

# Neglected aspects in the standardization of protein measurements. A standardized comparison method

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## **Abstract**

When immunoassay techniques are used for the measurement of protein concentrations, antigen-antibody reaction products - a two components metric system - are measured in immunocomplexes representing the quantities as well as the immunoreactivity qualities of both assay partners. The relationship between the measured signal size and the expected value result depends on many immunochemical and non immunochemical influences and effects. Especially variabilities in the molecular structure of proteins influence the signal size differently which can lead to wrong results.

The measurement range of a standardized comparison method (RID) is adapted to the assigned values of proteins in the new Certified Reference Material CRM 470 which can be used as calibrator for assigning target values in quality control preparations.

## **Introduction**

The determination of human proteins is usually performed by immunoassay techniques, today mainly by automated systems using the light scattering of immunocomplexes as a signal generator. It means: protein concentrations are measured by comparing antigen (Ag)-antibody (Ab) reaction products for a reference protein in standard preparations with those for the analyte protein in specimens. The immunometric measurement principle is a two components system on the contrary to the one component measurement system like weighing. This difference makes standardization measures complex and the understanding difficult. In some cases the correlation of the values obtained from method to method is quite low, in other cases the correlation is acceptable but the absolute values can be rather different. The early introduction of International Reference Preparations (WHO; 1) had not succeeded in the worldwide standardization expected for the protein analysis, because those materials were not suitable for all techniques. The establishment of the new Certified Reference Material [CRM 470 (2) through BCR/Brussels  $\cong$  RPPHS Lot 5 (3) through CAP/USA] by the Scientific Committee on Plasma Protein Standardization (PPS) of the International Federation of Clinical Chemistry (IFCC), available from 1993, makes a progressive harmonization of protein determination possible, a very helpful step for manufacturers and users.

Without any doubt the following points are important respectively necessary for a better harmonization of protein analysis and they need to be dealt with in the near future:

- Protein reference materials for standardization (as CRM 470)
- Antibody reference reagents for standardization (open)
- Reference methods (4) for measurement procedures (as RID)
- Validation of equipments for value transfer (open)
- Protocols for statistical evaluation (as described in 2, 5).

Nevertheless, there have further important and till yet neglected aspects and effects to be considered which can influence the signal sizes of measurements and which can lead to wrong evaluations of value results and with it to wrong clinical interpretations.

## 1. Measurement signal size versus value result

### 1.1. Ag - Ab reaction product

At first, it has to be realized that the common mental assumption "the result of an immunoassay measurement is identical with the expected protein concentration" is abridged, is not true (6).

A measurement result means a signal size (diameter for RID, voltage in "bit" for immunonephelometry, optical density for turbidimetry) and not a value result in the dimension g/l or IU/ml. Immunoassays measure the Ag - Ab reaction products as immunocomplexes or immunoprecipitates dependent on the time interval.

- a) beside the quantity of the Ag (the target part needed for the clinical application) also the second reaction partner Ab is measured in the immunocomplexes.
- b) furthermore the measurement signal size is not only influenced by the proportionality of both quantities Ag and Ab, but also by qualitative effects, that means by the reciprocal immunoreactivity or reaction quality of both assay partners.

### 1.2. The immunochemical property / immunoreaction quality

of the Ag depends on the molecular structure of the protein and is not constant in each case, something, which is often uncertain or unknown for the analyst. For the Ag the immunoreactivity is defined by the possibly present and/or available number of the epitopes (Ag-determinants) and their relationship, at least by the reacting ones in the actual measurement.

The immunoreactivity i.e. reaction quality of the Ab is defined by the number and relationship - it means by the population - of monoclonal antibodies (MAB's) in the antiserum used as reagent representing the possible, but not always available and/or assailable epitopes. The affinity respectively avidity of antisera is resulting from these properties.

### 1.3. Measurement and evaluation

For the relationship between signal sizes and value results the following definition is valid: Measurement (evaluation) of an analyte protein in specimen means the comparison of the signal size (value result) for the standard/Ag - Ab reaction product with the signal size (value result) for the specimen analyte / Ag - Ab reaction product (the same comparison sentence is valid for the evaluation, but with exchanging the signal size by value result)

#### Consequence:

Immunoassay techniques allow a correct evaluation for values resulting from signal sizes only for such measurement cases when the immunochemical properties of Ag in the standard preparation used as reference and those of analyte/Ag measured in specimen are identical or at least nearly similar. If this postulate for the reaction quality is not fulfilled, no exact value results are obtained. Such wrong results must be especially expected, if the immunochemical properties of the both proteins compared are various and if the antiserum used as reagent does not contain a constant population of antibodies from batch to batch and reacts differently with the various protein molecule of the Ag in the standard and analyte.

### 1.4. Further influences

Furthermore, it should be mentioned that the signal size is often not only representing the pure Ag - Ab reaction product, but also other factors like: complement components and interacting substances which have some affinity to Ag or Ab, or

supplementary reagents used in the techniques. The kind of starting complex (germ carrier) and the time for the stabilization of immunocomplexes are also variable from measurement to measurement.

There have to be reported also non immunochemical influences which can produce a false signal size: matrix effects, interfering substances, the principle of measurement, hardware and software of instruments and, finally, individual errors.

TABLE 1. Different Effect of Influence for Signal Size

Cause	Increase *	Influence for Signal Size		Examples
		RID (mm)	BNA (Bit)	
Immunoreactivity by epitope change **	++	↓	↑	Monoclonal Ig's BJ Proteins C3 → C3c α1A/α2M
Measurement principle by molecular mass	++	↓	↑	Hp-Types Aggregation (IgM)
Matrix effects Interfering substances	++	± 0	↑	lipemia hemolysis RF
Hardware / Software	-	?	?	all proteins

\*) reciprocal influence, if decrease

\*\*\*) if possible reflection by corresponding antibodies in reagent

## 2. Variability of molecular structure of proteins

The property of a protein as a native and physiologically active substance and its behaviour are determined by the type of primary / secondary / / tertiary / quarternary structures. Smallest influences (Table 1) can alterate the structure with a dramatic change of the immunoreactive properties of analytes:

- physiological processes  
(protein/protein complex formations, protein/component interactions, enzymatic digestions etc.)
- external influences  
(contamination by enzymes, alteration by temperature, storage, transport, production procedures, chemical and physical attacks)
- natural differences from individual to individual  
(polymorphism, different content of prosthetic groups like the degree of glycosilation, lipidation etc.)

This reaction variability of protein analyte can be compared with the changeable, characteristic behaviour of the animal chameleon!

Practical examples can be mentioned for Bence Jones proteins/polyclonal Ig L- chains (different epitopes), haptoglobin types (different molecular mass, Table 2), α<sub>1</sub>-antichymotrypsin (natural and recombinant preparation) demonstrating the summarized influences of molecular structure variabilities and the problematic consequence for the relationship between signal sizes and value results.

## 3. A standardized comparison method (RID)

Special RID plates were developed and manufactured for calibrating target values of proteins

TABLE 2. Signal Size vs haptoglobin type -for 200 mg/dl pure protein equilibrated by weighing

	Frequence*	MM/Dalton	RID Diameter/mm	BNA Signal/Bit	Value for RID vs BNA	Δ %
Hp 1-1	16 %	86.018 = x	6.5 ↑↑	700 ↓↓	greater	plus 20 to 30 %
Hp 2-1	48 %	x + polymers	5.8	1 400	similar	~
Hp 2-2	36 %	> 200 000 + polymers	5.6 ↓↓	1 500 ↑↑	lower	minus 5 to 15 %
Hp Mixture	natural **	~	5.9	1 300	similar	~

\*) approximately for Caucasians

\*\*\*) according to the relationship of frequence in Caucasians

in survey samples with reference to CRM 470. The measurement range of this technique (Table 3) was optimized and adapted to the assigned values of 14 proteins declared in CRM 470. It means: CRM 470 can be used as calibrator in the dilutions 1 + 3 / 1 + 2 / undiluted (for albumin: 1 + 19 / 1 + 9 / 1 + 5). The reference curve resulting from these three measurement points is suitable to establish target values, e.g. in external quality control materials. The source of antisera used for these plates (e.g. BEHRING / DAKO) shows no influence for comparative analyses of five selected proteins (IgG, IgA, IgM, albumin, transferrin) in seven different control specimens (7). This method has been evaluated for the calibration of proteins in survey samples of the German Society of Clinical Chemistry (GSCC). The target values assigned by this standardized comparison method were compared with the results of the survey participants. The data demonstrate a smaller mean variation for the target values than for the consensus values earlier performed for GSCC surveys, when comparing directly the values declared in g/l (IFCC standardization / CRM 470) or indirectly by converting the conventional results to the g/l (IFCC) by means of company dependent conversion factors. An improved commutability of the measurements could be achieved, if this very robust, easily changeable technique serves as a standardized comparison method.

TABLE 3. RID CRM 470 x Protein as "Standardized Comparison Method"

Radial Immunodiffusion Plates adapted to CRM 470 as Reference Standard / Calibrator

Protein X	CRM 470 Certified Value		RID Aims			RID Assay Range			
	g/l IFCC	mg/dl IFCC	D(mm)*	Slope	Intercept	from D(mm)	to D(mm)	from mg/dl IFCC	to mg/dl IFCC
TTR	0,243	24,3	8,5	2,521	11	4,0	9,3	1,98	29,9
ALB	39,7	3970	8,5	0,077	11	4,0	9,3	64,8	979
A1AG	0,656	65,6	8,5	0,934	11	4,0	9,3	5,36	80,9
A1AT	1,206	120,6	8,5	0,508	11	4,0	9,3	9,8	149
GER	0,205	20,5	8,5	2,988	11	4,0	9,3	1,67	25,3
A2M	1,64	164	8,0	0,323	11	4,0	9,3	15,5	234
HPT	0,893	89,3	8,5	0,686	11	4,0	9,3	7,3	110
TF	2,45	245	8,5	0,250	11	4,0	9,3	20,0	302
C3	1,091	109,1	8,5	0,561	11	4,0	9,3	8,91	134
C4	0,151	15,1	8,5	4,056	11	4,0	9,3	1,23	18,6
CRP	0,0392	3,92	9,5	17,92	20	5,0	10,0	0,28	4,5
IgG	9,68	968	8,5	0,063	11	4,0	9,3	79,0	1193
IgA	1,96	196	8,5	0,313	11	4,0	9,3	16,0	242
IgM	0,797	79,7	8,0	0,665	11	4,0	9,3	7,52	114

Volume per well: 5 microliter (CRP: 20 microliter)

Diffusion time: 3 days (A2M, IgM: 5 days)

\* for all proteins in CRM 470 after reconstitution with 1 ml aqua dest.: undiluted sample (for Albumin: 1 + 4) further general dilutions 1 + 1 / 1 + 3 (for Albumin: 1 + 9, 1 + 19)

## Conclusion

Recommendations for a further standardization of protein measurement can be given:

- Physiochemical and physiological properties representing the immunoreactivities of proteins must be characterized and known, to obtain exact analysis results by immunoassay techniques. If the immunoreactivities of proteins in standard preparations and specimens are identical then the correlation for two immunoassay techniques will be acceptable (slope near to 1, low midrange). If the correlation is rather good, but the absolute concentration differs (slope  $>/< 1$ ) this means that certain properties of the proteins have a different influence on both measurement principles.
- The use of antibodies containing reagents is not sufficiently certified; this deficit must be compensated. For the identification of an analyte an antiserum used as reagent should represent possibly many / all epitopes of an Ag. For the quantitation of an analyte the standardization is easier if the antiserum used as reagent represents only a few epitopes of the Ag, in extreme cases the aim of standardization can be obtained if only one MAB recognizing a stable, always present representative epitope is used.
- A standardized comparison method should not be influenced by non-immunochemical effects (matrix effects, interfering substances). Indeed it has to recognize / measure only the pure Ag - Ab reaction product.
- The same alteration of the Ag - immunoreactivity (increase) must not registered by the same kind of alteration of the signal size / value result (increase) using different immunoassays (and vice versa):  
better reactivity in RID means lower signal / lower value;  
better reactivity in immunonephelometry means higher signal / higher value
- The reliability of an analytical process can be only well assessed if the internal quality control material contains proteins with absolutely stable molecular structure. The values should be system assigned ones.
- Values obtained by a standardized comparison method should be preferred for external quality control materials and not consensus values resulting from measurements obtained by using many different assays.
- It is not the immunoreactivity size of analytes, but their mass value results per volume unit what is representative for the clinical relevance of proteins and important to apply the proteins for certain diagnostic purposes.

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