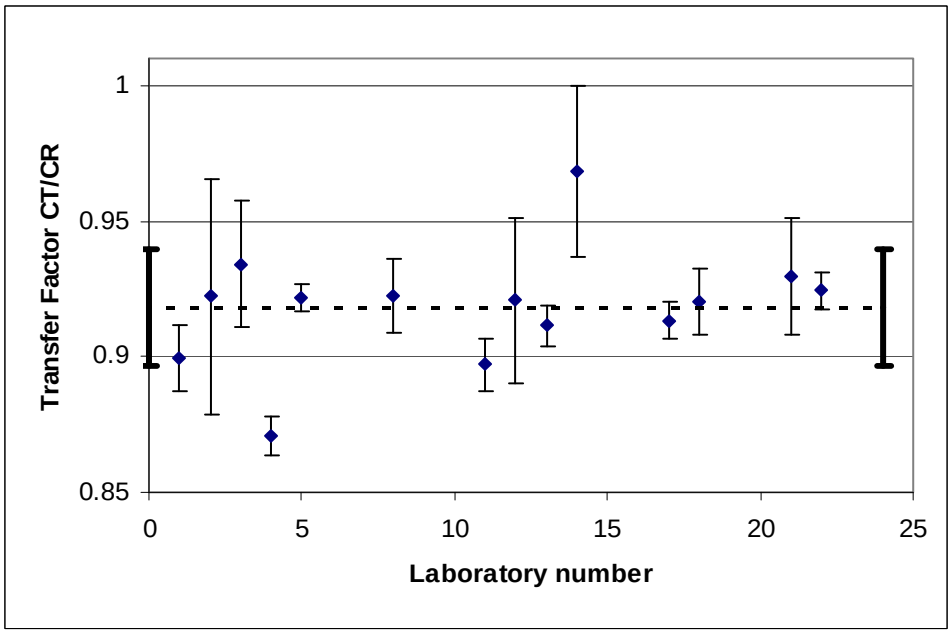

Figure 11: Results of the characterisation measurements for IgA The bars represent the laboratory means ± SD. The dotted line represents the mean of means, and the bars at the beginning and the end of the dotted line represents the mean of means ± SD.

IgG

The value assigned for IgG is the mean of means of the results of 14 laboratories (Fig. 12).

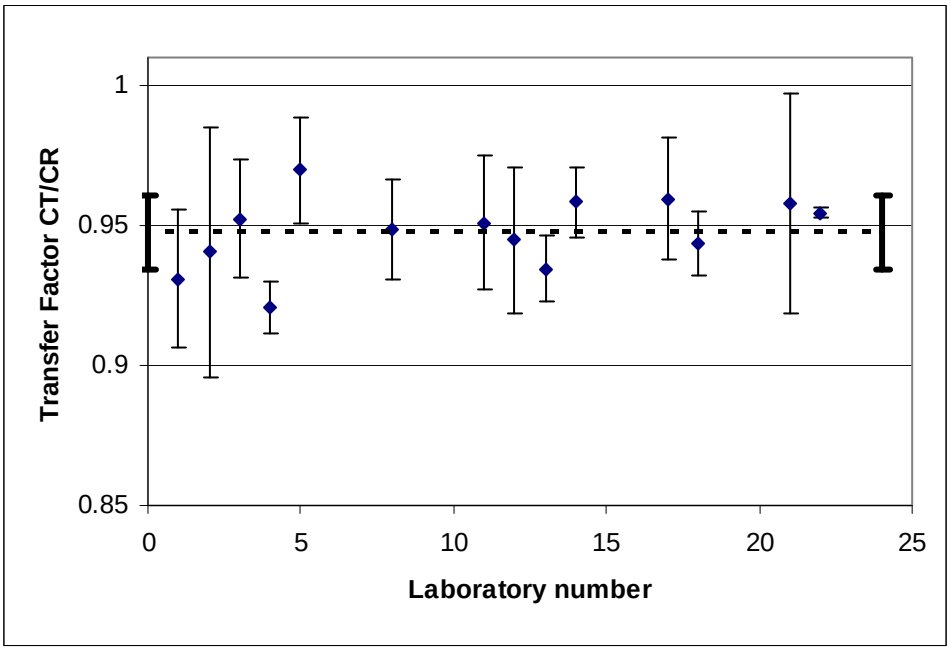

Figure 12: Results of the characterisation measurements for IgG The bars represent the laboratory means ± SD. The dotted line represents the mean of means, and the bars at the beginning and the end of the dotted line represents the mean of means ± SD.

IgM

The value assigned for IgM is the mean of means of the results of 11 laboratories (Fig. 13).

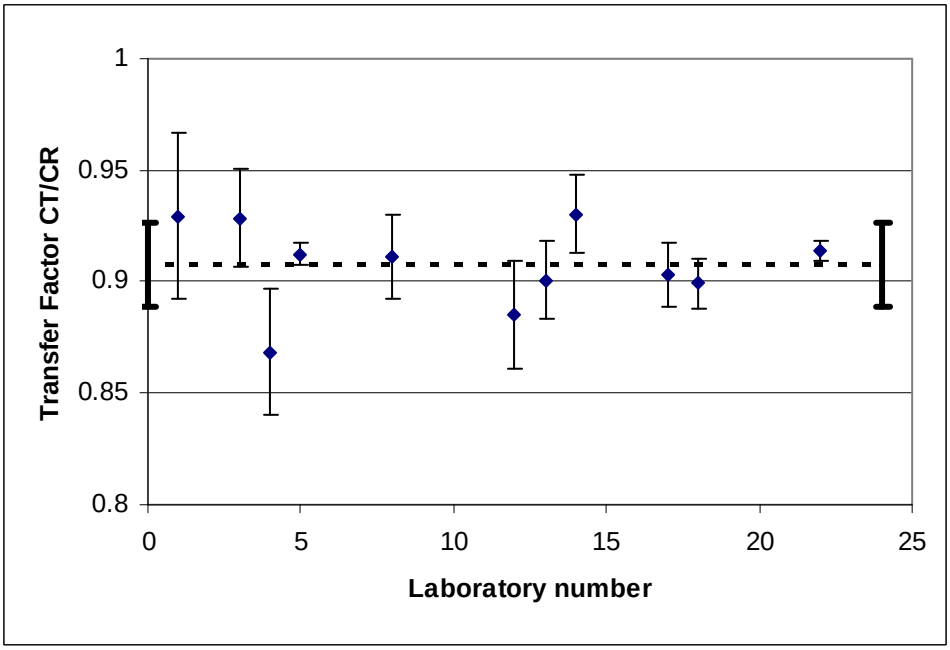

Figure 13: Results of the characterisation measurements for IgG The bars represent the laboratory means ± SD. The dotted line represents the mean of means, and the bars at the beginning and the end of the dotted line represents the mean of means ± SD.

TRF

The value assigned for TRF is the mean of means of the results of 11 laboratories (Fig. 14).

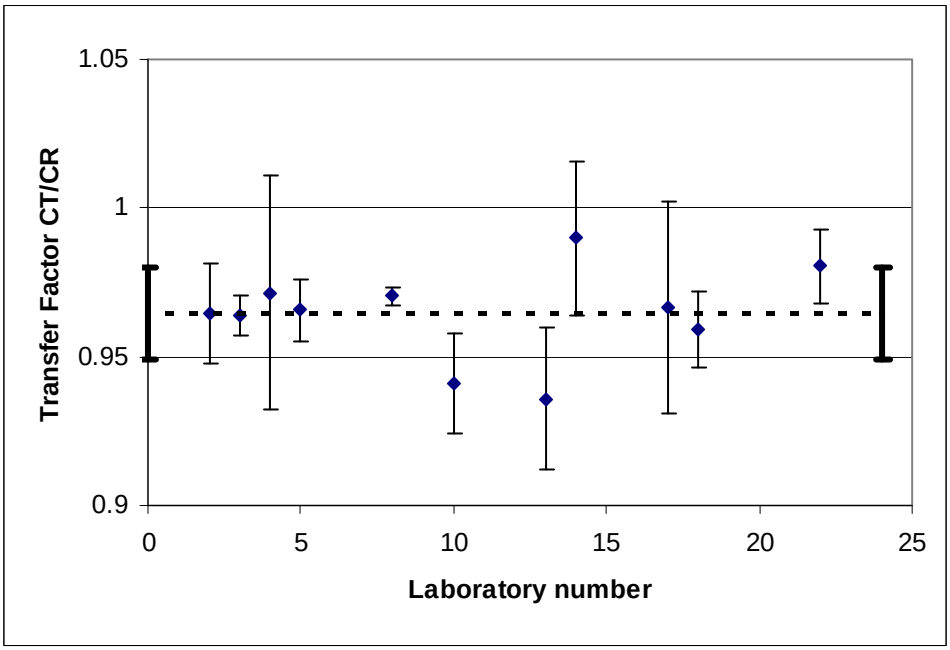

Figure 14: Results of the characterisation measurements for TRF The bars represent the laboratory means ± SD. The dotted line represents the mean of means, and the bars at the beginning and the end of the dotted line represents the mean of means ± SD.

TTR

The mean TF of Laboratory 12 is an outlier (Fig. 15) at the 95 % confidence level according to Grubbs test results, but as it does not represent a unique method, is within the 99 % confidence interval and within the 95 % tolerance interval the data of laboratory 12 were retained.

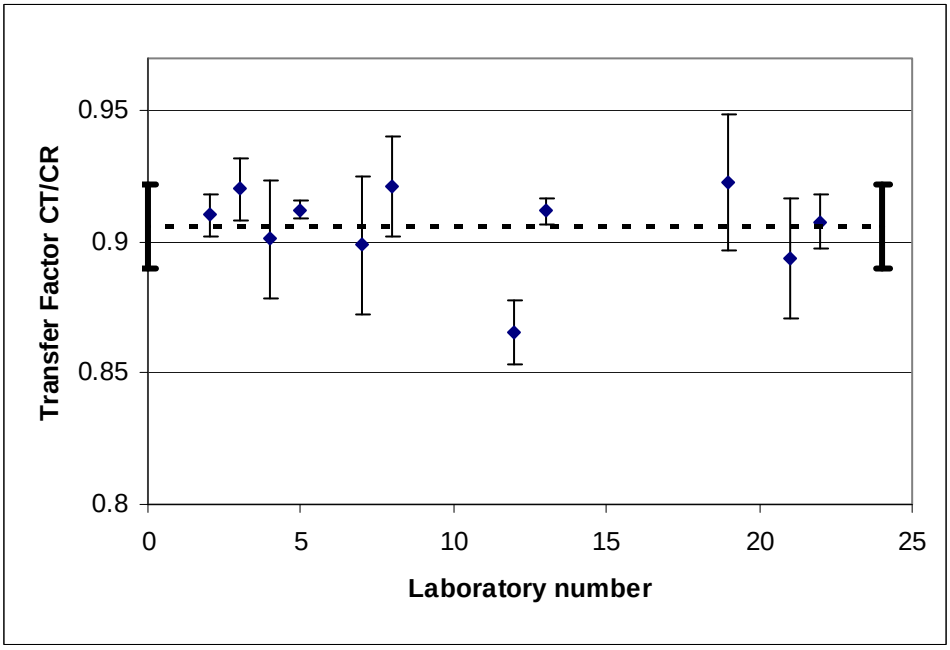

Figure 15: Results of the characterisation measurements for TTR The bars represent the laboratory means ± SD. The dotted line represents the mean of means, and the bars at the beginning and the end of the dotted line represents the mean of means ± SD.

6.5 Summary of the results of the characterisation study

The results of the characterisation study are the transfer factors TF = $C_T/C_{R,}$ determined for each protein. They are calculated as the mean of the means of the results per laboratory, using only the valid datasets. The relative standard uncertainty related to the characterisation u_{char} is taken as the relative standard error of the mean of the means of the valid datasets.

Table 12: Mean of means and uncertainty of the TFs, calculated using all valid results

Protein	TF		u_{char} [%]
	Mean of means	RSD [%]	
A2M	0.8711	2.047	0.836
AAG	0.9407	1.928	0.557
AAT	0.9293	3.026	0.874
ALB	0.9346	2.230	0.619
C3c	0.9197	1.828	0.528
C4	1.0704	3.022	0.873
HPT	0.9953	2.262	0.627
IgA	0.9183	2.364	0.632
IgG	0.9477	1.375	0.368
IgM	0.9076	2.109	0.636
TRF	0.9645	1.603	0.483
TTR	0.9060	1.801	0.543

7 Uncertainty budgets and certified values

7.1 Estimation of the uncertainties

The certified uncertainties consist of relative standard uncertainties related to characterisation (u_{char}), the relative standard uncertainties of the calibrant (u_{cal}), between-bottle heterogeneity (u_{bb}), and degradation during long-term storage (u_{lts}) [13].

- u_{char} was estimated as the relative standard error of the mean of laboratory means, i.e. SD/(p)$^{0.5}$ with RSD the relative standard deviation of the mean of means and p the number of datasets.
- u_{cal} was estimated as half the half-width of the 95 % confidence interval of the certified value of the protein in the calibrant, ERM-DA470, relative to the certified value.
- u_{bb} was estimated as the relative standard deviation between-units (s_{bb}) or the maximum heterogeneity potentially hidden by method repeatability (u^*_{bb}) as defined in Section 4.1 (Table 5). The higher of these two values was taken as a conservative estimate of potential heterogeneity.
- u_{lts} was estimated from stability tests and were taken from Table 8 (12 months stability study).

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

$$u_c = \sqrt{u_{char}^2 + u_{cal}^2 + u_{bb}^2 + u_{lts}^2}$$

The various uncertainty contributions and the relative combined standard uncertainty (u_c) are shown in Table 13.

Table 13: Uncertainty budget for ERM-DA470k/IFCC

Protein	u_{char} [%]	u_{cal} [%]	u_{bb} [%]	u_{lts} [%]	u_c [%]
A2M	0.836	1.53	0.517	0.669	1.93
AAG	0.557	0.382	0.484	0.632	1.04
AAT	0.874	0.456	0.675	0.410	1.26
ALB	0.619	1.01	0.612	0.567	1.45
C3c	0.528	1.24	0.557	0.445	1.52
C4	0.873	1.66	0.418	0.497	1.98
HPT	0.627	0.504	0.637	0.494	1.14
IgA	0.632	1.02	0.340	0.357	1.30
IgG	0.368	0.516	0.425	0.615	0.98
IgM	0.636	1.44	0.745	0.562	1.83
TRF	0.483	1.22	0.498	0.646	1.55
TTR	0.543	3.71	0.880	0.592	3.89

7.2 Certified values

For each protein the certified value (Table 14) was calculated from the TF (Table 12) and the certified value of the proteins in ERM-DA470 (Table 9) according to:

$$c_{\mathrm{ERM-DA470k}} = \mathrm{TF} \cdot c_{\mathrm{ERM-DA470}}$$

Relative expanded uncertainties were calculated from the relative combined standard uncertainty u_C (Table 13) by multiplication with a coverage factor k. This coverage factor was taken as 2. Relative expanded uncertainties were multiplied with the mean of retained dataset means to obtain absolute expanded uncertainties U_{CRM}.

Table14: Certified values and expanded uncertainties for mass fractions of proteins in ERM-DA470k/IFCC

Protein	Certified value [g/L]	U_{CRM} (k = 2) [g/L]
A2M	1.43	0.06
AAG	0.617	0.013
AAT	1.12	0.03
ALB	37.2	1.2
C3c	1.00	0.04
C4	0.162	0.007
HPT	0.889	0.021
IgA	1.80	0.05
IgG	9.17	0.18
IgM	0.723	0.027
TRF	2.36	0.08
TTR	0.220	0.018

8 Metrological traceability

The measurements of A2M, AAG, AAT, ALB, C3c, C4, HPT, IgA, IgG, IgM, TRF, and TTR were calibrated with ERM-DA470 applying the value transfer procedure 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 either immunochemical or visual spectrometry (only for ALB). Different combinations of reagents and platforms were used, and gave consistent results. Therefore the results are not dependent on the individual methods.

The value assignment measurements of A2M, ALB, C3c, C4, HPT, IgA, IgG, and IgM in ERM-DA470 had been calibrated with USNRP 12-0575C, also using immunochemical methods and a similar value transfer procedure [2]. The values of the proteins in the calibrant USNRP 12-0575C had been assigned on the basis of measurements by 24 laboratories using different in-house calibrants of unspecified origin and purity [3]. Therefore the traceability chain can not be extended further than to the USNRP 12-0575C, and the certified mass concentrations, of A2M, ALB, C3c, C4, HPT, IgA, IgG, and IgM in ERM-DA470k/IFCC are traceable to the stated values of the mass concentrations in the USNRP 12-0575C, applying the procedures described for the certification of ERM-DA470 and in the present report.

The certified values for AAG, AAT, TRF, and TTR in ERM-DA470 were obtained by calibration with pure proteins [14]. Consequently, the certified mass concentrations, for AAG, AAT, TRF and TTR in ERM-DA470k/IFCC are traceable to the International System of Units (SI), applying the procedures described in the certification report of ERM-DA470 [2] and in the present report.

9 Commutability

During the value assignment procedure it was found that there were no significant matrix effects. This was done by verifying that the regression line of the signal measured for the protein 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 methods (combinations of platforms and reagents) produced consistent results:

- Abbot Architect, Abbott reagents
- Beckman Immage, Beckman reagents
- BN II, Dade Behring reagents
- BN ProSpec, Dade Behring reagents
- Hitachi 717, MBL reagents
- Hitachi 917, DAKO reagents
- Hitachi 917, Denka Seiken reagents
- Hitachi 917, Roche reagents
- Hitachi 919, Nitto Boseki reagents
- LX-2200, Eiken reagents
- Roche Integra, Roche reagents

The equivalence of the material with patient samples has to be demonstrated for each particular assay. However, the results of external quality assurance schemes show that the inter-assay variation of the proteins certified has generally been significantly reduced during the long-term use of ERM-DA470 [4, 5]. As this is a strong indication of commutability ERM-DA470k/IFCC was produced in a similar manner, and it can be expected to be equally commutable.

10 Intended use and instructions for use

The material is primarily intended to be used for the calibration of immunoassay-based in-vitro diagnostic devices or control products for the proteins certified.

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

The entire content of the vial must be reconstituted.

Reconstitution of the material

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

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

Storage

Unopened ampoules should be stored at – (20 ± 2) °C. Under the condition that any microbial contamination during the reconstitution procedure has been excluded, the solution of ERM-DA470k/IFCC can be used for one week, as it was verified that changes to the certified concentration observed during that period are not significant. It is advisable to cover the vial with the original seal after use and to store it at 2 to 8 °C. 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 samples.

References and acknowledgements

References

1. H. Emons, T.P.J. Linsinger, and B.M. Gawlik
Reference Materials terminology and use. Can't one see the forest for the trees? Trends Anal. Chem. 23 (2004) 442 - 449

2. S. Baudner. J. Bienvenu, S. Blirup-Jensen, A. Carlström, A.M. Johnson, A. Milfor Ward, R. Ritchi, P.J. Svendsen, J.T. Whicher.
The certification of a matrix reference material for imunochemical measurement of 14 human serum proteins CRM470
EUR reports 15423 and 16882- European Community, Luxembourg - 1993

3. C. B. Reimer, S. J. Smith, T. W. Wells, R. M. Nakamura, P. W. Keitges, R. F. Ritchie, G. W. Williams, D. J. Hanson, D. B. Dorsey
Collaborative calibration of the U. S. National and the College of American Pathologists reference preparations for specific serum proteins. Am. J. Clin. Pathol. 77 (1982) 12 - 19

4. S.R. Goodall
Advances in plasma protein standardization. Ann. Clin. Biochem. 34 (1997) 582 - 587

5. T.B. Ledue and A.M. Johnson
Commutability of serum protein values: persisting bias among manufacturers using values assigned from the certified reference material 470 (CRM 470) in the United States. Clin. Chem. Lab. Med. 39 (2001) 1129 - 1233

6. S. Blirup-Jensen, A.M. Johnson, and M. Larsen
Protein standardization IV: Value transfer procedure for the assignment of serum protein values from a reference preparation to a target material. Clin. Chem. Lab. Med. 39 (2001) 1110 - 1122

7. H.G. Clarke and T. Freeman
Quantitative immunoelectrophoresis of human serum proteins. Clin. Sci. 35 (1968) 403 - 413

8. K. Fischer
Halbmikro-Bestimmung von Wasser. Pharmacopeia Deutsches Arzneibuch 10 (1991) 3.5.6

9. A. Lamberty, H. Schimmel, and J. Pauwels
The study of the stability of reference materials by isochronous measurements. Fresenius J. Anal. Chem. 360 (1998) 359 - 361

10. T. P. Linsinger , J. Pauwels, A. Lamberty , H. G. Schimmel , A. M. van der Veen , L. Siekmann
Estimating the uncertainty of stability for matrix CRMs. Fresenius J. Anal. Chem. 370 (2001) 183 - 188.A.

11 R.E. Lund
Tables for an Approximate Test for Outliers in Linear Models. Technometrics 17 (1975) 473 - 476

12. R. Beetham, P. White, P. Riches, D. Bullock, F. MacKenzie
Use of CRM 470/RPPHS has not achieved true consensus for ceruloplasmin measurement. Clin. Chem. 48 (2002) 2293 - 2294

13. J. Pauwels, H. Schimmel, and A. Lamberty
Criteria for the certification of internationally acceptable reference materials. Clin. Biochem. 31 (1998) 437 - 439

14. S. Blirup-Jensen
Protein standardization II: Dry mass determination procedure for the determination of the dry mass of a pure protein preparation. Clin. Chem. Lab. Med. 39 (2001) 1090 - 1097

Acknowledgements

The authors thank P. Corbisier and R. Zeleny from IRMM for the internal review of this report, and the experts of the Certification Advisory Panel ‘Biological Macromolecules and Biological/Biochemical Parameters’, R. Dybkaer (Frederiksberg Hospital, DK), A. Heissenberger (Umweltbundesamt, AT) and U. Örnemark (LGC Standards, SE) for reviewing of the certification documents. We also thank T. Linsinger (IRMM) for comments on the certification study. The authors are grateful for the active involvement of the participating laboratories in the characterisation study, and have appreciated the thoughtful discussions with Anders Larsson and Lars Hansson. The contribution of the Committee for Plasma Proteins of the IFCC throughout the certification project is gratefully acknowledged.

European Commission

EUR 23431 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: Certification of proteins in the human serum
Author(s): I. Zegers, W. Schreiber, J. Sheldon, S. Blirup-Jensen, A. Muñoz, G. Merlini, Y. Itoh, M. Johnson, S. Trapmann, H. Emons, H. Schimmel
Luxembourg: Office for Official Publications of the European Communities
2008 – 54 pp. – 21.0 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1018-5593
ISBN 978-92-79-09490-3
DOI 10.2787/63869

Abstract
The production and certification of ERM-DA470k/IFCC, a new serum protein reference material intended to replace ERM-DA470, 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 B2M and CRP and filled into vials (1 mL serum per vial). The serum was lyophilised in the vials and afterwards closed with rubber stoppers and screw caps and stored at -70 °C. The stability and homogeneity of the material were assessed for 14 proteins, including CER and B2M.
ERM-DA470k/IFCC was characterised for 12 proteins using the reference material ERM-DA470 as calibrant. This was achieved using a value transfer protocol that can be considered as reference procedure.
The techniques used to measure the protein concentrations were immunonephelometry, immunoturbidimetry, and for ALB also visible spectrometry. The measurements were performed with different platform/reagent combinations (Abbott Architect, Beckman Immage, BN II and BN ProSpec, different Hitachi instruments, Roche Integra, LX 2200, and Olympus AU640). In total 18 laboratories participated in the value assignment, 4 using open value transfer procedures, 12 using closed value transfer procedures, and 2 using both open and closed procedures.
The results show that open and closed value transfer procedures give very similar results, and lead to robust values for A2M, AAG, AAT, ALB, C3c, C4, HPT, IgA, IgG, IgM, TRF, and TTR.

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.

JRC
EUROPEAN COMMISSION

LA-NA-2431- EN- C

ISBN 978-92-79-09490-3

Publications Office
Publications.eu.int