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CERTIFICATION REPORT

Certification of cystatin C in the human serum reference material ERM®-DA471/IFCC

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Certification of cystatin C in the human serum reference material ERM®-DA471/IFCC

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Abstract

The production of ERM-DA471/IFCC, certified for the mass concentration of cystatin C, is described.
Serum was produced from blood collected in 2 collection centres according to a procedure ensuring that it was obtained from healthy donors, and that the lipid content of the serum was low. The serum was processed, spiked with recombinant cystatin C, and lyophilised. It was verified that the material is homogenous and stable.
The material was characterised using a pure protein primary reference preparation (PRP) as calibrant. The PRP was prepared from recombinant cystatin C, and its concentration determined by dry mass determination. The characterisation of ERM-DA471/IFCC was performed by particle enhanced immuno-nephelometry, particle enhanced immuno-turbidimetry and enzyme amplified single radial immuno-diffusion.
The certified cystatin C mass concentration in ERM-DA471/IFCC, if reconstituted according to the specified procedure, is 5.48 mg/L, the expanded uncertainty (k = 2) is 0.15 mg/L.

GLOSSARY

ANOVA	analysis of variance
b	slope in the equation of linear regression $y = a + bx$
β	slope of the linear regression $y = \beta x$
Bit	unit of the analog-to-digital converter of the light detector
c	mass concentration $c = m / V$ (mass / volume)
C3	complement 3
C3c	complement 3c
CASO	Casein Soy
CRM	Certified Reference Material
CysC	cystatin C
ERM	European Reference Material
GFR	glomerular filtration rate
HBV	hepatitis B virus
HBsAg	hepatitis B surface antigen
HCV	hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IRMM	Institute for Reference Materials and Measurements
IU	International Units
IVD	In Vitro Diagnostic
k	coverage factor
kDa	kilo Dalton
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
PAGE	polyacrylamide gel electrophoresis
PRP	primary reference preparation
RF	rheumatoid factor
RSD	relative standard deviation
s_{bb}	standard deviation between bottles
$s_{bb, rel}$	relative standard deviation between bottles
SDS	sodium dodecyl sulfate
SRID	single radial immuno-diffusion
s_{wb}	standard deviation within bottles
SI	International System of Units
TM	target material
TRIS	tris(hydroxymethyl)aminomethane
U	expanded uncertainty
$u_{bb, rel}$	relative standard uncertainty related to the between-bottle heterogeneity
$u^*_{bb, rel}$	relative standard uncertainty related to the between-bottle heterogeneity that can be hidden by the method repeatability
u^*_{bb}	relative standard uncertainty related to the between-bottle heterogeneity that can be hidden by the method repeatability
$u_{c, rel}$	relative combined standard uncertainty
$u_{dm, cal, rel}$	relative standard uncertainty related to the characterisation of the calibrant
$u_{purity, cal, rel}$	relative standard uncertainty related to the purity of the calibrant
$u_{char, rel}$	relative standard uncertainty related to the characterisation
$u_{lts, rel}$	relative standard uncertainty related to the long-term stability of the material
$u_{sts, rel}$	relative standard uncertainty related to the short-term stability of the material

1 Introduction and design of the project

1.1 Background: Need for the CRM

Cystatin C is a nonglycosylated, low molecular mass (13 kDa) protein produced by all nucleated cells. It is a protease inhibitor, and inhibits lysosomal cysteine proteases by binding reversibly to them. Its low molecular mass and its high isoeletric point allow it to be freely filtered by the renal glomerular membrane.

The immunoassays routinely used for measuring the mass concentration of cystatin C give results that show a high between-method variation in External Quality Assessment Schemes. The availability of a validated international calibrator for cystatin C would allow elimination of the problems generated by the use of different cystatin C calibrators and non-agreeing determination methods and thus promote the worldwide implementation of improved methods for non-invasive glomerular filtration rate (GFR) estimation, a measure of the capacity of the kidneys to filter plasma (GFR) [1, 2].

Plasma (or serum) cystatin C has been proposed as a marker for the GFR. Several studies (e.g. [3]), as well as one meta-analysis [4], have suggested that it is superior to serum creatinine for estimation of GFR. Particularly, cystatin C could be used for children, elderly, patients with low muscle mass, and in the early stages of kidney problems, where creatinine measurements do not perform well. Additionally cystatin C is used as a marker for cardiovascular risk [5] and pre-eclampsia [6]. Knowledge of the GFR is of crucial importance in the management of patients. In addition to a general evaluation of the kidney function, a more accurate assessment is valuable on many occasions, e.g., to allow correct dosage of drugs cleared by the kidneys, to detect early impairment of renal function, to prevent further deterioration, to manage renal transplant patients, and for the use of potentially nephrotoxic radiographic contrast media. The determination of GFR with high accuracy requires the use of invasive techniques based on measuring the clearance of injected substances that are exclusively excreted via glomerular filtration, *e.g.,* inulin, ^{51}Cr- ethylenediaminetetraacetic acid, ^{99m}Tc-diethylenetriaminepentaacetic acid, or radiographic contrast media such as ^{125}I-iothalamate and iohexol. These procedures are labour-intensive and not free of risks for the patients. The plasma or serum concentrations of endogenous substances, particularly creatinine, have therefore been used as markers for GFR for more than a century. However, it has become evident that the creatinine concentration is far from ideal as a GFR marker because it is significantly influenced not only by GFR but also by factors such as muscle mass, diet, gender, age, race and tubular secretion [7-9].

The level of cystatin C is less dependent upon anthropometric data than the level of creatinine and thus simpler cystatin C-based GFR prediction equations of the type GFR = A x cystatin C^{-B} can be used [7-9]. It is nevertheless problematic that a large number of different cystatin C-based GFR-prediction equations have been proposed. The reasons for the variability of the suggested cystatin C-based GFR-prediction equations are the use of different cystatin C calibrators, the use of different, non-agreeing methods for determination of cystatin C levels, the use of different mathematical models to generate the prediction equations and the use of different patient populations. Although the large number of proposed cystatin C-based GFR-prediction equations is problematic *per se* in the implementation of cystatin C as a GFR-marker in the clinical routine, an even bigger problem is that it may generate large errors in the GFR estimates produced. For when a prediction equation, based upon a specific cystatin C calibrator and method, is used to estimate GFR from cystatin C levels produced using another cystatin C calibrator and method, large errors in the resulting GFR-estimates may result even for similar patient populations.

The EU Directive on In Vitro Diagnostic Medical Devices (IVD-MD) (Directive 98/79/EC) requires the metrological traceability of calibrators and control materials through reference

measurement procedures and/or reference materials of higher order. The requirements for a calibrator for immunoassays are that the assignment of values is metrologically traceable, and is accompanied by an uncertainty statement, that the stability and homogeneity with respect to all the certified properties are verified, and that the material is commutable, i.e. resembles the patient samples [10].

1.2 Choice of the material

A material to be used to calibrate serum protein immunoassays has to be designed considering that:

- the concentration of the protein in the final material should be high enough so that dilutions of the material can cover a significant 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.

The certification project first involved the characterisation of a primary reference preparation (PRP), consisting of liquid frozen recombinant cystatin C. Values were assigned to this preparation by dry mass determination [11].

Serum was produced from blood collected in collection centres in Lund (Sweden) and Marburg (Germany).

The serum was processed, spiked with recombinant cystatin C and filled into vials (1 mL serum per vial) with screw caps. The serum was lyophilised in the vials and stored at (-70 ± 10) °C.

The material was characterised by transferring the values from the PRP to the candidate reference material (ERM-DA471/IFCC). This was achieved using a value transfer procedure that can be considered as a reference procedure [12].

The principles used to measure the protein concentrations were enzyme amplified single radial immuno-diffusion (SRID), particle enhanced immuno-nephelometry and particle enhanced immuno-turbidimetry, as implemented on different measuring systems (BN II™, and BN ProSpec®, Hitachi 917, Integra 800). In total 4 laboratories participated in the characterisation, and 7 datasets were used.

2 List of participants

Provision and characterisation of cystatin C

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3 Processing

3.1 Materials

3.1.1 Production of the serum

The starting material for the reference material was serum prepared from the blood of healthy donors, collected in Lund, SE and Marburg, DE (Table 1).

Selection of the donors:

- Sex — both sexes
- Age — 20 – 70 years
- Blood group — known
- RF — < 30 IU/mL
- Monoclonal components — absent (checked by zone electrophoresis)
- Bilirubin — serum colour visually normal
- Hemoglobin — serum colour visually normal
- Lipemia — no turbidity observed
- Infectious agents — negative for: HBsAg, anti HIV 1, anti HIV 2 and anti HCV

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

The following protocol was applied for preparing the serum:

a) Each whole blood sample was collected into a glass container that was suitable for centrifugation.
b) Clotting: at least 5 hours at room temperature.
c) First centrifugation at 1750 *g* for 35 minutes.
d) After centrifugation the supernatant of the serum was transferred immediately to another container.
e) Second centrifugation at 6850 *g* for 30 minutes.
f) The supernatant of the serum was removed rapidly and the benzamidinium chloride brought to 1 mmol/L and NaN_3 to 15 mmol/L.
g) 5 aliquots of 0.5 mL were taken from each donor for testing.
h) The serum was frozen immediately and stored individually at (-70 ± 10) °C until further processing, performed in 2006.

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

Collection centre	Donation period	No.	Male	Female	RF >30 IU/mL	Monoclonal components	Age	Final No.
Malmö (SE)	02/2004 to 05/2005	30	26 (25)	4 (4)	0	1	20-64 years	29
Marburg (DE)	09/2006	18	12 (11)	6 (4)	0	0	33-57 years	15

Tests of the serum: The blood collection centres tested each donation for anti HIV 1+2, HBsAg, and anti HCV. Monoclonal gammopathies were detected by gel electrophoresis.

One donation was discarded because of the presence of monoclonal components, two donations were discarded because information on the blood group were not available and one donation was discarded because the donation was found to be slightly opalescent upon visual inspection. 44 donations, of which 8 originated from female donors, were fulfilling the inclusion criteria, and were released for further processing.

3.1.2 Production of cystatin C and the Primary Reference Preparation

Recombinant human cystatin C was produced by expression in *E. Coli* according to [13]. Cystatin C was purified from the cell extract using dialysis, anion- and cation exchange chromatography and gelfiltration. The purified cystatin C was filled into bottles and lyophilised. The lyophilised cystatin C was reconstituted using 0.1 mol/L KCl, dialysed against 0.1 mol/L KCl for 4 days with repeated replacements of the solvent and finally adjusted to a mass concentration of approximately 5.2 g/L as measured by refractometry. For use in the characterisation procedure the solution was aliquoted in vials of 0.2 mL and labelled Lot 11082006.

5835 mg of a recombinant cystatin C solution (cystatin C spiking material Lot: 11082006-2; mass concentration about 5.2 g/L cystatin C (PRP) Dako Denmark, Glostrup (DK)) were used for spiking. The material was stored at (-70 ± 10) °C and thawed at 37 °C with occasional inversions. Before spiking the stabilised serum matrix, the cystatin C solution was gently mixed while avoiding any turbulence.

3.2 Processing of the serum

Overview of the procedure

The serum donations from both collection centres were pooled and processed as one pool. The processing procedure followed largely the one used for CRM 470 (ERM-DA470) [14]. The major differences were that there was no conversion of C3 to C3c performed, that after the delipidation step a dialysis membrane with a cut-off of 10 kDa was used instead of 12-14 kDa, and that the final sodium azide mass concentration was kept below 1 g/L. Particulate matter and lipids were removed and preservatives (sodium azide, aprotinin and benzamidine hydrochloride) were added. The stabilised serum matrix was spiked with recombinant cystatin C, sterile filtrated, filled into bottles and lyophilised.

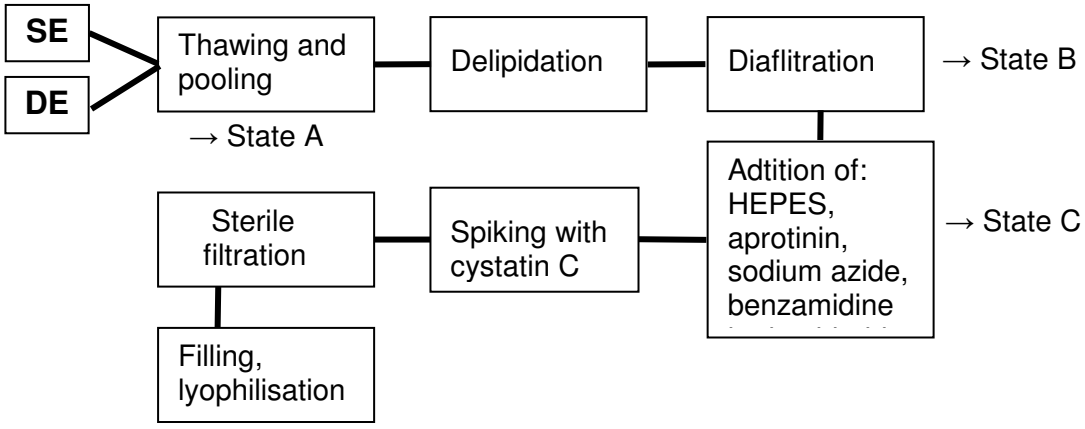

Figure 1: Overview of the processing of the serum.

3.2.1 Preparation of the stabilised serum matrix

Donations fulfilling the criteria were processed as follows:

- thawing at 25 °C in a water bath
- centrifugation at 11000 *g* for 35 minutes
- pooling of the supernatant of all accepted donations
- removal of aliquots for the measurement of the concentrations of cholesterol, triglycerides, apolipoprotein A-I, apolipoprotein B and total protein (Biuret reaction).

The material is now in state A.

Delipidation with Aerosil

- addition of sodium chloride (crystalline; Merck, Darmstadt, DE) to a final concentration of 50 g/L while stirring constantly
- adjustment of the pool to pH 8.5 ± 0.1 with a saturated tris(hydroxymethyl)aminomethane (TRIS) solution (Merck, Darmstadt, DE)
- calculation of the required amount of Aerosil 200 (Degussa, Frankfurt, DE) based on the total protein concentration of the pool (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 60 minutes
- removal of Aerosil (Degussa, DE) and protein precipitates by centrifugation for 30 minutes at about 10000 *g* using 500 mL centrifugation bottles
- determination of the total volume of the clear supernatant
- determination of the concentrations of cholesterol, triglycerides and apolipoproteins A-I and B to verify the successful lipid removal.

Filtration and diafiltration

- filtration of the supernatant using a folded filter S&S Type 520½ (GE Healthcare Europe GmbH, Freiburg, DE) to remove small particles interfering with the following diafiltration
- diafiltration (Centrasette with Omega membrane with a relative molecular mass cut-off of 10000; Pall, Hauppauge, USA) using an isotonic sodium chloride solution to remove TRIS and Aerosil-derived free silica; this step combines buffer exchange and adjustment to the desired volume of the serum
- removal of aliquots for the measurement of total protein and for the measurement and physicochemical analysis of selected proteins.

The material is now in state B

Preservation

- addition of sodium azide (Merck, Darmstadt, DE) to achieve a final amount of substance concentration of 14.6 mmol/L (0.95 g/L)
- addition of aprotinin (Trasylol 500000 KIU/L, Bayer, Leverkusen, DE) to achieve a final concentration of 80000 KIU/L
- addition of benzamidine hydrochloride monohydrate (Merck, Darmstadt, DE) to a final amount of substance concentration of 1 mmol/L
- adjustment of pH to 7.2 ± 0.1 with saturated 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Calbiochem, Merck Biosciences, Schwalbach, DE)
- removal of 200 mL for the preparation of the pilot batch PB001
- preparation of 500 mL aliquots of the stabilised serum matrix; these aliquots were stored at (-70 ± 10) °C until processing to the final reference material.

The material is now in state C.

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

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

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

Blank signal: According to the specifications for the measuring system concerned an empty cuvette is considered adequately inserted and optically clear if the scattered light gives a signal between 20 and 600 Bit.

3.2.2 Preparation of the candidate reference material

- thawing and pooling of the aliquots of stabilised serum matrix (state C) at room temperature (24 hours)
- preparation of the recombinant cystatin C as described under 3.1.2
- slow addition of the total amount of the solution of recombinant cystatin C while constantly stirring
- sterile filtration (0.2 µm)
- sterile removal of aliquots for further analysis

Filling was performed under controlled conditions. 1 mL serum was filled into uncoloured GW 2,5 threaded and siliconised glass bottles (ISO Gesellschaft für Arzneimittel mbH; Bad Königshofen, Germany), grey chlorobutylcaoutchouc GT rubber stoppers (West Pharmaceutical Services, Eschweiler, Germany) were used.
A part of this material was not lyophilised, but left in liquid form and stored frozen at (-70 ± 10)°C.

Within one hour after filling the lyophilisation program was started with cooling the serum to -40 °C over one hour. The vacuum equilibration was 10 to 30 Pa over 18 hours. Thereafter temperature was changed in successive steps to +25 °C over 22 hours. Capping of the bottles was performed under a low-pressure nitrogen atmosphere.

Final capping was performed using yellow polypropylene screw caps, GL14 x 2.5 (Ki-Si-Co GmbH, Oestrich-Winkel, Germany) The vials were finally labelled according to the filling order and stored frozen at (–70 ± 10) °C.

3.2.3 Processing control

Total protein mass concentration: In line with the protocol of the preparation of ERM-DA470 a total protein mass concentration between 60 and 80 g/L is considered acceptable for the starting material for processing, and the Aerosil treated pool should be adjusted to between 55 and 75 g/L. The total protein mass concentration of the pool was 78 g/L in state A and 64 g/L in state C, within the defined tolerance limits.

Delipidation: The delipidation by the Aerosil treatment is considered successful when the final mass concentrations of cholesterol and apolipoprotein B relative to the concentration in the starting material are reduced to below 1 %. 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 2, these criteria were fulfilled. The reduction of the blank value obtained by measuring the signal of undiluted sample using a BN100 System demonstrated the effective removal of lipoproteins. The Aerosil treatment also reduced the cystatin C concentration in the material (Table 3).

Table 2: Lipoproteins mass concentrations and blank signal measured with a nephelometer before processing (state A) and after Aerosil treatment (state B)[1]

state A					state B				
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]
1969	1094	1550	857	846	ND	ND	ND	ND	83

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

Table 3: Cystatin C mass concentrations before and after Aerosil treatment

cystatin C [mg/L]	
state A	state B
0.79	0.24

4 Homogeneity

4.1 Homogeneity

The homogeneity of ERM-DA471/IFCC was verified by measuring mass concentrations of cystatin C in triplicate in 20 vials taken randomly over the whole batch. The measurements were performed with a Hitachi 917 instrument and Dako reagents. The protein concentrations were corrected for deviations from the reconstitution protocol using the mass of the water used to reconstitute each sample.

It was checked whether the data followed a normal or other unimodal distribution using normal probability plots and histograms. The individual data and the bottle means were unimodal, except for one outlying value.

Grubbs tests were performed to detect outlying individual results as well as means measured for each vial. For one vial the mean was an outlier at the 95 % confidence level, but was retained in the analysis. There was no significant trend either in the analysis sequence or in the filling sequence.

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: $s_{bb,\,rel}$ = 0.294 %, and $u^*_{bb,\,rel}$ = 0.151 %. The larger of both values was included into the calculation of the overall uncertainty of the certified values (Section 7.1).

4.2 Minimum sample intake for analysis

The reconstituted material forms a clear solution, and a true solution is not expected to have any relevant heterogeneity in protein concentration at sample intakes even below nL volumes. The sample intake of the homogeneity study on the material measured with a Hitachi 917 platform was 2 µL. The standard deviation within a bottle (s_{wb}) is lower or equal to the expected method variability (data not given), so there is no indication of intrinsic heterogeneity 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 [15] that consists of the simultaneous analysis of reference and test samples. For each study a defined set of samples was exposed to elevated temperatures for different periods of time and then brought back to the reference temperature. At the end of the study all samples were analysed for the concentrations of cystatin C within one analytical run. The data were analysed by determining the regression line for the protein concentration as a function of time [10].

5.1 Short-term stability

A short-term stability study was performed in order to assess the possible effect of transport at different temperatures on the stability of the material. 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 -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 cystatin C using a Hitachi 917 measuring system with Dako reagents. The values were corrected for the actual reconstitution volume. The results are shown in Table 4.

Table 4: Short-term stability study: slope (*b*), test for significance of the slope ($|b/u_b|$), and relative standard uncertainty after two weeks storage at the specified temperature $u_{sts,\,rel}$

temperature [°C]	*b* [(mg/L)/week]	$\lvert b/u_b \rvert$	$u_{sts,\,rel}$ [%]
-20	-0.018	3.66	0.39
4	-0.003	0.38	0.26
60	-0.022	5.50	0.46

For each temperature there were 24 measurements, or 22 degrees of freedom for the linear regression. The slope of the protein concentration as a function of time is significantly different from 0 when the absolute value of slope *b* divided by its uncertainty u_b ($|b/u_b|$) is larger than $t_{0.05,\,22} = 2.07$. When samples were kept at 4 °C the slope was not significantly different from 0. At -20 and 60 °C significant (at a 95 % confidence level) negative slopes were found although even at 60 °C the added uncertainty 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-DA471/IFCC has been produced following the procedures for ERM-DA470 [14] 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.

6 months and 1 year stability studies were performed in order to confirm the stability of the new material upon storage at -20 and 4 °C. The reference temperature was (-70 ± 10) °C. The test samples were kept for 2, 4, and 6 months at -20 and 4 °C for the 6 months stability study, and for 4, 8, and 12 months for the 1 year stability study. For each combination of time and temperature 2 samples were analysed in triplicate. The samples were analysed for cystatin C using a Hitachi 917 measuring system with Dako reagents.

For each temperature there were 24 measurements, or 22 degrees of freedom for the linear regression. The slope of the protein concentration as a function of time is significantly

different from 0 when the absolute value of slope *b* divided by its uncertainty u_b ($|b/u_b|$) is larger than $t_{0.05, 22} = 2.07$. None of the slopes (protein mass concentration versus time) was significantly different from 0 at a 95 % confidence level (Table 5). 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 or 12 months. The results indicate that it is safe to store the material at either -20 or 4 °C.

Table 5: Long-term stability study: slope (b), test for significance of the slope ($|b/u_b|$), and relative standard uncertainty contribution $u_{lts, rel}$ after storage for 6 months or 1 year at the specified temperature

6 months				1 year			
tempe-rature [°C]	*b* [(mg/L)/month]	$\|b/u_b\|$	$u_{lts, rel}$ [%]	tempe-rature °C	*b* [(mg/L)/month]	$\|b/u_b\|$	$u_{lts, rel}$ [%]
-20	0.002	0.28	0.678	-20	-0.002	0.55	0.733
4	0.009	1.85	0.491	4	-0.004	1.45	0.591

6 Characterisation

6.1 General principles for the value assignment

The characterisation of cystatin C in the human serum reference material ERM-DA471/IFCC is performed according to the theoretical and practical principles as documented in [12] and [16]. The characterisation is achieved by transferring values from the primary reference preparation (PRP) to the candidate reference material. The PRP is a solution of the pure protein in 0.1 M KCl. It is spiked at different concentrations in a matrix background to prepare dilutions that are measured as samples in the measuring system. The dose-response function of the measuring system is determined by using dilutions of the candidate reference material.

The practical transfer protocol is based on multiple measurements of 6 dilutions of the primary reference preparation and 6 dilutions of the candidate reference material under repeatability conditions. The transfer protocol requires several measurements a day repeated on several days, an important prerequisite being that all reconstitutions and dilutions are controlled by weighing, thus reducing uncertainty in the transfer.

The reference preparation is defined as the protein preparation with known concentration value. It can either be an international reference material or a pure protein preparation. For this characterisation study a special preparation of pure cystatin C was produced and the concentration of cystatin C was obtained using dry mass determination according to [11].

Target material is defined as the serum protein matrix material with unknown concentration value. In this case it is the new matrix material ERM-DA471/IFCC.

The transfer method should be based on well established and recognised methods used in clinical chemistry. In this characterisation study particle enhanced immuno-turbidimetry, particle enhanced immuno-nephelometry and enzyme amplified single radial immuno-diffusion were used.

Slight variations in the assay conditions, in the programming of the instruments or in the reagents may lead to different results. This has lead to the prerequisite of method standardization [17]. To minimise all of these factors contributing to variation, an optimised and practical transfer protocol was made with detailed instructions and emphasis on weighing all solutions used to prepare dilutions. To record the obtained data, special registration forms were made also containing the intended dilution schemes. These raw data forms were subsequently sent for data treatment.

When assigning a value to a target material (a serum) using a pure protein preparation the indirect value transfer procedure is used [12]. In this procedure dilutions of the target material (TM) are used to determine the dose-response function for the instrument or the assay. Dilutions of the primary reference preparation (PRP) are then measured against this calibration curve in the following way:

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

$$C_{TM}(i) = F_{TM}(i) \times C_{TM} \quad (1)$$

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

2. A series of 6 test samples are prepared by adding increasing amounts of the primary reference preparation to 6 different test tubes. The final volume of all preparations is then adjusted to be the same by adding the dilution solution. The dilutions are made in such a

way that the signals will fall within the signal interval of the dose-response curve. The concentration of the samples will be:

$$C_S(j) = F_{PRP}(j) \times C_{PRP} \quad (2)$$

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

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

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

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

5. Since the unknown concentrations in $C_S(j)$ can be either calculated using equation (2) or found by interpolation on the dose-response curve (3) a combination of (2) and (3) will give:

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

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

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

6. The different dilution factors $F_{TM}(j)$ obtained by the interpolation are now plotted against the different dilution factors $F_{PRP}(j)$ used for the initial dilution of PRP. Since all dilutions are controlled by weighing the uncertainty of $F_{PRP}(j)$ is negligible.
If the two materials behave similarly in the method (i.e. there are no matrix effects) the regression line will pass through zero with a slope (β) equal to the ratio of the concentrations of the specific protein in the two materials:

$$\beta = C_{PRP} / C_{TM} \quad (5)$$

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

$$C_{TM} = C_{PRP} / \beta \quad (6)$$

The final characterisation study was conducted using the PRP to assign values to ERM-DA471/IFCC. The methods including instruments, reagents and laboratories are listed in Table 7.

The practical procedures (the transfer protocols) are based on the following concept, where the basic set-up is repeated 3 times each day over 4 days:

Target material: 6 different dilution levels of the ERM-DA471/IFCC are used as standards for the dose-response curve, covering the actual measuring interval.

Reference preparation: 6 different dilution levels of the primary reference preparation for cystatin C are measured as samples. The dilutions are made in such a way that the signals will fall within the signals of the dose-response curve i.e. within the measuring interval. The samples are measured in duplicate.

Control: One selected dilution of the target material is measured in duplicate.

Each of the 4 days (6 days for dataset 2) a new vial of the reference preparation and the target material is used and a new set of dilutions is prepared. Each day 3 calibrations are performed together with determinations of samples and controls (i.e. 3 runs).

For SRID measurements a slightly modified procedure was developed. Five dilutions of target and reference materials were measured in triplicate, in one run per day.

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

6.2 Characterisation of the primary reference preparation

Dry mass determination

Dry mass determination was started immediately after filling the vials of PRP. It was performed according to [11] and documented in [18]. All weighings were carried out on an analytical balance. Pyrex glass vials with fitted lids were filled with 3 mL of either protein solution or solvent. The sequence of weighing of vials *(Empty – Protein Solution – Solvent)* was repeated four times.

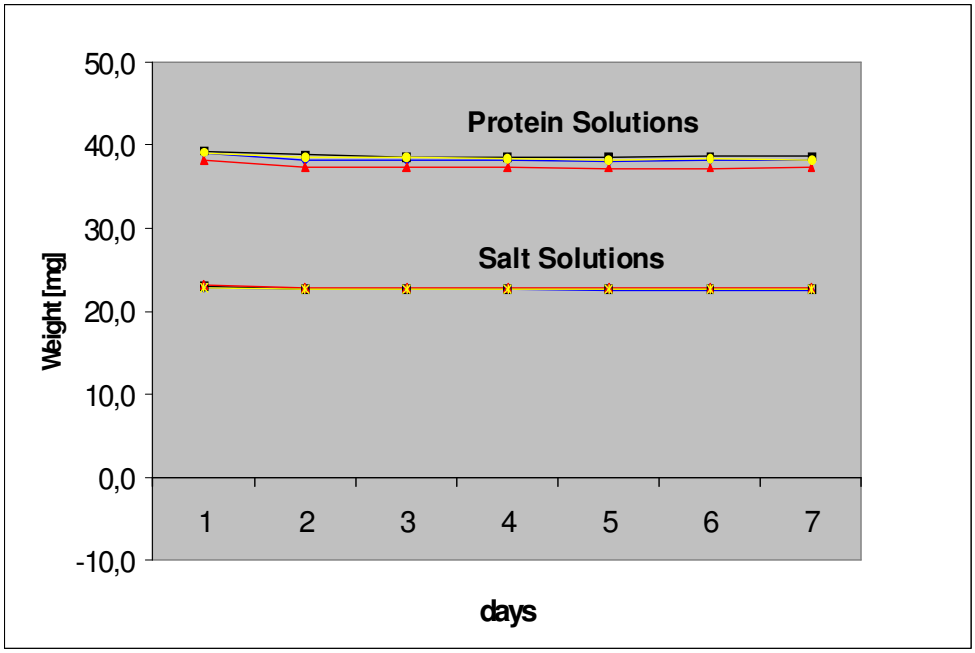

Figure 1: Mass of protein solutions and solvents recorded over a period of 7 days

Drying was performed in an oven at 90 °C with a slight vacuum of 27 kPa over 7 days. Figure 1 shows the mass of the protein solution and the solvent as it was recorded over the 7 days.

The mass concentration of cystatin C in the primary reference preparation was then calculated according to [11] and found to be: C_p = 5.1971 ± 0.033 g/L, with $u_{dm,\,cal,\,rel}$ = 0.635 %.

Physico-chemical characterisation
The primary reference preparation for cystatin C was further characterised using:

- Agarose screen electrophoresis: cystatin C was migrating at the expected position.
- SDS-PAGE. These measurements have a limit of detection of 0.5 % (relative mass concentration). The results showed that no other protein contaminants could be observed when denaturated proteins were separated on the basis of molecular weight. As an example an SDS-PAGE is shown in Figure 2.
- Crossed immunoelectrophoresis: no other serum proteins were detected by poly-specific antibodies to human serum proteins, cystatin C was detected in the PRP.
- Double immunodiffusion (Ouchterlony): the precipitation pattern obtained with antibodies against cystatin C were consistent with the presence of cystatin C at the expected concentration.
- FPLC Superdex 75 GL and Superdex 200 HR 10/30. These measurements have a limit of detection for mass concentrations between 1 and 5 µg/mL, depending on the identity of contaminating proteins. No contaminants were found upon the injection of cystatin C at a mass concentration of 2 mg/mL (limit of detection for impurities 0.25 % in relative mass concentration).
- N-terminal sequencing (Edman and Mass Spectrometry): the protein was identified as cystatin C with an intact N-terminus.

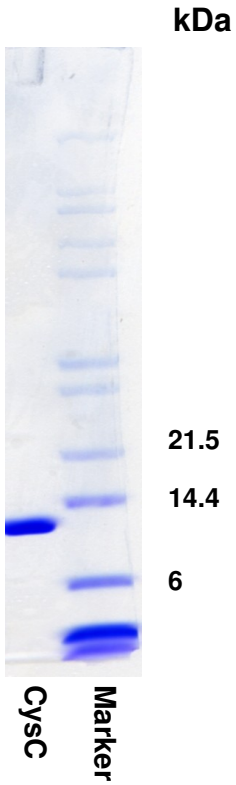

Figure 2: SDS-PAGE of the Primary Reference Preparation for Cystatin C (left) and a molecular mass marker (right). Cystatin C has the expected molecular weight, and no impurities can be detected in the PRP.

A summary of all characterisations showed that the primary reference preparation for cystatin C is monomeric, highly pure, has the expected mobility, has the expected molecular mass, and shows high homogeneity. No truncated cystatin C or other non-cystatin C proteins were detected. As no impurities could be detected with any of the methods, the purity of the

material is 100 % with an estimated relative standard uncertainty of 0.3 %, the value of the limit of detection of the size exclusion chromatography measurements rounded up.

This primary preparation was therefore judged qualified to be used in the value assignment of the Target Material ERM-DA471/IFCC.

6.3 Trial run of the characterisation measurements

In order to test the value assignment procedure and to qualify the different methods and laboratories a trial run was conducted using a pure preparation of cystatin C similar to the primary reference preparation and with a cystatin C mass concentration of 5.20 g/L, and the newly prepared reference material for cystatin C ERM-DA471/IFCC.

The methods, including instruments, reagents and laboratories, are summarised in Table 6.

Table 6 Set-up for the trial run

Principle	Instrument	Reagent	Laboratory
Single Radial Immunodiffusion (enzyme amplified)		• Dako • Siemens	L1
Immuno-turbidimetry (Particle enhanced)	Hitachi 917	• Dako	L2
	Hitachi 917	• Roche	L3
Immuno-nephelometry (Particle enhanced)	BN ProSpec	• Siemens	L4

A practical protocol was used based on the original principles for an indirect value transfer using a carrier matrix for the pure protein. It was found that a solution containing 0.1 mol/L KCl and 4 % bovine serum albumin gave the same results as serum when used as a carrier matrix. Therefore that solution was used both for pre-dilutions and as a carrier matrix for the value assignment. The same solution could be used in all the selected immunochemical methods (particle enhanced immuno-turbidimetry and immuno-nephelometry plus enzyme amplified single radial immuno-diffusion).

Based on the obtained data the practical protocol was revised accordingly and final registration forms were made.

All laboratories qualified for the final value assignment.

6.4 Results of the characterisation measurements

All four laboratories having participated produced satisfactory results in the trial run and participated also in the final characterisation. These laboratories produced a total of 7 independent datasets.

The laboratories used the dilutions of the target material to construct the dose-response 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-DA471/IFCC, expressed in percent. These relative concentrations were calculated from the masses of the solutions. Then for each run, the laboratories measured the dilutions of the target material in duplicate.

Outliers were only rejected if there was a technical reason for doing so (a transcription error, wrong dilution, etc.). The different dilution factors $F_{TM}(j)$ obtained by the interpolation were plotted against the different dilution factors $F_{PRP}(j)$ used for the initial dilution of PRP. The cystatin C concentration in the target material was calculated from the slope of the linear regression line.

The following acceptance criteria were applied:

(1) The mean control value (measured concentration of cystatin C in a control dilution of ERM-DA471/IFCC relative to the concentration in the control sample expected on the basis of the dilution, calculated from the mass values obtained by weighing) is within the interval 1.00 ± 0.05 for turbidimetric and nephelometric methods, and within the interval 1.00 ± 0.10 for SRID.

(2) Tests for linearity (visual inspection and evaluation of the correlation coefficient): the correlation coefficient of the linear regression should be higher than 0.98.

(3) Data from at least 4 dilutions must be available, and the completeness of the data must at least be 50 % for each particular day.

(4) Data from at least two days must be valid.

In total six measurement days were excluded because mean control values were outside the tolerance limit: three from dataset 2, one from dataset 1, and two from dataset 4. For all other measurement days valid data were obtained (Figure 3).

Table 7: Summary of the measurements of the cystatin C mass concentrations performed by the participating laboratories

Dataset	Laboratory	Principle	Instrument	Reagents	cystatin C [mg/L]
1	L1	SRID	-	Dako	5.484
2	L1	SRID	-	Siemens	5.650
3	L2	turbidimetry	Hitachi 917	Dako	5.526
4	L3	turbidimetry	Hitachi 917	Roche	5.453
5	L3	turbidimetry	Integra 800	Roche	5.518
6	L4	nephelometry	BN ProSpec	Siemens	5.313
7	L4	nephelometry	BNII	Siemens	5.391
Mean of means					5.477
RSD [%]					1.96

The individual daily measured values and the laboratory means are normally distributed at $\alpha = 0.05$ (error probability upon rejection of the zero hypothesis). There were no outlying laboratory means according to Dixon's, Nalimovs *t*-test, and Grubbs test.

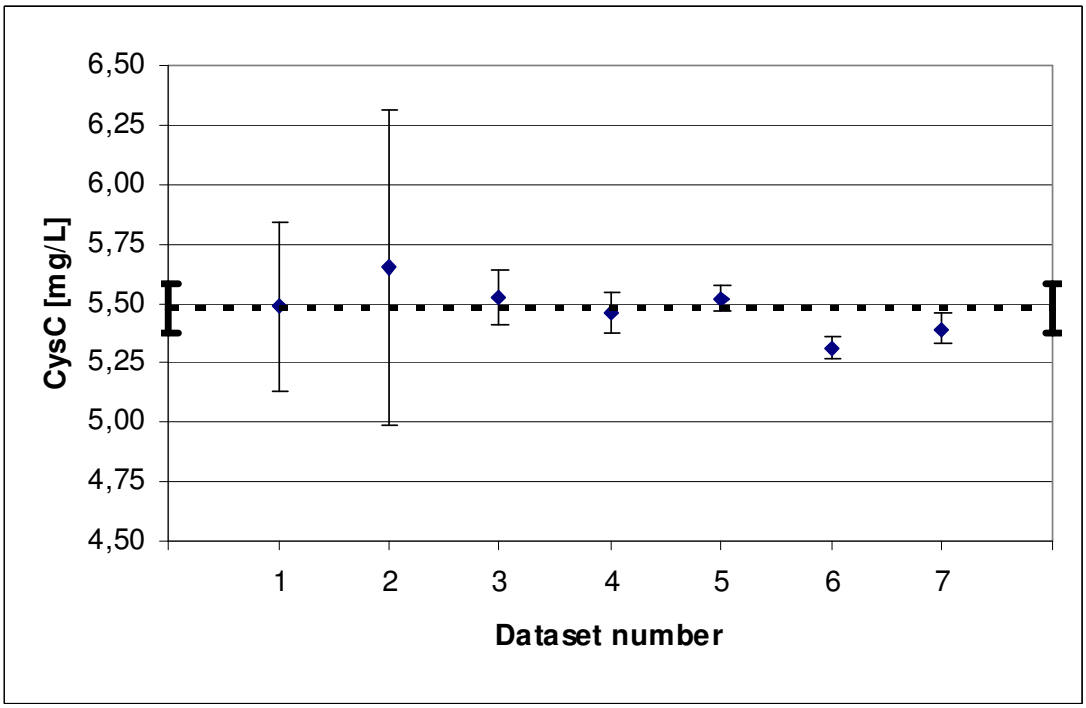

Figure 3: Results of the characterisation measurements of the mass concentration of cystatin C. The bars represent the dataset means ± *s*. The dotted line represents the mean of means, and the bars at the beginning and the end of the dotted line represents the mean of means ± *s*.

7 Uncertainty budget and certified value

7.1 Estimation of the uncertainty

The uncertainty components consist of the relative standard uncertainty related to characterisation ($u_{char, rel}$), the relative standard uncertainty of the dry mass determination and the purity of the PRP ($u_{dm, cal, rel}$ and $u_{purity, cal, rel}$), and the uncertainties due to the between-bottle heterogeneity ($u_{bb, rel}$), and possible degradation during long-term storage ($u_{lts, rel}$) [19].

- $u_{char, rel}$ was estimated as the relative standard uncertainty of the mean of laboratory means, i.e. $s/(p)^{0.5}$ with s the relative standard deviation of the mean of laboratory means and p the number of datasets, converted into a relative value
- $u_{dm, cal, rel}$ was taken as the relative standard uncertainty of the dry mass determination
- $u_{purity, cal, rel}$ was estimated from the uncertainty of the purity assessment of the PRP by size exclusion chromatography
- $u_{bb, rel}$ was taken as the $s_{bb, rel}$ as defined in Section 4.1.
- $u_{lts, rel}$ was estimated from stability tests and were taken from Table 5 (12 month stability study).

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

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

The various uncertainty contributions and the relative combined standard uncertainty ($u_{c, rel}$) are shown in Table 8.

Table 8: Uncertainty budget for ERM-DA471/IFCC

$u_{char, rel}$ [%]	$U_{dm, cal, rel}$ [%]	$u_{purity, cal, rel}$ [%]	$u_{bb, rel}$ [%]	$u_{lts, rel}$ [%]	$u_{c, rel}$ [%]
0.74	0.64	0.3	0.29	0.73	1.29

7.2 Certified values

The certified value (Table 9) was taken as the mean of means of the laboratory results (Table 7).

The relative expanded uncertainty was calculated from the relative combined standard uncertainty $u_{c, rel}$ (Table 8) by multiplication with a coverage factor k. This coverage factor was taken as 2, corresponding to a level of confidence of approximately 95 %. The relative expanded uncertainty was multiplied with the mean of dataset means to obtain the expanded uncertainty U_{CRM}.

Table 9: Certified value and expanded uncertainty for the mass concentration of cystatin C in ERM-DA471/IFCC

Certified value [mg/L]	U_{CRM} (k = 2) [mg/L]
5.48	0.15

8 Metrological traceability

The certified values for cystatin C in ERM-DA471/IFCC was assigned by transferring values from a pure protein reference preparation 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 was verified. The methods used were all immunochemical ones. Different combinations of reagents and measuring systems were used, and gave consistent results. Therefore the results are not dependent on the individual measurement procedures.

The concentration of cystatin C in the pure protein reference preparation had been assigned using a procedure based entirely on weighings, and is traceable to the SI.

Therefore the value of cystatin C in ERM-DA471/IFCC is traceable to the pure protein reference preparation, and thus to the SI, applying the procedures described in the present report.

9 Commutability

If ERM-DA471/IFCC is used for the calibration of other cystatin C assays it should be verified by the user that the material or its dilution used is commutable for that particular method in combination with other methods.
ERM-DA471/IFCC and its dilutions prepared using the assay diluent have been shown to be commutable for combinations of the Siemens N Latex Cystatin C Test Kit run on a BN ProSpec, the Sentinel CH assay run on an Architect c16000, and the Gentian cystatin C immunoassay run on an Architect. Dilutions with a mass fraction of ERM-DA471/IFCC in the dilution buffer of the relevant assay kit equal to or below 0.6 are commutable for combinations of these three assays and the Roche tina quant cystatin C assay run on a Cobas c501.

10 Intended use and instructions for use

The material is primarily intended to be used for the calibration of immunoassay-based IVD products for the measurement of cystatin C.

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. The minimum sample intake for which the material was shown to be homogenous was 2 µL.

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 during the afternoon of the day before use and place the vial for 1 hour in the room where the balance is located.
- After one 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. Document the mass or press the “TARE” button on the balance. Lift the rubber stopper with care until air is allowed to enter the vial and the groove in the rubber stopper becomes accessible.
- Add 1.00 mL of water through the groove, and press the rubber stopper back into place. Weigh the vial and document the mass. If you have used the “TARE” function, the value can be used directly for the mass *m*. Otherwise the first mass value must be subtracted from the second to obtain *m*.
- The concentration of cystatin C in the solution can be calculated using the actual reconstitution mass *m* and the intended reconstitution mass (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.

Storage

Unopened ampoules should be stored at or below -20 °C. Under the condition that any microbial contamination during the reconstitution procedure has been excluded, the solution of ERM-DA471/IFCC can be used for one week, as it was verified that changes in 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 - 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.

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Abstract

The production of ERM-DA471/IFCC, certified for the mass concentration of cystatin C, is described. Serum was produced from blood collected in 2 collection centres according to a procedure ensuring that it was obtained from healthy donors, and that the lipid content of the serum was low. The serum was processed, spiked with recombinant cystatin C, and lyophilised. It was verified that the material is homogenous and stable.
The material was characterised using a pure protein primary reference preparation (PRP) as calibrant. The PRP was prepared from recombinant cystatin C, and its concentration determined by dry mass determination. The characterisation of ERM-DA471/IFCC was performed by particle enhanced immuno-nephelometry, particle enhanced immuno-turbidimetry and enzyme amplified single radial immuno-diffusion.
The certified cystatin C mass concentration in ERM-DA471/IFCC, if reconstituted according to the specified procedure, is 5.48 mg/L, the expanded uncertainty ($k = 2$) is 0.15 mg/L.

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