Traceability and heterogenous analytes – what more?

IRMM - Institute for Reference Materials and Measurements
Geel - Belgium

http://irmm.jrc.ec.europa.eu/
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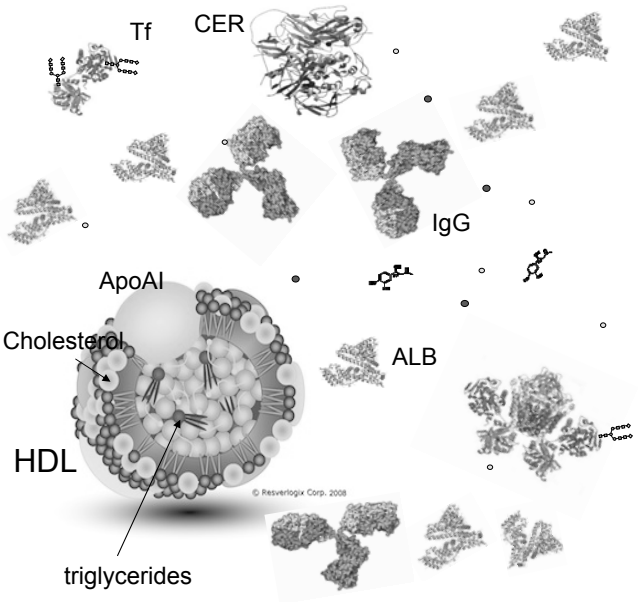
1. **heterogenous analytes and traceability**
2. **the serum protein system for traceability of heterogenous analytes**
3. **CRP: the consequences of oligomeric heterogeneity in the traceability chain**
4. **moving from broadly specific methods and heterogenous measurands to highly specific methods and (recombinant) protein preparations**

1 Heterogenous proteins



Complex mixture:

- cations/anions
- proteins (~ 2000)
  enzymes
- glycosylation
- lipids
- …

And these components evolve:

- binding
- denaturation
- proteolysis
- large structures
- …

Heterogenous proteins

Covalent:

- sequence (isoforms)
- splicing
- degradation (N-terminal, …)
- chemical modification (oxidation, …)

Scorpion toxin HsTx1 (NMR)

Non-covalent:

- oligomeric state
- ligand binding (metals, other proteins, co-factors, …
- degree of structuration (partial denaturation)
- different conformational states, unstructured proteins

Traceability: underlying assumption

Being **traceable** to a **common standard** or stated reference should ensure that **independently obtained measurement results will overlap** within their stated uncertainties and at a certain level of confidence with the true value and consequently with each other

- provided measurement procedures applied in the traceability chain determine the **same measurand**
- if the comparison measurements do not introduce **unrecognised bias** (e.g. matrix effects, differential extraction etc.)
- if **all relevant uncertainty components** are included in the estimate of the combined uncertainties

Measurand

Definition

« quantity intended to be measured»

1- **The specification of a measurand requires knowledge of the kind of quantity, description of the state of the phenomenon, body, or substance carrying the quantity, including any relevant component, and the chemical entities involved**
2- **In the 2nd edition of the VIM and in IEC 60050-300:2001, the measurand is defined as the 'quantity subject to measurement'**
3- **The measurement, including the measuring system and the conditions under which the measurement is carried out, might change the phenomenon, body, or substance such that the quantity being measured may differ for the measurand as defined. In this case adequate correction is necessary.**
4- **...**

ISO/IEC Guide 99-12:2007, International Vocabulary of Metrology - Basic and General Concepts and Associated Terms, VIM

Structural definition of a protein measurand ?

The ensemble of entities with sequence X, with on position 42 a Q instead of a E in 14 % of the molecules, with an oxidated cysteine in position 98 in 65 % of the molecules, with a three-dimensional structure such that the RMS deviation between the atom positions and the atom positions of the crystal structure is smaller that 2.5 Å, where 98 % of the molecules have a calcium ion bound, with 20 % of the molecules in the dimeric form, ...

2 Serum protein CRM

1993-1994: A huge effort by a very dedicated group of clinicians and scientists led to the development of CRM470, later renamed ERM-DA470

1st USNRP

(ALB, CER, A2M, HPT, C3c, C4, IgA, IgG, IgM)

WHO 85/506

(CRP)

SI

Pure proteins

(TTR, AAG, AAT, TRF, ACT)

CRM470

ERM-DA470k/IFCC

12 proteins, July 08

ERM-DA472/IFCC

CRP, Feb 09

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traceability chain for ERM-DA470k

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• all isoforms
• heterogenous,
• high yield purification
• verified by immunoassay

definition of SI unit

AAA, refractive index, dry mass determinations

"Pure" protein

value transfer procedure, immunoassay

ERM-DA470/ ERM-DA470k

value transfer procedure, immunoassay

manufacturer's working calibrator

value transfer procedure, immunoassay

manufacturer's product calibrator

Immunoassay

• all isoforms recognised in immunoassay,
• heterogenous

routine sample

result (U including pre-analytical and IQC contributions)

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Structures of C3, C3b, C3c

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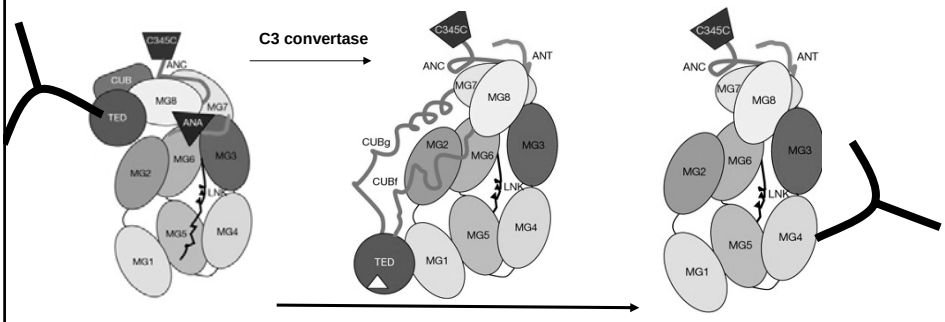
C3 | C3b | C3c



MW 185000 Da

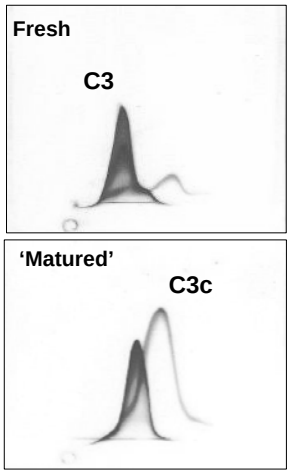
MS signal = or 0

Immunoassay signal

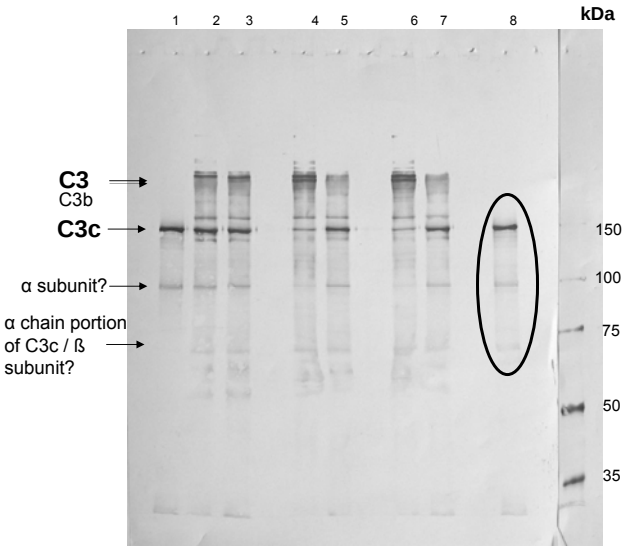
Mass concentration

conversion of C3 to C3c during the CRM production

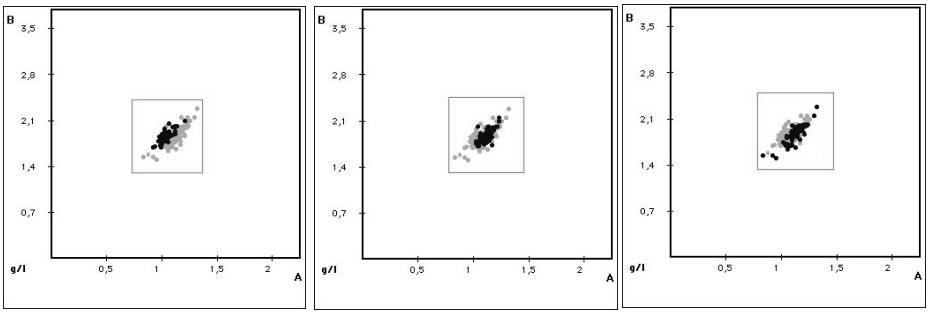
Crossed immuno-electrophoresis of C3 in serum



Western blotting of non-reducing gel



RfB/DGKL EQAS IG2/08 results C3/C3C



Nephelometry (Beckman)

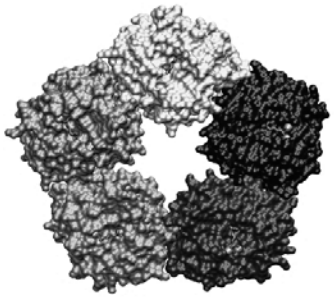
Nephelometry (Siemens, former DB)

Turbidimetry

In spite of the complexity of the system the ERM-DA470 works well

Problems observed for CRP in the pilot studies:

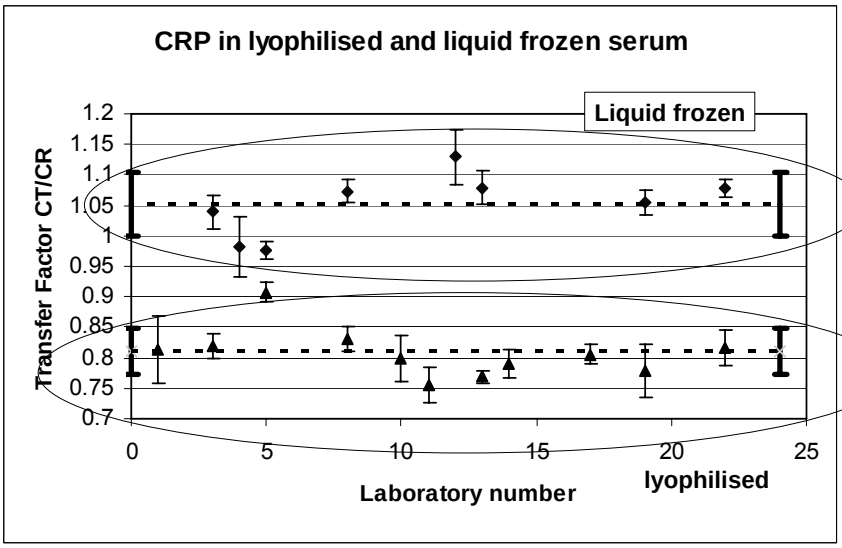
- **Only 75 % recovery after freeze-drying**
- **High between bottle heterogeneity in freeze-dried material**
- **Presence of different oligomeric forms in freeze-dried material**



Pentameric protein
Monomer: 25 106 Da
Binds two Ca2+

Physicochemical state verified by:

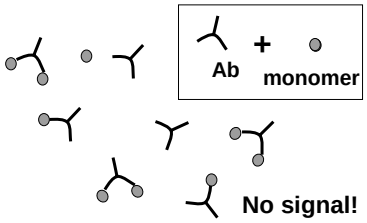
Gel filtration followed by SDS PAGE and Western blotting
Semi-native gel electrophoresis followed by western blotting



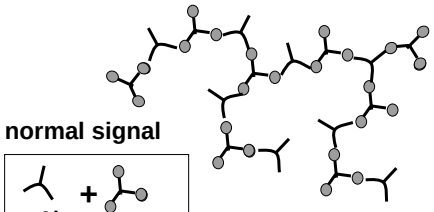
Different oligomeric forms of CRP

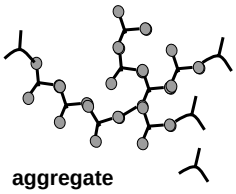
Clinical analysers: most measure the formation of aggregates between target protein and antibodies

→ Relevance of aggregation state of protein

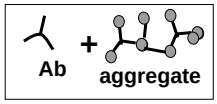


normal signal





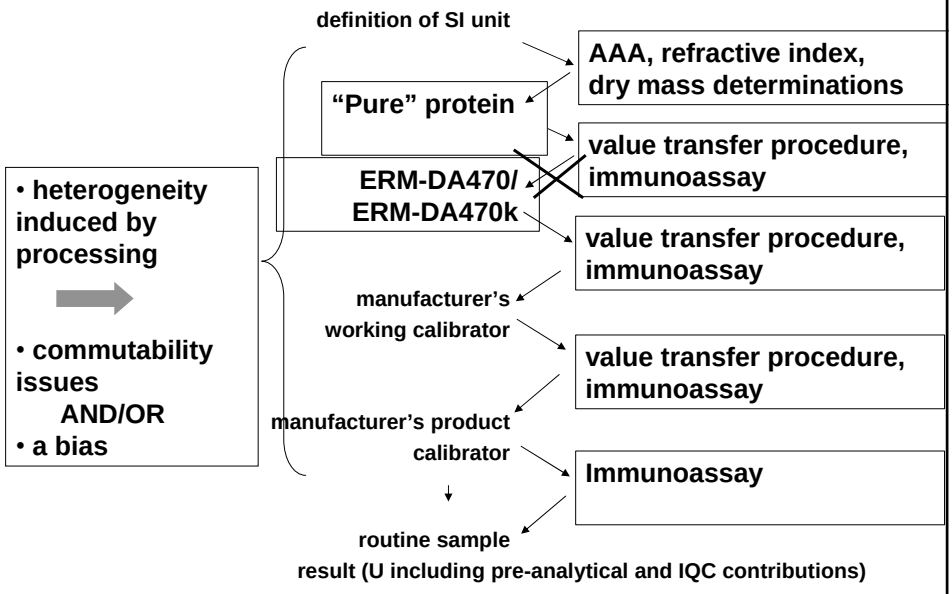
Variable signal



Processing of calibrant may cause changes in oligomeric state

→ Breaks in traceability chain depending on technique

traceability chain ERM-DA470k



Different measurement procedures

Multimeric heterogeneity will affect different measurements procedures to a different extent:

- **LC-MS: all polypeptides chains measured, also 'non-native' ones**
- **Immunoassay: differences depending on many factors.**

→ **In the case of CRP a small multimeric heterogeneity could lead to a significant bias (2-20 %) if LC-MS methods would have been used anywhere in the chain**

February 2009: ERM-DA472/IFCC

→ **Certification of a liquid frozen material that is commutable, with CRP entirely in the native state:**

CERTIFICATE OF ANALYSIS

ERM®- DA472/IFCC

HUMAN SERUM

	Mass concentration	
	Certified value [2] [mg/L]	Uncertainty [3] [mg/L]
C-reactive protein (CRP) [1]	41.8	2.5

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

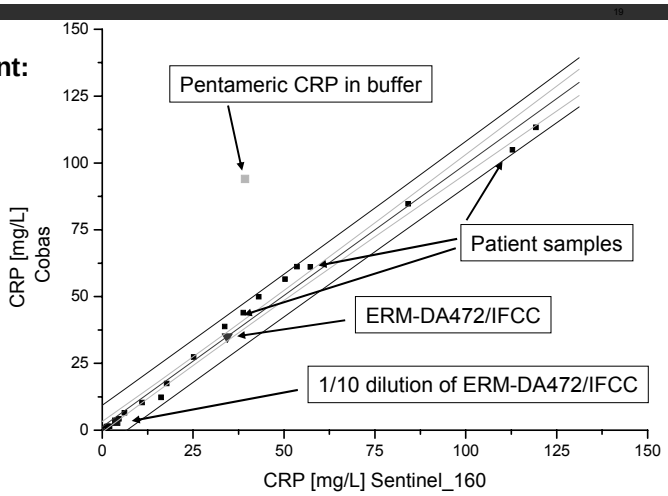
2) The value is the unweighted mean of 8 accepted mean values, independently obtained by 8 laboratories. The certified mass concentration is traceable to the SI, via ERM-DA470, 1st Int. St. for CRP Code 85/506, and the pure protein preparation used as calibrant.

3) Expanded uncertainty *U* 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.

This certificate is valid for 6 months after purchase.

CRP commutability studies

Method or instrument:
Beckman Immage
Dako
Sentinel
Abbott Architect
Cobas
Integra
BN ProSpec



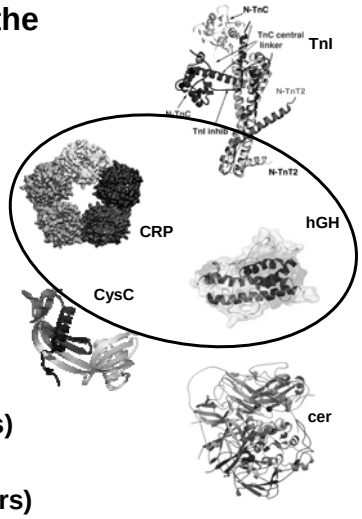
ERM-DA472/IFCC is commutable
A 1/10 dilution of ERM-DA472/IFCC is commutable
CRP without matrix is not commutable
Non-pentameric CRP is not recognised

4 ClinBioTrace

Collaborative research project on the traceability of values for complex biomolecules:

'amount of substance'

Functional activitity

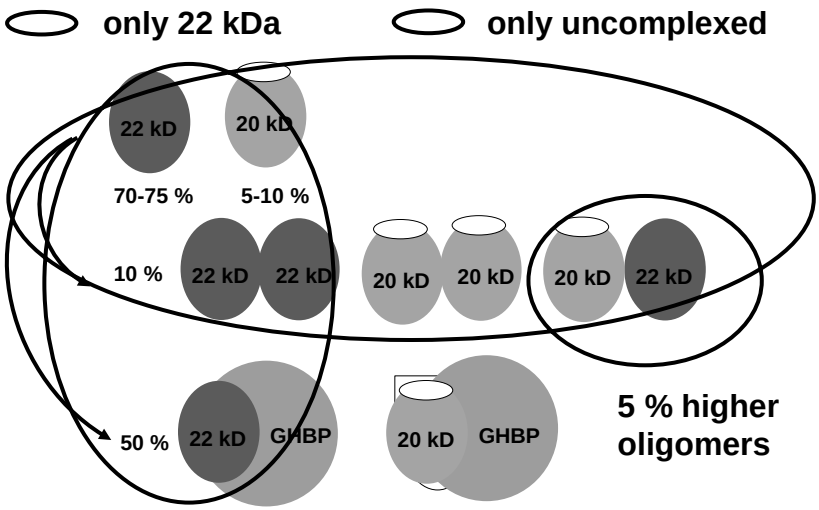


Partners:

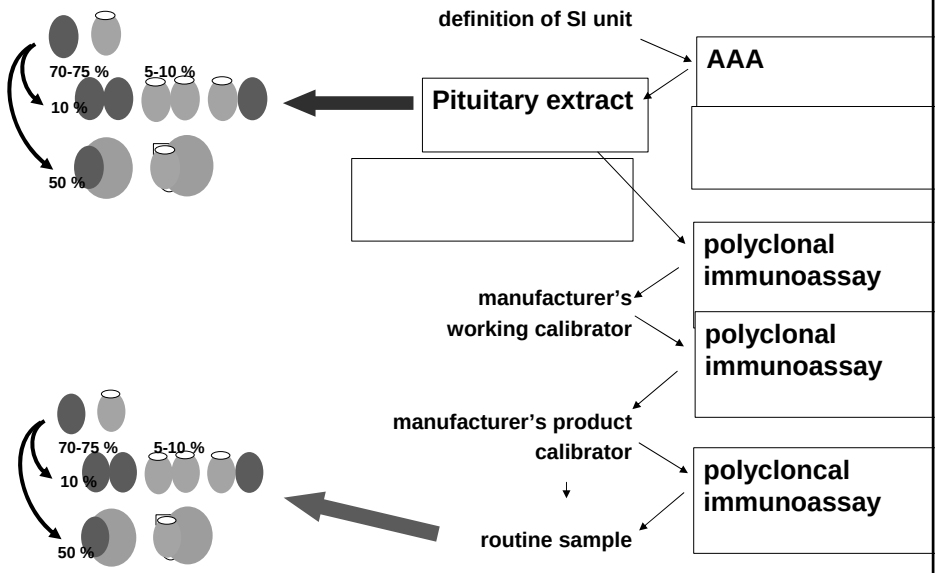
- **LGC (Gavin O'Connor, Helen Parkes)**
- **PTB (André Henrion)**
- **IRMM (Heinz Schimmel, Ingrid Zegers)**
- **NPL (Anna Hills)**

Different forms of hGH

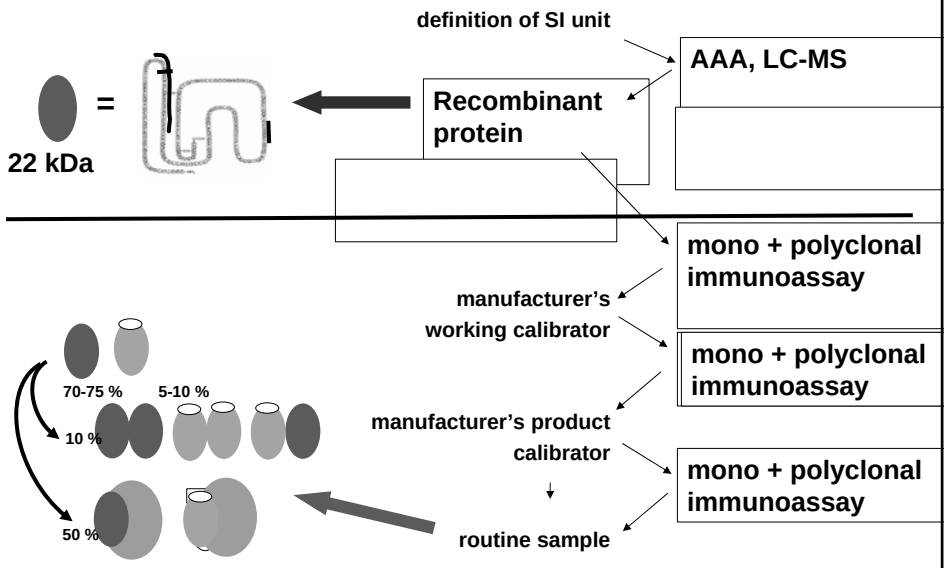
Different isoforms, oligomers and protein complexes recognised to different extent by immunoassays



hGH



hGH



conclusions

- **Traceability: whole chain should be considered**
  - **Change of method, change of measurand can cause a break in traceability chain**

- **Even minor processing can affect the properties of protein preparations**

- **Not all heterogeneities need to be characterised – only those that influence commutability and trueness**

- **The use of highly specific methods and recombinant calibrants should go together with studies of commutability and bias, as for any other calibrant**

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Thanks!

irmm Institute for Reference Materials and Measurements

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(IFCC CPP)

Commutability CRP in ERM-DA472/IFCC:

St. Georges Hospital
Siemens
Sentinel
DAKO
Klagenfurt Hospital