Joint Research Centre (JRC)

Standardization of cTnI: Aspects to be considered for a model which will (is expected to) work

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Definition of common reference ranges or decision limits for eventually critical diagnostic decisions

According to EQAS schemes for proteins inter-assay variability tends to be relatively high, in particular for TnI

Diagnostic power of particular biomarkers can eventually not be exploited to the full extend

Standardisation taken up in legislation (IVD-MD Directive 98/79/EC) and by ISO (ISO 17511 / ISO 18153) requiring 'traceability to higher order reference methods and/or materials'

ISO 17511

Uc

definition of SI unit

primary reference measurement procedure

primary calibrator

secondary reference measurement procedure

secondary calibrator

manufacturer's selected measurement procedure

manufacturer's working calibrator

manufacturers standing measurement procedure

manufacturer's product calibrator

end user's routine measurement procedure

routine sample

result (U including pre-analytical and IQC contributions)

Underlying concept

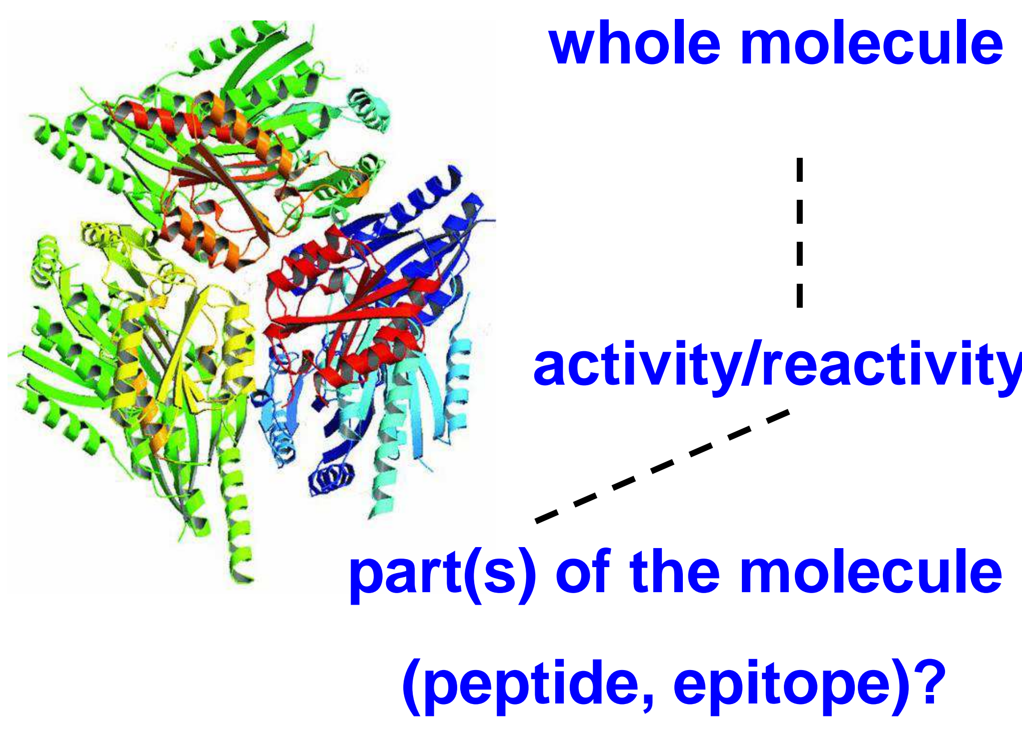
- **Metrological traceability to a stable reference, i.e. SI or a stable materialized standard**
- **Traceability via an unbroken chain of unbiased comparisons with known uncertainty**
- **Only then comparable measurement results can be achieved over space and long time periods**
- **Not a purpose on its own but means to achieve comparability of measurement results!**

ISO 17511 technical requirements

To make sure that traceability is not a purpose on its own but leads to comparability of measurement results:

- **Definition of the measurand (VIM2) - VIM3 definition of measurand (‘quantity intended to be measured’) ambiguous in this context**
- **Calibrators (stable, homogenous) commutable for routine methods and reference methods**
- **Elimination of bias and control of all parameters which may cause bias**
- **Maintain the measurand (VIM2) or know the relationships through traceability chain (equal relationship in patient samples and calibrators required, otherwise traceability chain broken)**

Biomolecules: what to measure ?



whole molecule ?

activity/reactivity

part(s) of the molecule

(peptide, epitope)?

“straight forward” for:

total amount of defined molecules

Less evident: amount of a protein occurring in various forms / fragments

Problem: Defining the analytical target & unit

- **The critical parameter is not only the “amount of substance”, rather functionality of the molecule or its parts in analytical systems that matters (not identical to biological activity)**

Heterogeneous proteins

Scorpion toxin HsTx1 (NMR)

Covalent:

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

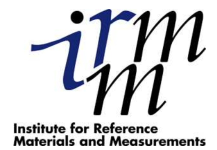
Non-covalent:

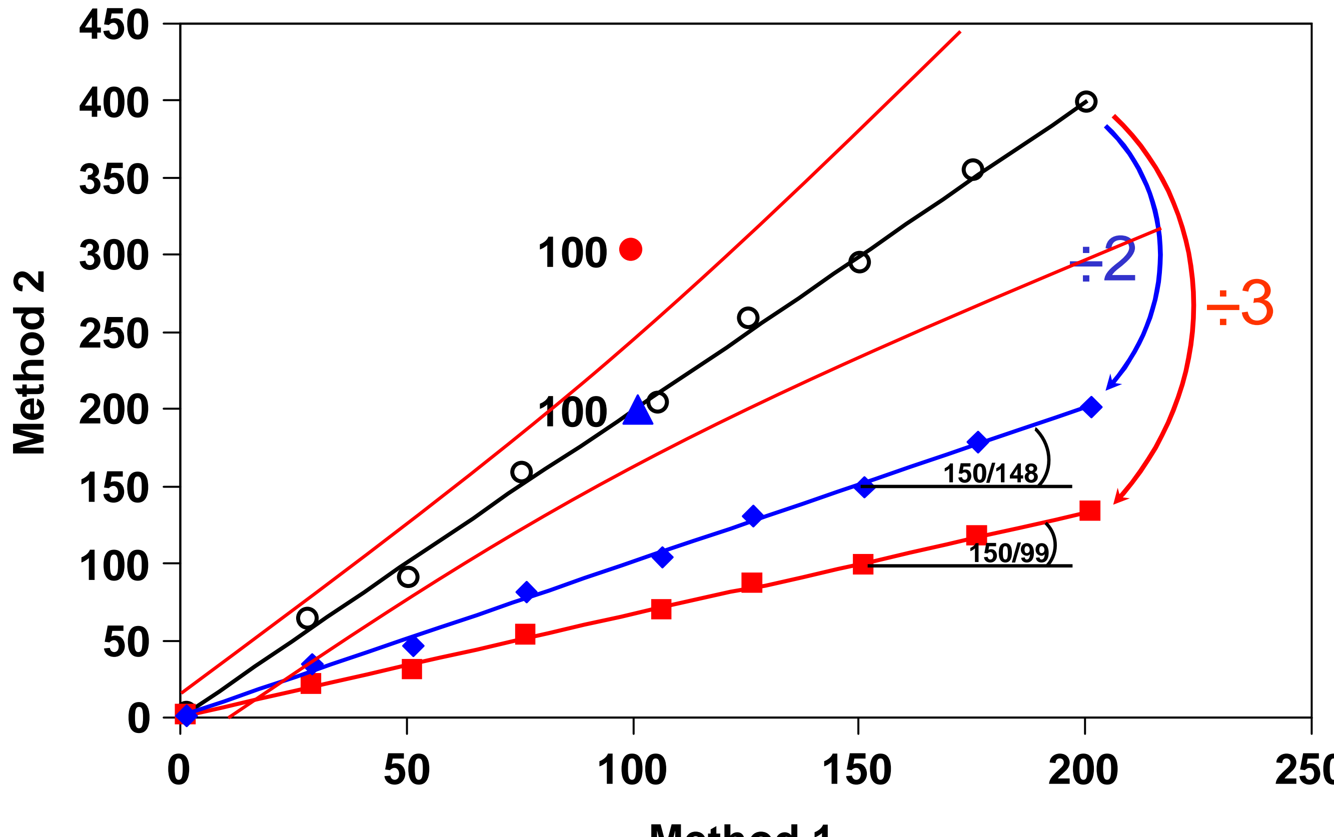
- oligomeric state
- ligand binding (metals, other proteins, co-factors, …)
- degree of structuration (partial denaturation)
- different conformational states, unstructured proteins
- aggregation

Issues with the definition of the measurand

- Proteins are typically quantified by measuring the amount of substance of a certain part of all isoforms or of particular isoforms (epitopes for immunoassays, peptides for mass spectrometry methods)
- A very specific and strictly species-limited definition of the measurand would mean that the measurand of the different methods would mostly be different without necessary having different clinical significance
- By definition results on different quantities cannot be compared
- Correlating measurands can be harmonised and standardised

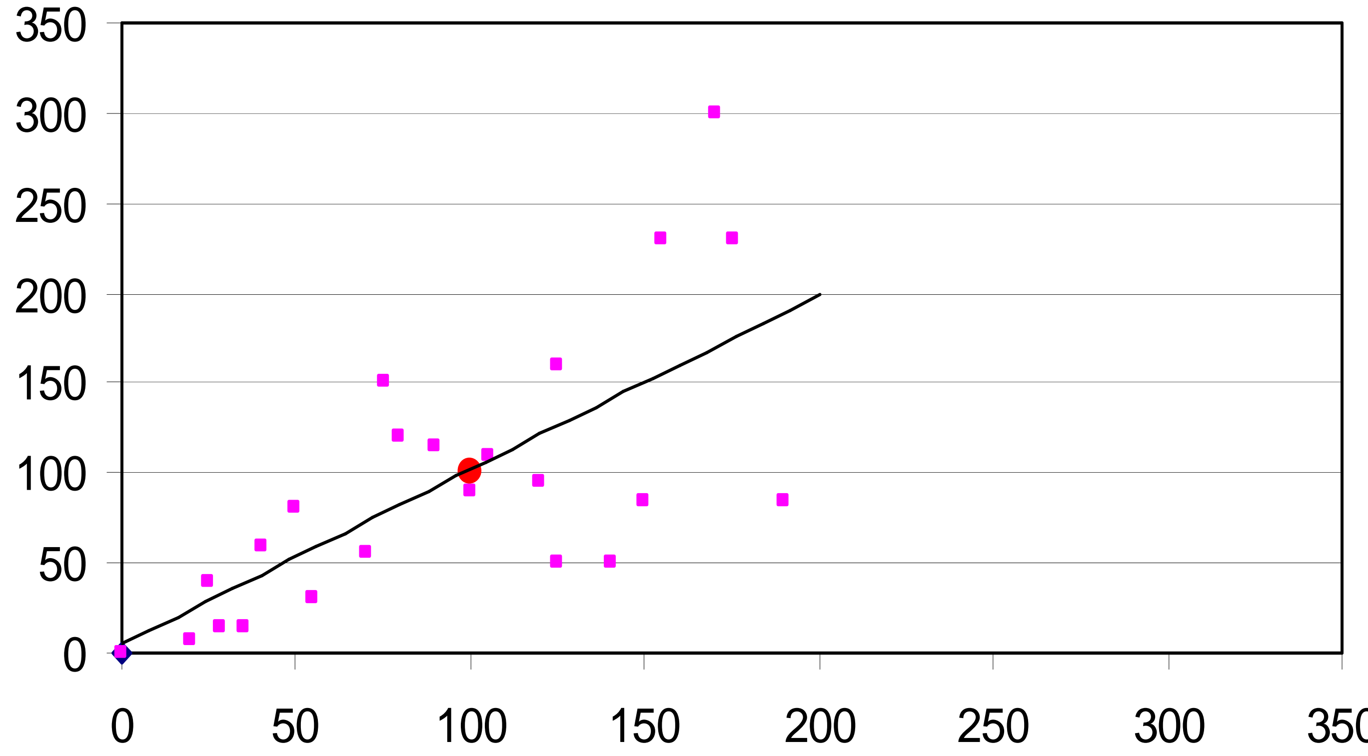
Correlation / commutability studies





- Patient samples
- Non-commutable RM, assigned value 100
- Calibration with non-commutable RM
- Commutable RM, assigned value 100
- Calibration with commutable RM
- 95 % prediction interval

Different selectivity / commutable calibrator



Outcome of commutability study

- **Lacking correlation reveals different method selectivity and non-constant relationship between different measurands of the methods applied**
- **Only averages of sample populations could be harmonized**
- **Pronounced differences between results for individual routine samples will persist after recalibration**
- **If data scatter is not acceptable the question has to be clarified which method gives the more clinically significant results and accordingly the measurand should be defined**

- **Reasons for lacking commutability**
  - **Matrix interactions, differences between routine samples and reference material**
  - **Differences in method selectivity. Although routine methods may correlate well on typical routine samples the presence of atypical isoforms or isoform patterns, differently detected by routine methods in the reference material, may cause (eventually uncontrolled) bias**

Ceruloplasmin

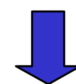
[mg/L] Method 2

- ERM-DA470 (freeze-dried)
- ERM-DA470k (freeze-dried)
- ERM-DA472 (liquid frozen)
- Clinical samples

CRM used to calibrate both methods

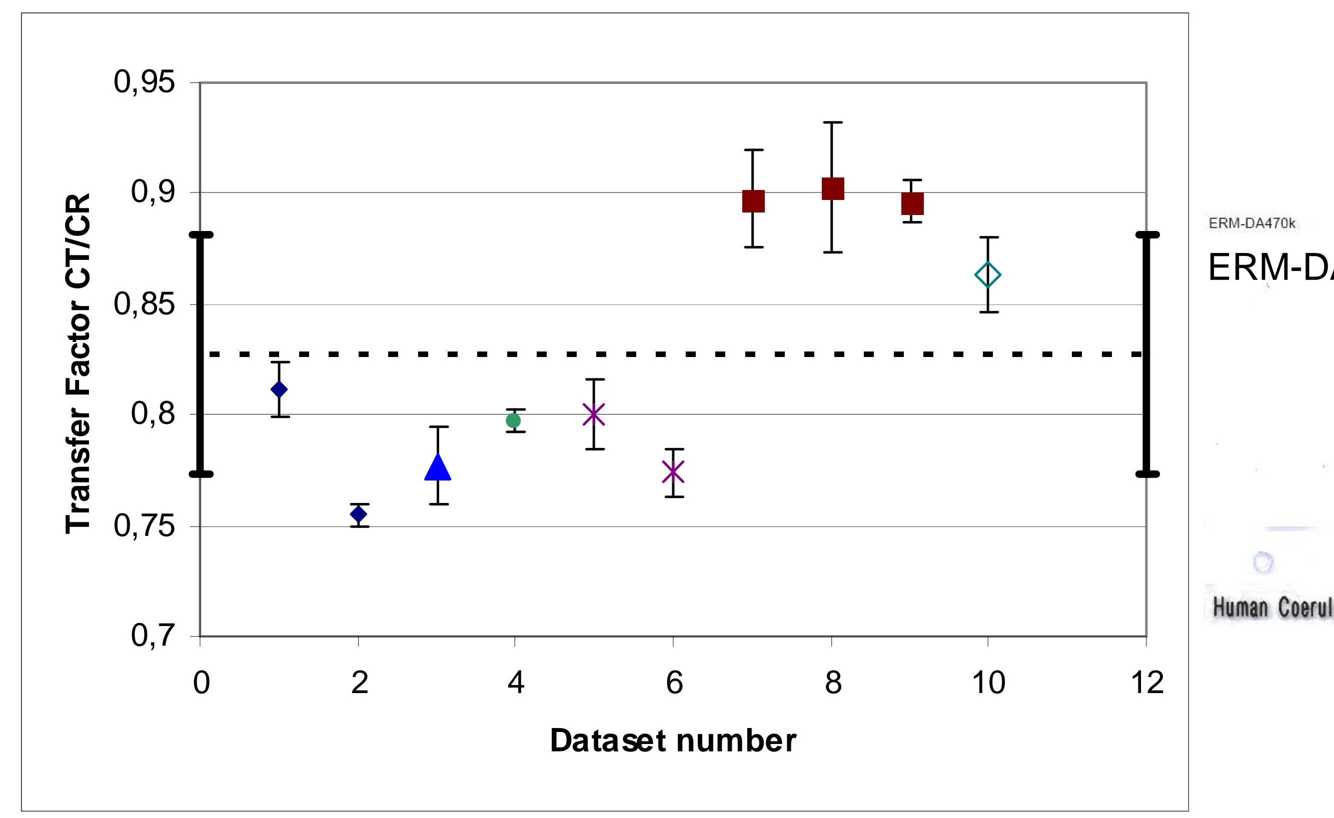
[mg/L] Method 1

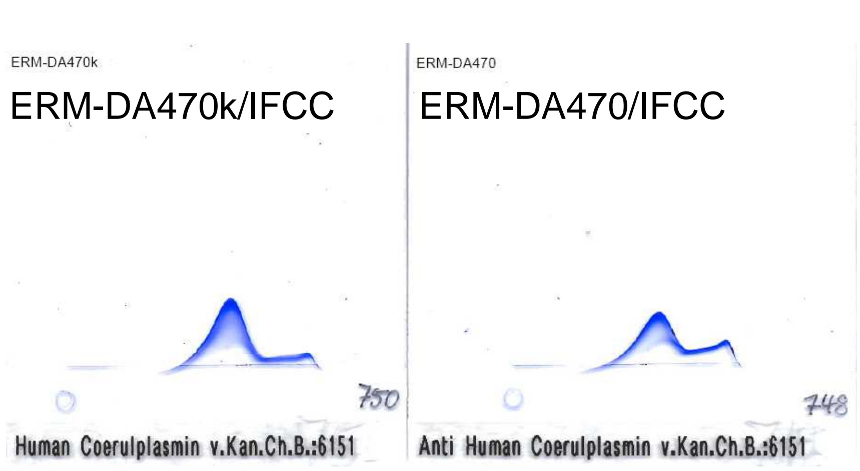
- Both methods were **using ERM-DA470** for calibration
- **Result** for ceruloplasmin in ERM-DA470 is reasonable with both method 1 and 2 (certified concentration 205 mg/L)
- **ERM-DA470 is not commutable** for this combination of methods



Discrepant results for clinical samples

Ceruloplasmin in ERM-DA470/IFCC





C-reactive protein

CRP in lyophilised and liquid frozen serum

Transfer Factor CT/CR

1.2
1.15
1.1
1.05
1
0.95
0.9
0.85
0.8
0.75
0.7

Liquid frozen

lyophilised

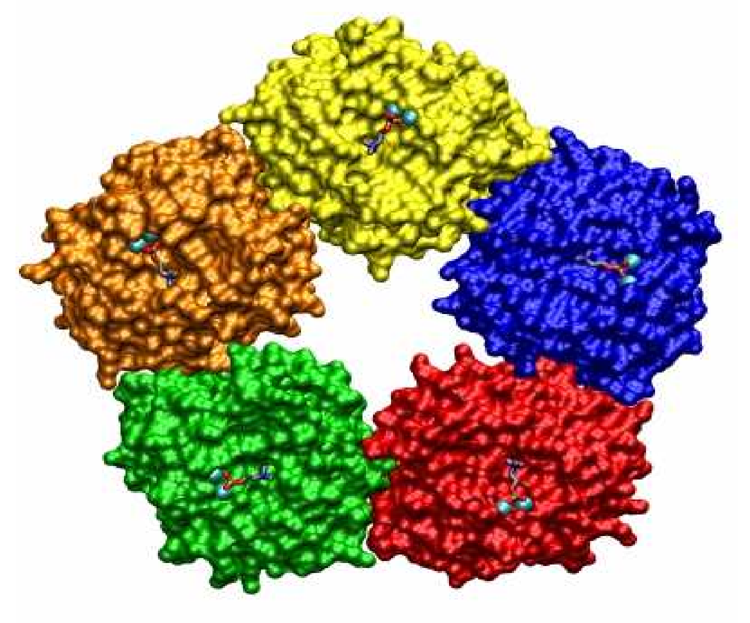
0 5 10 15 20 25

Laboratory number

The issue: Lyophilisation results in a loss of about 20 % of measurable CRP compared to the non-lyophilised material and both formats appear commutable!

Problems observed for CRP:

- Only 80 % detected by routine assays after freeze-drying
- High between bottle heterogeneity in freeze-dried material
- Presence of different oligomeric forms in freeze-dried material

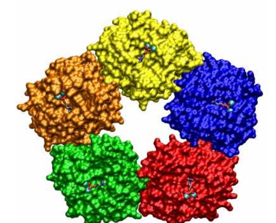


Pentameric protein
Monomer: 25 106 Da
Binds two Ca2+

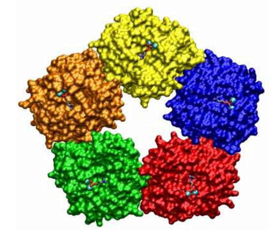
Physicochemical state verified by:

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

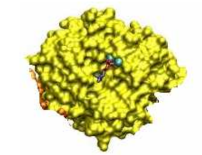
CRP oligomeric state and homogeneous immunoassay response



Pentamer in serum matrix



Pentamer in buffer



Monomer in buffer

Material	nominal conc	CRP mass concentration measured with different methods (n=3), relative to the nominal concentration							
	%	IA1 %	IA2 %	IA3 %	IA4 %	IA5 %	IA6 %	IA7 %	IA8 %
ERM-DA472/IFCC	100	101	89	107	100	89	98		100
Pentamer In buffer	100	103	49	59	79	96	118		100
Monomer In buffer	100	12	1	<1	<13	<8	2	3	2

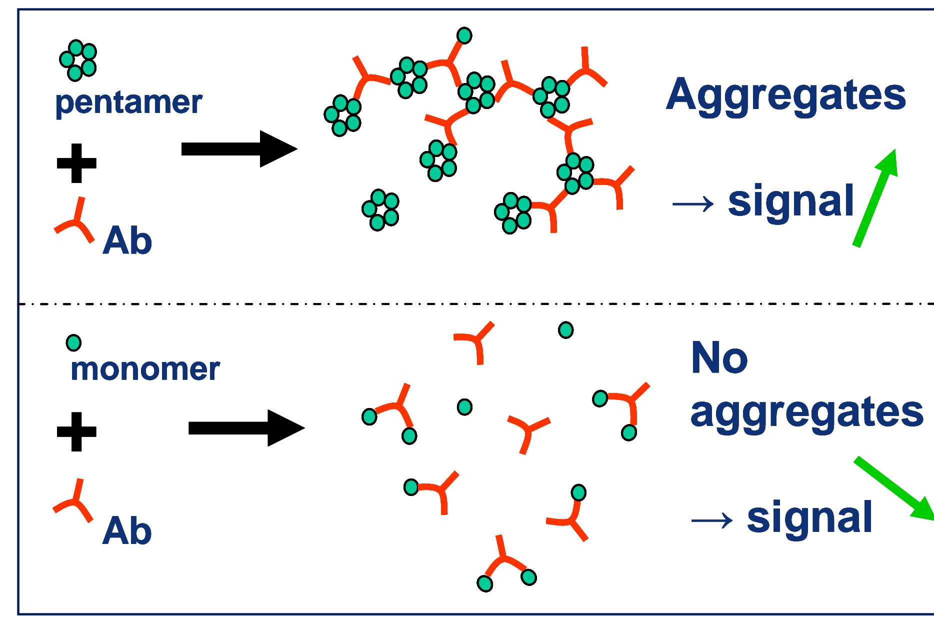
- mCRP gives < 10 % response in homogeneous immunoassays compared to nCRP

Impact of mCRP

The loss in measured CRP found for ERM-DA470k/IFCC is due to the partial dissociation of CRP into monomers which are not recognised
The presence of 2 mM calcium can prevent the dissociation
Liquid frozen ERM-DA472/IFCC does not contain detectable amounts of mCRP

M. Rzychon, I. Zegers, H. Schimmel Analysis of the physico-chemical state of CRP in different preparations including two reference materials Clinical Chemistry 2010;56:1475-82

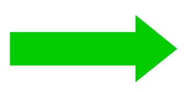
Impact of the oligomeric form on the homogeneous immunoassay response? Impact depends on immunoassay format!



CRP commutability studies

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

CRP [mg/L] Cobas
150
125
100
75
50
25
0
Pentameric CRP in buffer
Patient samples
ERM-DA472/IFCC
1/10 dilution of ERM-DA472/IFCC
0 25 50 75 100 125 150
CRP [mg/L] Sentinel_160



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

Biochemistry of hGH

hGH

22 kD 20 kD

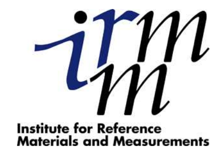
70-75 % 5-10 %

10 % 22 kD 22 kD 20 kD 20 kD 20 kD 22 kD

up to 50 % 22 kD GHBP 20 kD GHBP

5 % higher oligomers

Immunoassays results



Methods evaluated: Main routine immunoassays

Manufacturer	Calibration	Symbol
Siemens®	WHO 98/574	A
Roche®	WHO 98/574	B
Dia Sorin®	WHO 98/574	C
Mediagnost®	WHO 98/574	D
IDS®	WHO 98/574	E

Immunoassays	A	B	C	D	E
A		✓	✓	✓	✓
B			✓	✓	✓
C				✓	✓
D					✓
E					

→ **10 comparisons**

Immunoassay C *versus* A

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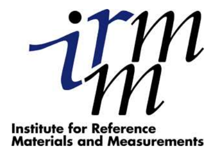
hCG



Methods evaluated: Main routine immunoassays

→ **10 comparisons**

Patient samples: ○

Reconstitution: ■ (green) Phys. buffer
■ (orange) Phys. buffer - Albumin
Blank controls: ■ (red)

Spiking:
- ○ Phys. buffer
- □ Phys. buffer - Albumin
- ■ hGH depleted serum
- ◆ Charcoal stripped serum
- ◇ SRM 971 male
- ▲ Sheep serum

Immunoassay A *versus* B

Reconstitution with Albumin
95-130 % "recovery"
Reconstitution without Albumin
Around 30 % "recovery"

0 2 4 6 8 10 12 14

$hGH_{Immunoassay\ A}$, [µg/L]

$hGH_{Immunoassay\ B}$, [µg/L]

- **Measurands have to be strictly defined or need to correlate for the routine and reference methods in order to allow harmonization / standardisation**

- **Methods determining the amount of substance of a protein only (such as mass spectrometry on peptides or the protein backbone) may not be sufficient to control the traceability chain well enough (see CER, CRP, hGH)**
  - A well understood immunoassay based reference method is a valid option for standardizing methods with equal specificity and/or correlating measurands

- **Control (and quantification) of all relevant influence parameters with the related uncertainties (e.g. matrix properties and interaction, structure, aggregation, oligomerisation, isoform profile, metal binding) is a prerequisite for being able to reproducibly produce reference materials / calibrators and to use them for calibration in a way that comparable measurement results can be achieved on long term.**
  - Highly fluctuating isoform patterns in patient samples require equal response of the routine methods and in particular of the reference method to allow for a maximum degree of harmonisation / standardisation

Lessons learnt: commutability

- Non-commutability of a reference material means that using it for calibration will introduce a bias for at least one of the methods, whereas commutability means that it can be used for achieving comparable results but it does not guarantee the absence of bias (see CRP and hGH). Commutability is absolutely required!
- Careful evaluation of the impact of processing steps on the properties of quality control materials / PT samples / reference material is crucial
- If necessary uniform calibration protocols using commutable calibrators and commutable dilutions thereof need to be developed (e.g. for the calibration of high sensitivity assays)

Conclusions

- A higher order reference material or reference measurement procedure cannot stand alone, they are integral parts of reference measurement systems

- Application of quality control / reference materials outside their validated intended use requires verification of their usability for the extended purpose

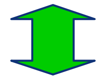
- Standardisation of protein biomarkers is challenging, however, often possible with reasonable efforts and within acceptable timelines

- Correlation is key!

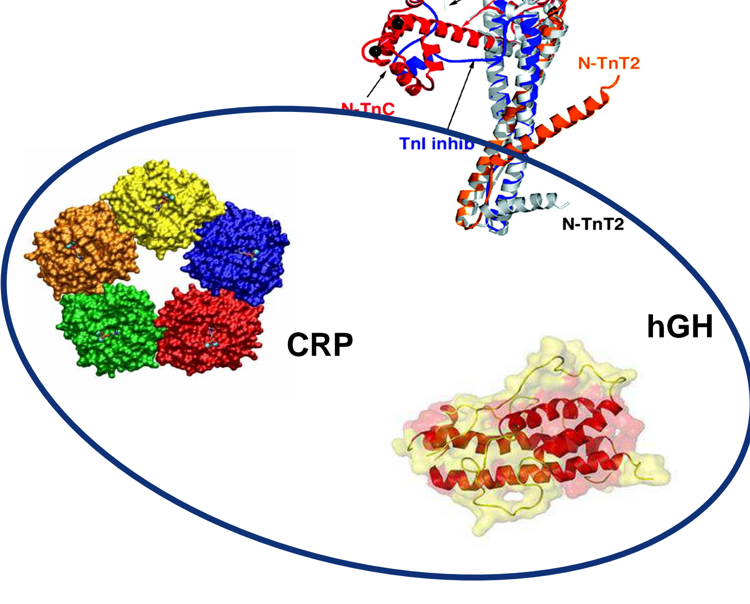
Acknowledgement: CLINBIOTRACE

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

‘amount of substance’



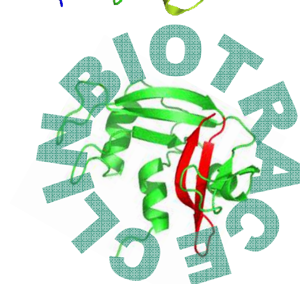
Functional activitity



Partners and collaborators:

- PTB (A. Henrion, C. Arsene, R. Ohlendorf)
- NPL (M. Ryadnov, P. Rakowska, A. Hills)
- LGC (G. O’Connor, M. Quaglia, H. Parkes, C. Pritchard, S. Biesenbruch)
- IRMM (I. Zegers, H. Schimmel, A. Munoz-Pineiro, M. Ryzchon, G. Auclair, K. Hanisch, S. Boulo)
- IFCC-WG hGH: C. Sturgeon, M. Bidlingmaier

CysC



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