Plasma proteins standardization: an accomplished fact?

Centro Interdipartimentale per la Riferibilità Metrologica in Medicina di Laboratorio

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PRU St. George's London
(IFCC Committee on Plasma Proteins)

Protein standardisation

We think we are so clever!

★we have labs full of fancy equipment
★we run a huge selection of tests 24/7
★we use primary tube samples, bar codes, pre-prepared reagents
★we have standards, QC, QA, accreditation, GLP, delta checking, reflex testing etc.

A question

What is acceptable variation from the "true" value of a test?

±10%

± 7%

± 5%

± 2%

± 1%

0%?

Now think of pay day

What is acceptable variation in your pay?

± 10%, ± 7%, ± 5%, ± 2%, ± 1%, 0%?

…..does it matter – it should even out in the long run

…..what happens if it is consistent bias?

Standardisation – why bother?

- ★ Result today will be the same as tomorrow
- ★ Result in Milan will be the same as the result in London
- ★ We can set reference ranges and decision points
- ★ We all measure to the same set of rules

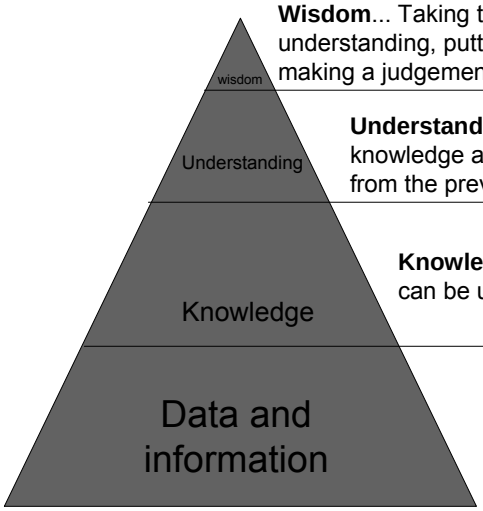
.....so we can diagnose, monitor and treat patients appropriately

Protein standardisation

Who is from a lab where they measure plasma protein concentrations?

Do you know….
…what your assays are calibrated against?
…when your reference ranges were set?
…how your reference ranges were set?
…where your standards are from?
…where your antiserum is from?

Protein standardisation



Wisdom... Taking the data, knowledge and understanding, putting it into a context and making a judgement

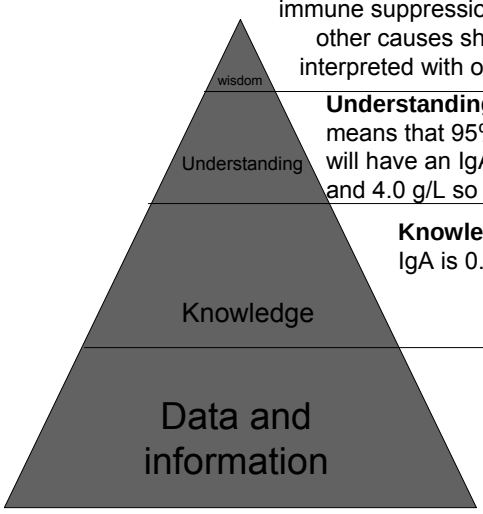
Understanding... the process of taking knowledge and synthesize new knowledge from the previously held knowledge.

Knowledge... a collection of information so it can be useful.

Data... it simply exists and has no significance beyond its existence.
Information... data that has been given meaning

Protein standardisation

Wisdom... an IgA of 0.06 g/L could be due to an analytical error, to the patient being IgA deficient or having secondary immune suppression. IgA deficiency is fairly common but other causes should be considered and the results interpreted with other immunoglobulin results and EP.



Understanding... The reference range means that 95% of people who are normal will have an IgA concentration of between 0.8 and 4.0 g/L so an IgA of e.g. 0.06g/L is low

Knowledge... The adult reference range for IgA is 0.8 – 4.0 g/L

Data... 0.8, 4.0

Protein standardisation

With all the fancy analyses and analysers we still have issues with standardisation.

A recent UKNEQAS distribution the method means for IgA varied from:

0.012 g/L (low) to 5.89 g/L (high)

The lower limit of detection was between 0.02 g/L and 0.7 g/L

Some labs reported the IgA concentration to 4 decimal places!

So what **is** the problem?

Protein standardisation

Not easy!

- ★there is molecular heterogeneity and genetic variability
- ★presence of binding proteins
- ★the protein needs to be purified from human serum
- ★this needs precipitation, heating, alcohol, solvents etc. all can denature the protein.
- ★the pure preparation needs to be dried – further degradation and variable water of hydration
- ★the pure preparation needs to be weighed and made up accurately – accurate balances and volumetric flasks

We can't just buy pure IgG and make a 10g/L solution

Protein standardisation

★Whole process is likely to change the protein from its native form

★minimal processing is desirable.

★Now we have recombinant proteins…

- ★best for proteins with minimal molecular variation
- ★but these can vary from native proteins

…… so are of limited use.

Protein standardisation

History

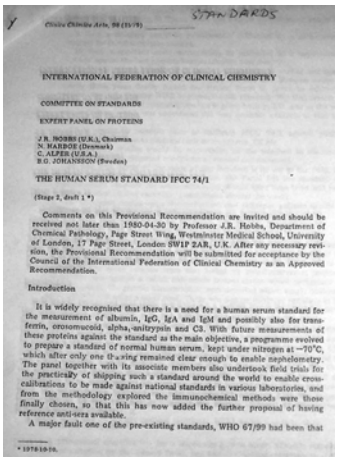
WHO 67/86

★used in the early days of protein quantification

★Freeze dried preparation

★Turbid on reconstitution - o.k. for RID or rockets but not good for nephelometry

Protein standardisation



INTERNATIONAL FEDERATION OF CLINICAL CHEMISTRY

COMMITTEE ON STANDARDS

EXPERT PANEL ON PROTEINS

J.R. HOBBS (U.K.), Chairman
N. HARBOE (Denmark)
C. ALPER (U.S.A.)
B.G. JOHANSSON (Sweden)

THE HUMAN SERUM STANDARD IFCC 74/1

(Stage 2, draft 1 *)

Comments on this Provisional Recommendation are invited and should be received not later than 1980-04-30 by Professor J.R. Hobbs, Department of Chemical Pathology, Page Street Wing, Westminster Medical School, University of London, 17 Page Street, London SW1P 2AR, U.K. After any necessary revision, the Provisional Recommendation will be submitted for acceptance by the Council of the International Federation of Clinical Chemistry as an Approved Recommendation.

Introduction

It is widely recognised that there is a need for a human serum standard for the measurement of albumin, IgG, IgA and IgM and possibly also for transferrin, orosomucoid, alpha$_1$-antitrypsin and C3. With future measurements of these proteins against the standard as the main objective, a programme evolved to prepare a standard of normal human serum, kept under nitrogen at −70°C, which after only one thawing remained clear enough to enable nephelometry. The panel together with its associate members also undertook field trials for the practicality of shipping such a standard around the world to enable cross-calibrations to be made against national standards in various laboratories, and from the methodology explored the immunochemical methods were those finally chosen, so that this has now added the further proposal of having reference anti-sera available.

A major fault one of the pre-existing standards, WHO 67/99 had been that

In 1979 the IFCC expert panel on proteins published details of the new protein standard IFCC 74/1

Protein standardisation

History
IFCC 74/1

★Prepared to replace WHO 67/99
★Young male donors 20-30 years
★Screened for known viruses, hyperlipidaemia and genetic variation e.g. AAT, AAG, C3, TRF
★Fasted for 18 hours before donation
★Discard first 20 mls of collection
★Separate sample from cells within 40 mins
★Handled aseptically
★Sodium azide added
★Liquid frozen in glass ampoules at -70^{o}C

IFCC 74/1

Calibrated for

★Albumin

★IgG, IgA and IgM

★Other proteins gradually added

★Local material cross calibrated to IFCC 74/1 in 18 countries

IFCC 74/1

Introduction of IFCC 74/1 showed that:

★a major cause of variation in protein analysed had been the methodologies used

★using recommended methods with IFCC 74/1 gave between lab variation never before achieved

★Proposal to designate reference antiserum

Other factors

★there were other reference preps
- WHO 6HSP and USNRP

★there were big changes in method
- Automated rate nephelometers

★BUT by the late 80s we should have seen excellent comparability between methods

Unfortunately…..

★EQA showed values for some proteins varied by 100%

★This was often due to variations in the calibrants

★A single international reference preparation was needed

IFCC working group on plasma protein standardisation

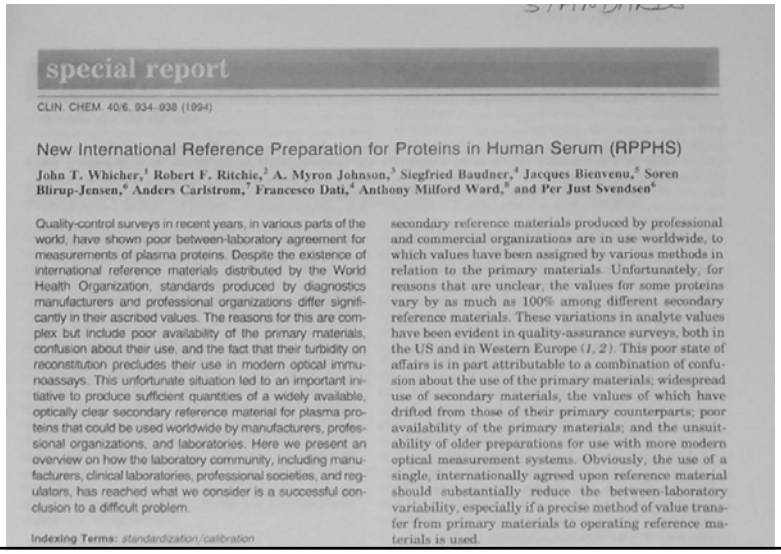
★Project to make a new reference material

★Produced by the Community Bureau of Reference of the Commission of the European Communities

★Managed by the IFCC Committee for Plasma Protein Standardisation and the College of American Pathologists

★Certified Reference Material (CRM) 470

CRM 470 Released in 1993



special report

CLIN. CHEM. 40/6, 934–938 (1994)

New International Reference Preparation for Proteins in Human Serum (RPPHS)

John T. Whicher,[1] Robert F. Ritchie,[2] A. Myron Johnson,[3] Siegfried Baudner,[4] Jacques Bienvenu,[5] Soren Blirup-Jensen,[6] Anders Carlstrom,[7] Francesco Dati,[4] Anthony Milford Ward,[8] and Per Just Svendsen[6]

Quality-control surveys in recent years, in various parts of the world, have shown poor between-laboratory agreement for measurements of plasma proteins. Despite the existence of international reference materials distributed by the World Health Organization, standards produced by diagnostics manufacturers and professional organizations differ significantly in their ascribed values. The reasons for this are complex but include poor availability of the primary materials, confusion about their use, and the fact that their turbidity on reconstitution precludes their use in modern optical immunoassays. This unfortunate situation led to an important initiative to produce sufficient quantities of a widely available, optically clear secondary reference material for plasma proteins that could be used worldwide by manufacturers, professional organizations, and laboratories. Here we present an overview on how the laboratory community, including manufacturers, clinical laboratories, professional societies, and regulators, has reached what we consider is a successful conclusion to a difficult problem.

Indexing Terms: *standardization/calibration*

secondary reference materials produced by professional and commercial organizations are in use worldwide, to which values have been assigned by various methods in relation to the primary materials. Unfortunately, for reasons that are unclear, the values for some proteins vary by as much as 100% among different secondary reference materials. These variations in analyte values have been evident in quality-assurance surveys, both in the US and in Western Europe (*1, 2*). This poor state of affairs is in part attributable to a combination of confusion about the use of the primary materials; widespread use of secondary materials, the values of which have drifted from those of their primary counterparts; poor availability of the primary materials; and the unsuitability of older preparations for use with more modern optical measurement systems. Obviously, the use of a single, internationally agreed upon reference material should substantially reduce the between-laboratory variability, especially if a precise method of value transfer from primary materials to operating reference materials is used.

CRM 470

★Fresh serum, naturally clotted
★Several hundred donors across Europe
★Patient demographics noted
★Tested for known viruses and RhF, monoclonal proteins
★Alpha-1 antitrypsin and haptoglobin phenotypes
★Haemolysis, bilirubin and turbidity
★Sodium azide added and then frozen to be sent for processing

CRM 470

★Collections thawed, pooled, delipidated and stabilised
★Pure CRP added
★Buffered
★Sterile filtered
★Bottled and freeze dried (40,000 ampoules)
★It was expected that this batch of CRM 470 would last for some years

CRM 470

★Values assigned against WHO 6HSP and USNRP and WHO CRP

★27 labs participated

★Albumin, ceruloplasmin, C3, C4, IgG, IgA IgM, α2 macroglobulin, α antitrypsin, haptoglobin, CRP

★Where possible, checked against pure preparations

★There were some marked differences from existing standards and consequent changes in reference ranges

★CRM470 was adopted by reagent manufacturers across the world

Effect of CRM 470 on Protein Assay Quality Control

★Two serum samples with ~35% difference in concentrations were sent in 1992 to national QC programs in Europe and the South Pacific

★Two new but similar samples were distributed in 2002-3

★Results are shown as Youdin plots, with the axes the same for both graphs in each case

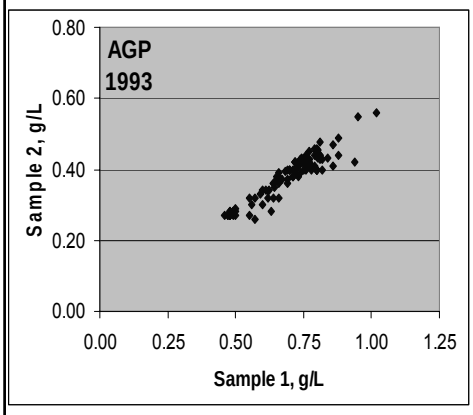
with thanks to Myron Johnson

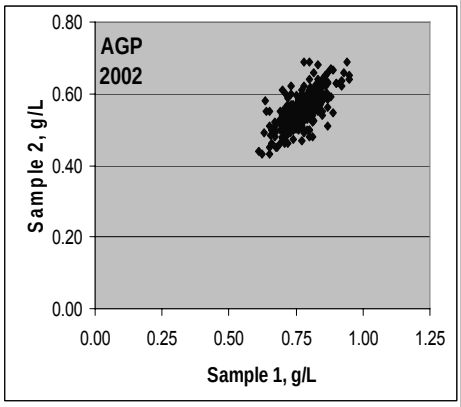
Effect of CRM 470 on Protein Assay Quality Control

★With general usage of CRM 470, uncertainty for the plasma proteins

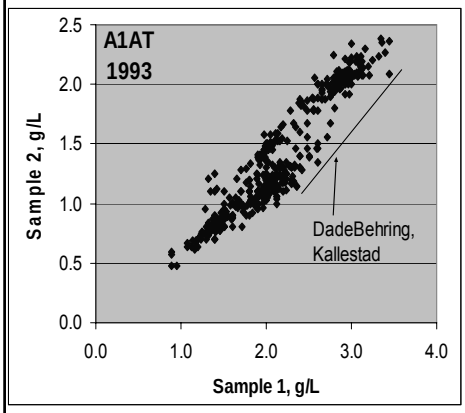
- Decreased markedly: α_1-antitrypsin, haptoglobin, transferrin,C3, C4, IgA, IgG, IgM
- Remained essentially unchanged: orosomucoid (α_1-acid glycoprotein), α_2-macroglobulin, ceruloplasmin
- Increased: C-reactive protein

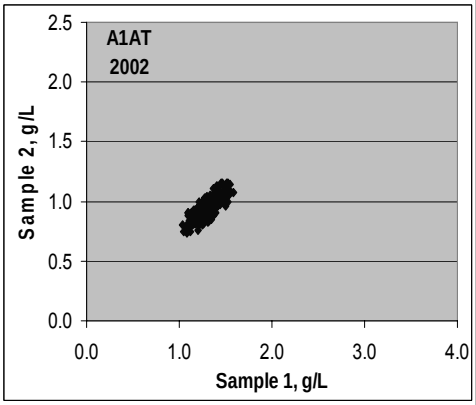
α_1-Acid Glycoprotein



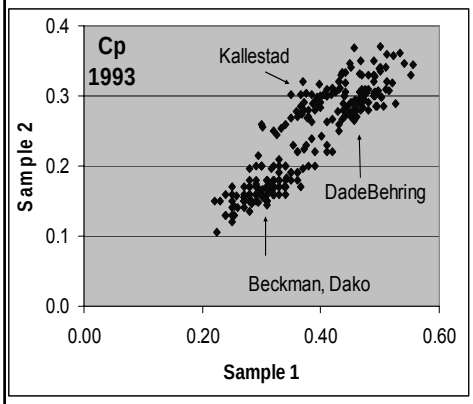


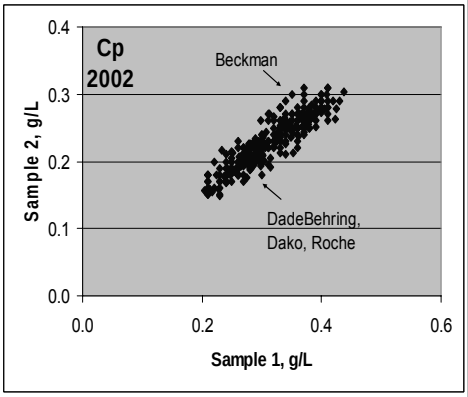
α_1-Antitrypsin



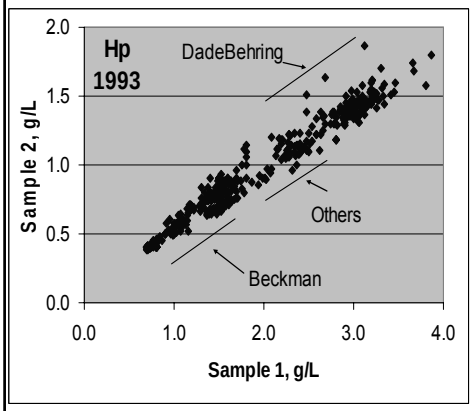


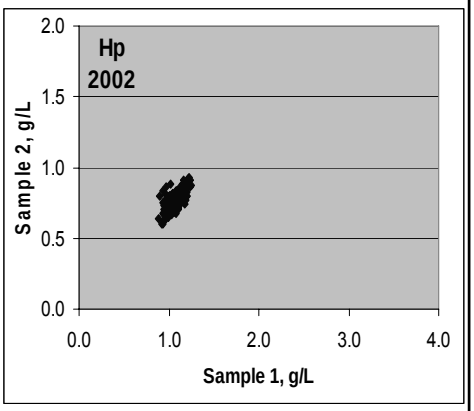
Ceruloplasmin



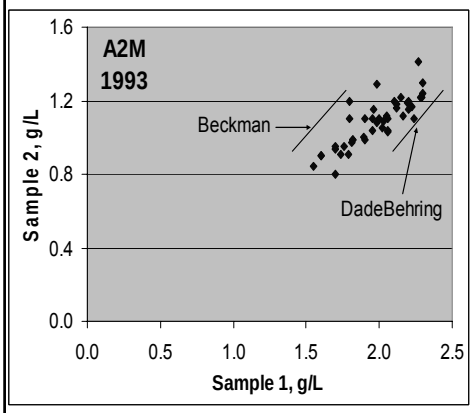


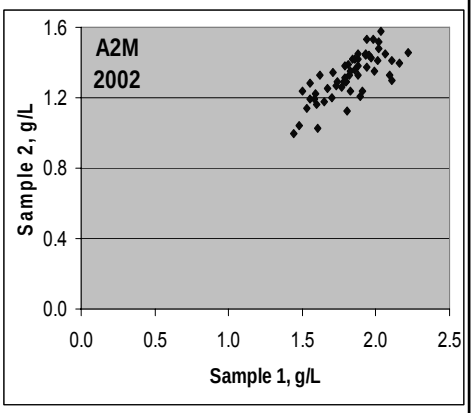
Haptoglobin



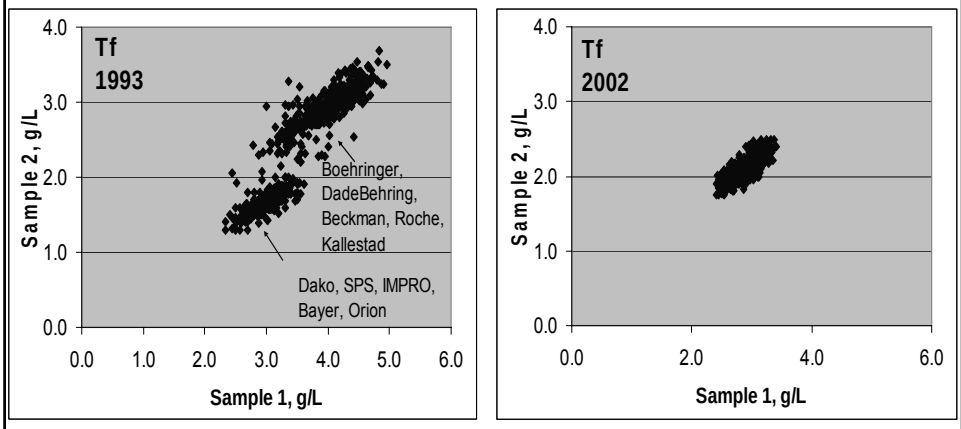


α_2-Macroglobulin

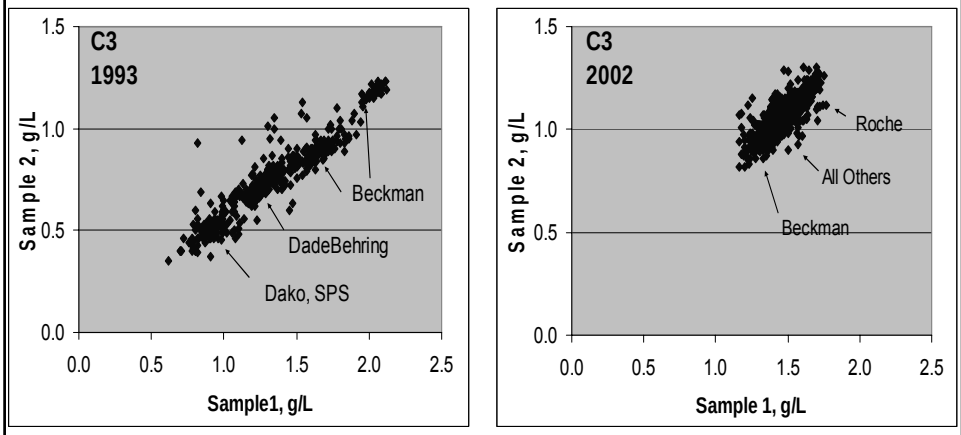




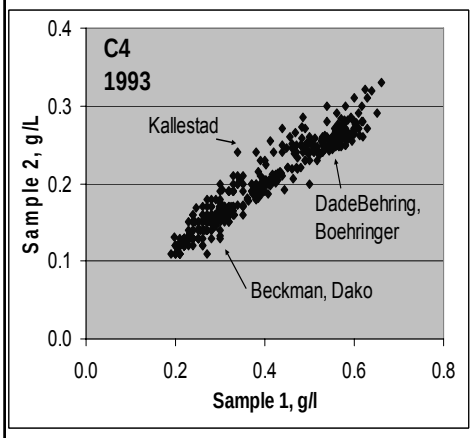
Transferrin

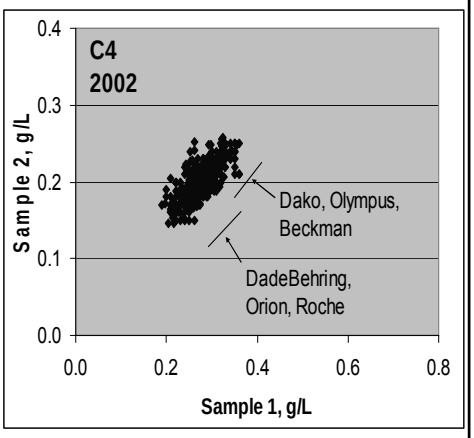


C3

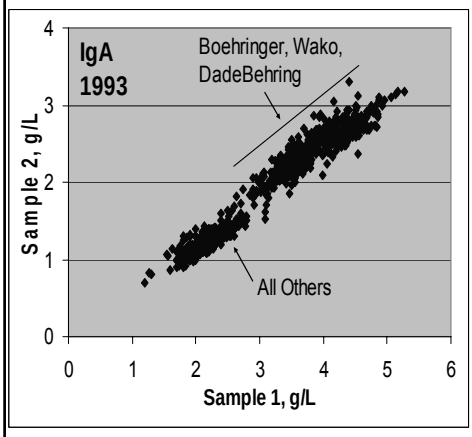


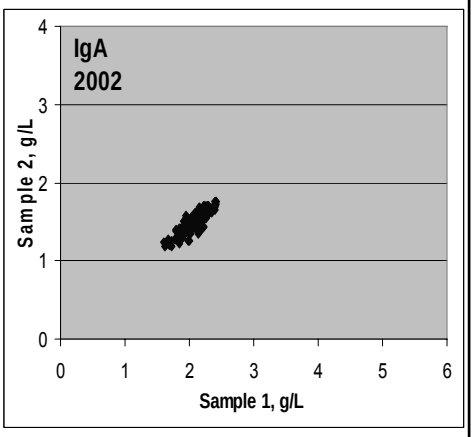
C4


C4
1993
Kallestad
DadeBehring,
Boehringer
Beckman, Dako
Sample 2, g/L
Sample 1, g/l
0.0
0.1
0.2
0.3
0.4
0.0
0.2
0.4
0.6
0.8

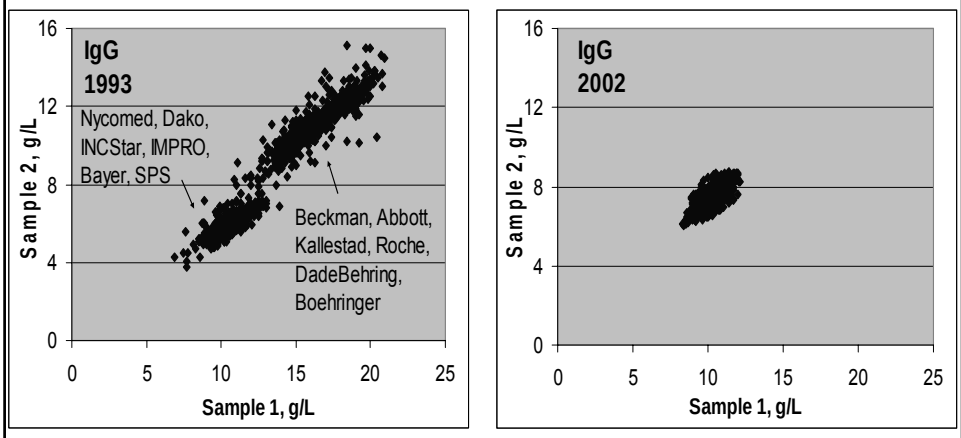

C4
2002
Dako, Olympus,
Beckman
DadeBehring,
Orion, Roche
Sample 2, g/L
Sample 1, g/L
0.0
0.1
0.2
0.3
0.4
0.0
0.2
0.4
0.6
0.8

IgA

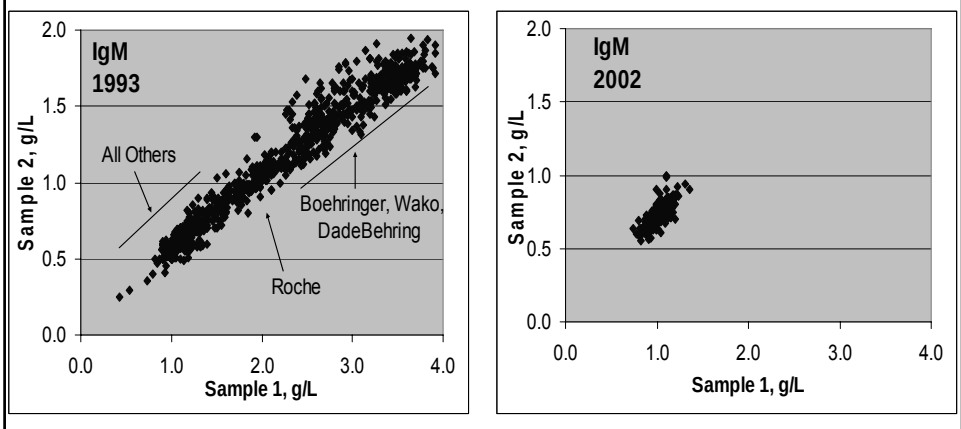

IgA
1993
Boehringer, Wako,
DadeBehring
All Others
Sample 2, g/L
Sample 1, g/L
0
1
2
3
4
0
1
2
3
4
5
6


IgA
2002
Sample 2, g/L
Sample 1, g/L
0
1
2
3
4
0
1
2
3
4
5
6

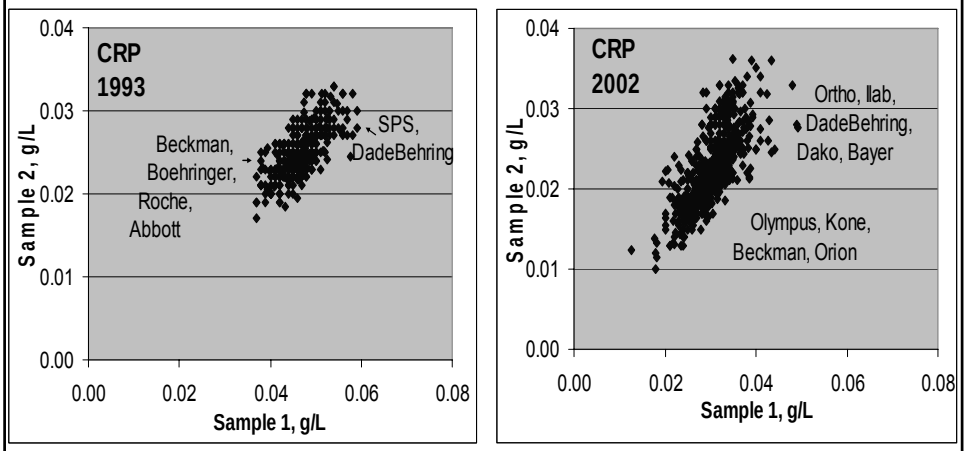
IgG


IgG
1993
Nycomed, Dako,
INCStar, IMPRO,
Bayer, SPS
Beckman, Abbott,
Kallestad, Roche,
DadeBehring,
Boehringer
Sample 2, g/L
Sample 1, g/L
0
4
8
12
16
0
5
10
15
20
25
IgG
2002
Sample 2, g/L
Sample 1, g/L

IgM


IgM
1993
All Others
Boehringer, Wako,
DadeBehring
Roche
Sample 2, g/L
Sample 1, g/L
0.0
0.5
1.0
1.5
2.0
0.0
1.0
2.0
3.0
4.0
IgM
2002
Sample 2, g/L
Sample 1, g/L

C-Reactive Protein



Some Reasons High Uncertainty

- ★ Inadequate accuracy and precision of transfer of values by manufacturers from CRM 470 to controls and calibrators
- ★ Matrix differences among reference materials (CRM 460 is a delipidated pool of normal serum)
- ★ Epitopic differences in proteins (genetic variants, partial catabolism, etc.)

Some recent examples of poor standardisation

- ★ Ceruloplasmin – marked difference between various methods – probably related to how the antisera behave with fresh and aged sera
- ★ C4 – a manufacturer incorrectly transferred C4 values to its calibrants giving their C4s significant bias
- ★ IgG – a manufacturer NEVER checked its value transfers back to CRM 470 and gradually generated a 10% positive bias
- ★ AAT – a manufacturer consistently shows 10% negative bias

A huge step forward…since 1993

- ★ 2006 we knew that CRM 470 was running out
  - ★ It remained stable and valid but we needed a new preparation
- ★ IFCC committee for plasma proteins met to collaborate with the IRMM to produce a new reference material
- ★ Discussions on
  - ★ which proteins to include
  - ★ whether to use recombinant proteins where necessary
  - ★ sample collection protocols

2008 ERM-DA470k

- ★ Produced by the IRMM
  - ★ Similar protocols to CRM470
  - ★ Normal human serum with added
    - ★ CRP
    - ★ Recombinant β2 microglobulin
  - ★ Processed, virus tested, pre-tested to proteins, bottled, lyophilised.
  - ★ Value assigned against CRM470 and where possible the pure proteins
  - ★ Value transfer protocols developed
  - ★ Tested as calibrant with samples
  - ★ Stability tested - ongoing

2008 ERM-DA470k

- ★ Produced by the IRMM
  - ★ Collaboration with Dade Behring (Marburg) and 20 laboratories across Europe
- ★ Vials of ERM-DA470K distributed under strict transport guidelines to participating labs
- ★ Value transfer protocol detailed and strict
  - ★ Storage, reconstitution, pipettes, balances, volumes, timing, operators, reagents, QC, assay performance etc.
- ★ Closed and open systems used for value transfer
- ★ Specific investigations on particular issues

Some issues with ERM-DA470k

- ★We are doing some final investigations for some proteins
- ★The ceruloplasmin problem seen with CRM470 is still seen –likely related to a difference in how the assays recognise fresh or aged serum
- ★β2 microglobulin will be value assigned soon

CERTIFICATE OF ANALYSIS

ERM®- DA470k/IFCC

HUMAN SERUM		
Proteins in the reconstituted material [1]	Mass concentration	
	Certified value [2] [g/L]	Uncertainty [3] [g/L]
α_2 macroglobulin (A2M)	1.43 [4]	0.06
α_1 acid glycoprotein (AAG)	0.617 [5]	0.013
α_1 antitrypsin (AAT)	1.12 [5]	0.03
albumin (ALB)	37.2 [4]	1.2
complement 3c (C3c)	1.00 [4]	0.04
complement 4 (C4)	0.162 [4]	0.007
haptoglobin (HPT)	0.889 [4]	0.021
immunoglobulin A (IgA)	1.89 [4]	0.05
immunoglobulin G (IgG)	9.17 [4]	0.18
immunoglobulin M (IgM)	0.723 [4]	0.027
transferrin (TRF)	2.36 [5]	0.08
transthyretin (TTR)	0.220 [5]	0.018

1) When the material is reconstituted according to the specified procedure (see page 3).
2) The certified values are the unweighted means of 6-14 accepted mean values, independently obtained by 5-14 laboratories, using ERM-DA470 as calibrant (Bauchner et al., EUR reports 15423 and 16882 European Communities, Luxembourg (1993)).
3) Expanded uncertainty with a coverage factor $k = 2$ corresponding to a level of confidence of about 95 % estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM), ISO, 1995.
4) This certified mass concentration is traceable to the stated value of the mass concentration in IRMM/IFCC 12-0575C (Reimer et al., Am. J. Clin. Pathol. 77 (1982) 12-19) used as calibrant for assigning values to ERM-DA470, applying the procedures described for the certification of ERM-DA470 and in the report for ERM-DA470k/IFCC.
5) The certified value in the calibrant ERM-DA470 was obtained by calibration with a pure protein preparation (Blirup-Jensen, Clin. Chem. Lab. Med. 39 (2001) 1090 - 1097). Consequently, the certified value in ERM-DA470k/IFCC is traceable to the International System of Units (SI) via ERM-DA470, applying the procedures described in the certification report of ERM-DA470 (see point 2) and in the report for ERM-DA470k/IFCC.

This certificate is valid for one year after purchase.
Sales date:
The minimum amount of sample to be used is 2 µL.
Accepted as an ERM®, Geel, July 2008

Signed:

Prof. Dr. Hendrik Emons
Unit for Reference Materials
EC-DG JRC-IRMM
Retieseweg 111
2440 Geel, Belgium

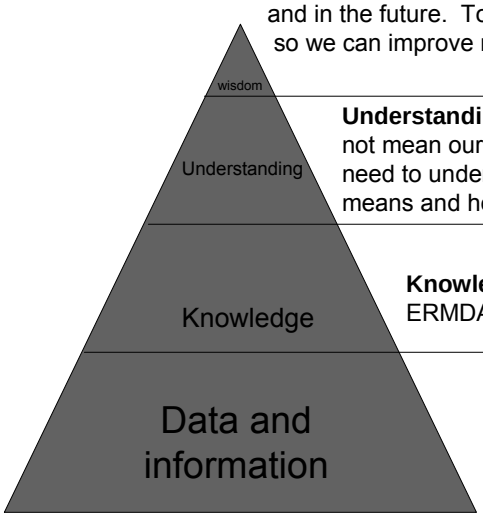
JRC EUROPEAN COMMISSION

Registration No. 200-TEST
ISO Guide 34 for the production of reference materials

All following pages are an integral part of the certificate.
Page 1 of 4

ERMDA470K

Wisdom... Considering all the components of an analysis to make a judgement about the validity – for the patient today, and in the future. To continually assess our standardisation so we can improve rather than always aiming for the mean



Understanding... having a standards does not mean our assays are standardised – we need to understand what standardisation means and how to maintain it

Knowledge... We know the values for ERMDA470K

Data... We know it exists √

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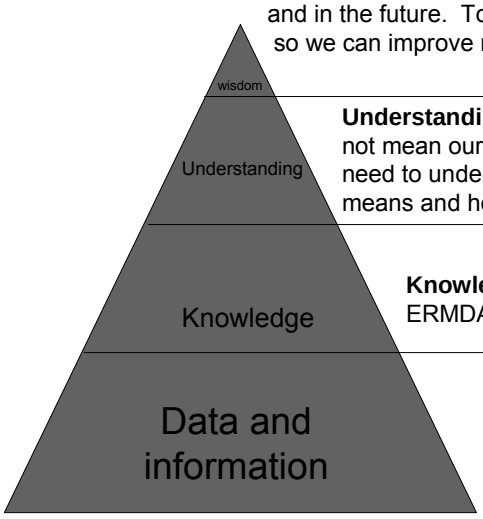

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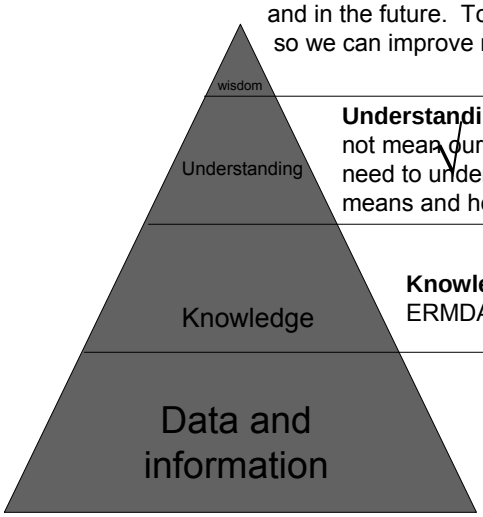

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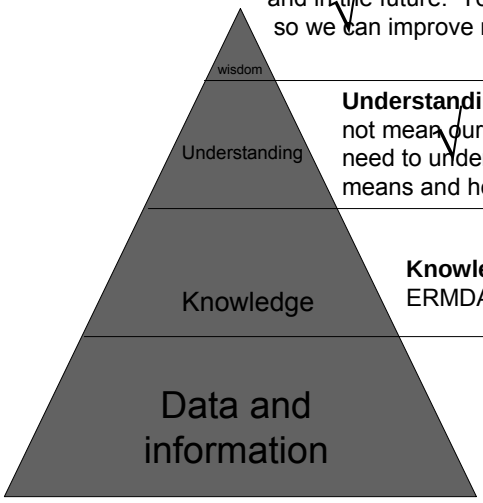

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Summary

- ★ Standardisation is vital
- ★ We have to keep it as a high priority in our labs
- ★ It is the first step in generating quality results
- ★ It should be the main reason we select an analyser – not speed or throughput
- ★ It should mean that we generate high quality results for our patients….and we can all be the patient!