

## REVIEW

# Metrological sharp shooting for plasma proteins and peptides: The need for reference materials for accurate measurements in clinical proteomics and *in vitro* diagnostics to generate reliable results

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Reliable study results are necessary for the assessment of discoveries, including those from proteomics. Reliable study results are also crucial to increase the likelihood of making a successful choice of biomarker candidates for verification and subsequent validation studies, a current bottleneck for the transition to *in vitro* diagnostic (IVD). In this respect, a major need for improvement in proteomics appears to be accuracy of measurements, including both trueness and precision of measurement. Standardization and total quality management systems (TQMS) help to provide accurate measurements and reliable results. Reference materials are an essential part of standardization and TQMS in IVD and are crucial to provide metrological correct measurements and for the overall quality assurance process. In this article we give an overview on how reference materials are defined, prepared and what role they play in standardization and TQMS to support the generation of reliable results. We discuss how proteomics can support the establishment of reference materials and biomarker tests for IVD applications, how current reference materials used in IVD may be beneficially applied in proteomics, and we provide considerations on the establishment of reference materials specific for proteomics. For clarity, we solely focus on reference materials related to serum and plasma.

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**Abbreviations:** CRM, certified reference material; ID-MS, isotope dilution mass spectrometry; IS, international standard; IU, international unit; IVD, *in vitro* diagnostic; QA, quality assurance; QC, quality control; SI, Système International d'Unités; SOP, standard operating procedure; TQMS, total quality management systems

## 1 Introduction

Clinical proteomics studies help to increase the knowledge on states of human health and disease, as well as on physiological and pathological processes. Among many other useful aims, clinical proteomics is applied to determine whether proteins and peptides are potential biomarker candidates. Biomarkers change in response to physiological variations and pathological alterations of a biological system [1, 2].

Determination of the absolute or relative concentration of biomarkers can aid in decision making during drug development or in the decision on medical treatments. For drug development, biomarker assays do not need to be approved and registered as *in vitro* diagnostics (IVDs). In contrast, these assays need to be approved and registered when used for IVD purposes [3]. Furthermore, technologies applied in clinical proteomics research, in particular MS and protein arrays, are being proposed as useful tools for IVD laboratories for the routine analyses or determination of relevant proteins [4–6].

Proteomics technologies and studies need to provide reliable information, as is the case with any other analytical approach. Reliable information depends upon comparability and constancy of results across time and space as well as a known and acceptable level of variability of all processes involved, including preanalytical, analytical and postanalytical steps [7, 8]. Results need to be reliable to increase the likelihood of making the right choice for further investigations, *e.g.*, verification of promising biomarker candidates in clinical proteomics. Comparability of results is of particular importance when multisite verification trials and subsequent validation studies are performed.

Currently, it appears that a major impediment for a smooth and swift transition of biomarkers from proteomics research to health care, in particular IVD, is due to a bottleneck of verification and validation of large numbers of candidates. Technologies applied for verification or validation usually exhibit limitations, leading to this bottleneck.

For example, when immunoassays are to be applied, the transfer from proteomics technologies to immunoassays is often hampered due to (i) the heterogeneity of distinct proteins and peptides and the complexes they form, (ii) differences in the functional activity of isoforms with identical primary structure, (iii) the lack of understanding of which form(s) ultimately correlate with a disease, as well as (iv) the limited availability of specific antibodies and antibody pairs. Antibody generation is time consuming, costly and not always successful. The same applies to the implementation of immunoassays with adequate performance characteristics. In addition, resource constraints (experienced personnel, time and funding) usually limit the number of analytes and their variations that can be verified and validated by conventional immunoassay techniques.

Proteomics technologies used for verification studies allow for the investigation of multiple proteins or peptides and their variations at once. However, aspects like choice and availability of appropriate samples, throughput, costs, installed base, availability of sufficiently educated staff to run respective tests in multicentric settings, standardization, lacking comparability of results, insufficient analytical and limited test or measurement accuracy can be prohibitive [3, 7].

Demands on measurement reliability and comparability may be different depending on the scope of a study, *i.e.*, discovery, verification and validation as well as the medical context. This also applies to what is deemed to be a sufficient accuracy of measurement, especially in discovery and research, at least within the limits of statistical requirements to have a certain level of confidence in the data generated (see below).

Standardization and quality management help to improve measurement accuracy and to provide reliable as well as comparable results. The implementation of appropriate protocols or standard operating procedures (SOPs) and compliance to them help to reduce variation and error and increase precision, reproducibility and comparability [9]. When protocols are not only specific to a distinct laboratory but rather written standard documents, *e.g.*, general SOPs, rules, guidelines or recommendations these protocols can be a helpful tool towards harmonization or even better standardization, if bias and method dependence of the measurand can be excluded. The implementation of such written standards, *i.e.*, an authoritative document setting forth criteria for performance and characteristics agreed upon by laboratories and national or international institutions, is time consuming and costly. Consensus documents produced by the Clinical Laboratory Standards Institute (CLSI) are examples of this type of written standard ([www.clsi.org](http://www.clsi.org)).

Apart from SOPs and document standards, reference materials are an essential part of standardization and quality management, and allowing for trueness control during the validation process of newly developed methods, laboratory performance monitoring, and interlaboratory comparison. Reference materials have been considered to be the best guarantee for correct calibration and thus trueness of measurement [10]. In this respect, the reference material must be internationally accepted to be a common calibrator or trueness control material. Such common reference materials significantly improve comparability of measurements as, for example, shown by the use of internationally agreed upon certified reference material (CRM) ERM-DA 470 (formerly known as CRM 470) for distinct plasma proteins (see below) [11].

Reference materials, whose values have been determined by a reference measurement procedure, whereas procedure refers to an implemented method, represent a comparatively easy way to directly calibrate or control a routine measurement procedure (assay or test) [12]. In contrast, the implementation of a distinct reference measurement procedure is usually associated with high expense and/or low throughput and often not applicable for analysis in a routine laboratory. In addition, reference measurement procedures generally do not exist in the proteomics field. The development of reference materials may also involve great expense. However, once established, reference materials are available universally and their use at a given laboratory is cost effective and simple. In conclusion, usage of established reference

materials appears to be a promising and beneficial improvement for clinical proteomics to determine and improve the performance of technologies and methods, reliability and comparability of results, and to perform clinical verification, and validation studies. For the application of emerging biomarkers and the use of proteomics technologies in IVD, the application of reference materials is mandatory [7, 13].

We thus set out to outline how reference materials are defined, prepared and applied, what the rationale of their application in IVD and other analytical disciplines, such as proteomics, is, and how proteomics may benefit by supporting the development of reference materials for IVD applications. In addition, we explain why reference materials should be applied in proteomics and how current reference materials used in IVD may be beneficially applied in clinical proteomics. We also provide considerations on the development of reference materials specific for proteomic applications. We focus on reference materials related to serum and plasma. These specimens serve as examples as they are thought to reflect the whole human proteome and represent the specimens analysed most frequently in IVD [14, 15].

## 2 Reference materials are crucial for standardization and quality management to improve reliability of results

It is helpful to take a look at the need for and benefits of standardization and total quality management systems (TQMS), where reference materials play an essential role. Standardization and TQMS (www.iso.org: ISO 13485; ISO 9001) are essential in IVD to support the generation of reliable results and for the correct interpretation of the assay results and their diagnostic or prognostic use [16–18]. This helps to provide a high level of quality for patient care.

TQMS encompass the complete laboratory process, *e.g.*, organization and management, personnel, premises and environment, equipment, material, and reagents *etc.* [16–18]. TQMS rely on a comprehensive system of quality development, maintenance and assurance, control and improvement.

To achieve adequate TQMS in IVD laboratories it is necessary to guarantee quality assurance (QA) of the analytical systems that are applied. QA may include preanalytical, analytical and postanalytical steps like preparation of the patient, specimen collection and processing, performance characteristics of the measurement, validation of results, their reporting and interpretation. Of note, the initial concept of QA was based on quality control (QC), which mainly focused on the assessment of analytical performance characteristics, like analytical sensitivity and specificity and measurement accuracy. To guarantee QA, reference measurement systems that rely on reference materials are used (see Sections 4 and 5). Reference materials are therefore an indispensable part of harmonization, standardization and TQMS.

## 3 Reference materials are used as calibrators and controls

Being an essential part of reference measurement systems, reference materials are applied in a variety of analytical procedures as calibrators for the calibration of a measuring system for assigning values, *e.g.*, concentrations, to certain properties, *e.g.*, analytes, of a material, *e.g.*, plasma or serum. Reference materials are also applied as external QCs for the assessment of the performance characteristics of a measurement procedure.

A reference material is defined as a material or substance, one or more of whose property values are sufficiently homogeneous and well established (www.iso.org: ISO 15195) to be used for the calibration of an apparatus, the assessment of a measuring method, or for assigning values to materials (www.iso.org: ISO Guide 30 (E/F)). The term 'homogeneous' refers to the physical homogeneity between macroscopic parts of the material. It does not refer to any microheterogeneity between molecules of the analyte, *e.g.*, the potential inherent microheterogeneity of a protein or peptide due to the presence of isoforms, PTMs, *etc.* Properties can be quantitative or qualitative ones. An assigned property value can be the activity, potency or amount of an analyte, *e.g.*, the concentration of a protein, as determined by the analysis with a measuring system. A measuring system is a complete set of measuring instruments and other equipment assembled to carry out specified measurements. The system may include material measures, *e.g.*, the instrument, and chemical reagents, *e.g.*, diluents, controls and calibrators.

A calibration is a set of operations that establishes, under specified conditions, the relationship between values for the quantities of a property indicated by a measuring instrument or measuring system, or values represented by a material measure or a reference material, and the corresponding values realized by standards. The result of a calibration permits either the assignment of values of measurands to the indications or the determination of corrections with respect to indications.

As an aside, the term 'measurand' has a broader meaning than 'analyte'. A measurand is a particular quantity of a property that is subject to a measurement, for example, vapour pressure of a distinct sample under certain conditions of the measurement, *e.g.*, temperature [19]. The measurand describes what is causing the result of the measurement, whereas the analyte describes the particular component of interest. An analyte is a component represented in the name of a measurable quantity of a property (www.iso.org: ISO 17511). For example, 'the catalytic concentration of lactate dehydrogenase isoenzyme 1 in plasma' is the measurand, whereas the 'lactate dehydrogenase isoenzyme 1' is the analyte. In 'bottom up' proteomics, a peptide specific for a distinct protein is the measurand, whereas the protein from which that specific peptide was produced is the analyte.

A calibration may also determine other metrological properties such as the effect of influence quantities, *e.g.*, such as a chemical interference. Metrology is the science of measurement and includes all aspects both theoretical and practical with reference to measurements, whatever their uncertainty, and in whatever fields of science or technology they occur. Calibrators with different quantities of a property or analytes may be used to establish a quantity-response dependence curve over a range of interest, *e.g.*, as is the case in isotope dilution MS (ID-MS) [20]. Of note, the same reference material may not be used for both purposes, *i.e.*, as a calibrator and a trueness control, in a given situation in a particular laboratory.

Reference materials are used as external controls for most laboratory tests in conjunction with internal and procedural controls for QC purposes [21]. Internal and procedural controls are designed or integrated into a measurement system to monitor one or more components for unacceptable variation or error. Such components may be (i) elements of an analytical process like technical failures, the functionality of the reagents, *etc.*; (ii) operator variation, *e.g.*, in the processing of samples; and (iii) environmental factors like temperature and humidity. For example, test results of hemolytic or lipemic samples are to be flagged, or results that may be false due to unacceptable temperature changes will not be provided.

External controls are not designed into a measurement system. These controls are tested throughout all analytic test phases in the same manner as the actual samples. In this sense, compounds added to a material as 'internal standards' are used as an external control reference material. External controls are thus principally useful to monitor all the analytical components of a measurement system. They will also provide information on how long a test system maintains stable performance specifications, *e.g.*, how long the calibration is stable.

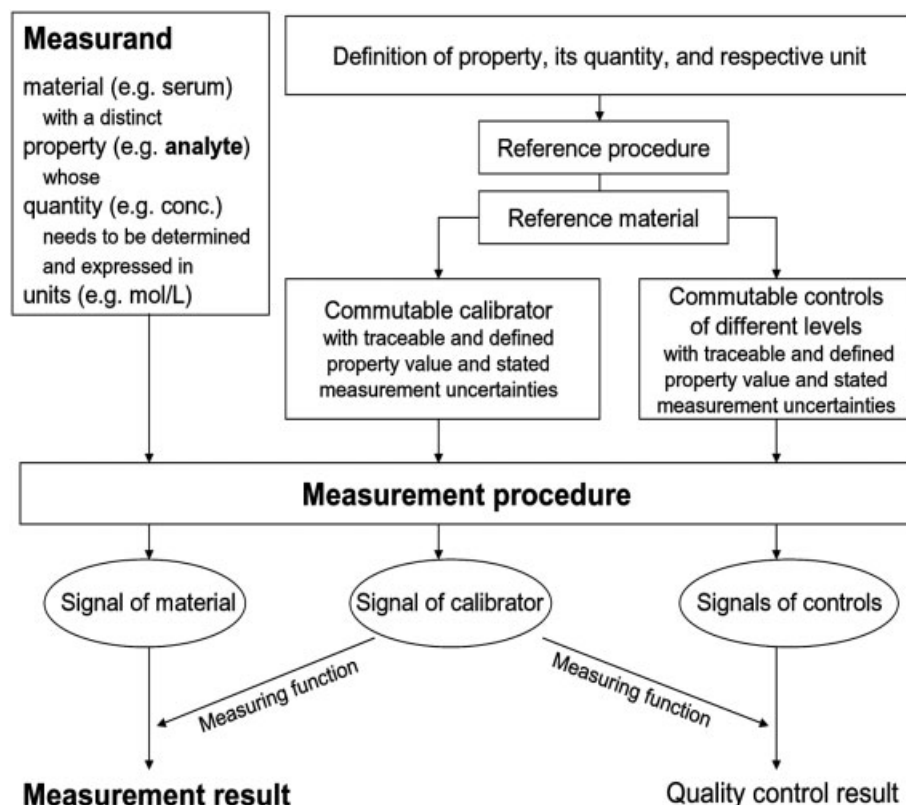
Materials that may be used as external controls include reference materials, internal standards, commercially prepared or in-house prepared samples, proficiency test samples with confirmed results and previously tested patient specimens with established values. For example, control plasmas are a preparation of fresh, frozen or lyophilized plasma, like standard human plasma [22], collected from human or animal blood, or artificially derived material, intended for use in a calibration or QC process, *e.g.*, to monitor all aspects of a laboratory test system, including the reagents, instruments, reconstituting and diluting fluids and pipettes. Normal controls should give test results within the reference interval. Abnormal control plasmas should give values within the clinically relevant abnormal range. In summary, the use of reference material based controls allows for the monitoring of the analytical performance of a system. The use of reference material-based calibrators allows for the trueness of measurement.

#### 4 Traceable and commutable calibrators and controls are essential for metrological correct measurement procedures and QC, respectively

To further assess the importance of and basis for calibration and control of measurement procedures by reference material, it is helpful to recall the basic process of a measurement (Fig. 1) [12]. The purpose of a measurement is to describe a property of a material. A quantity, *e.g.*, a concentration, and respective units, *e.g.*, mol/L, may be chosen that express the property, *e.g.*, the analyte. A critical step of a measurement is the calibration, because it permits the expression of the measured signal in units of the selected quantity [23]. This requires a calibrator with established values of the property. A measurement procedure can denote a set of operations that are described specifically and used in the performance of particular measurements, *e.g.*, a distinct assay, according to a given measurement method. A method of measurement is a logical sequence of operations, described generically, used in the performance of measurements, which are performed to determine values of quantities of properties. The calibration of a measurement procedure (with a common reference material) is basically a refined form of (a reference) measurement [24]. For this purpose, reference materials contain traceable and commutable quantities of distinct analytes. The expected reaction or concentration of the property or analytes of interest is known within limits ascertained during preparation and confirmed in use.

Traceability is the ability to trace the history, application or location of an entity by means of recorded identifications. More specifically, measurement traceability refers to a (metrological) property of the result of a measurement or the value of a standard, which can be related to stated references, usually national or international measurement standards, through an unbroken chain of comparisons all having stated uncertainties [19]. The unbroken chain of comparisons is called a traceability chain. The traceability of values assigned to a reference material must be assured through reference measurement procedures or reference materials of higher order, *e.g.*, internationally agreed, common and certified primary or secondary reference materials with true or conventional true concentrations or values. Traceability is to be established to the highest international standards (IS) by a comprehensive reference measurement system (see Section 5). Apart from being traceable, results obtained with a reference material need to be commutable to the samples tested or in case of an internal calibrator or control to the respective analyte.

Commutability is a consistent relationship of the signals generated by the calibrator or control to those generated by the specimen. The commutability of a material is its ability to yield the same numerical relationships between results of measurement by a given set of measurement procedures, claiming to measure the same quantity as those between the expectations of the relationships obtained when the same



**Figure 1.** Metrological correct measurement procedure and QC.

procedures are applied to other relevant types of material. This means that a calibrator must behave in a similar fashion as all the samples tested in different methods. The samples must behave consistently in any one method, but may behave differently in different methods. Commutability is thus a concept of similar analyte behaviour and consistent relationship between methods [11, 25].

Consequently, commutable calibrators and controls with traceable and defined property values and stated measurement uncertainties are essential for metrological correct measurement procedures and QC. To provide respective calibrators and controls it is necessary to implement reference measurement systems.

## 5 Reference materials are key to reference measurement systems that allow for true and traceable measurement results

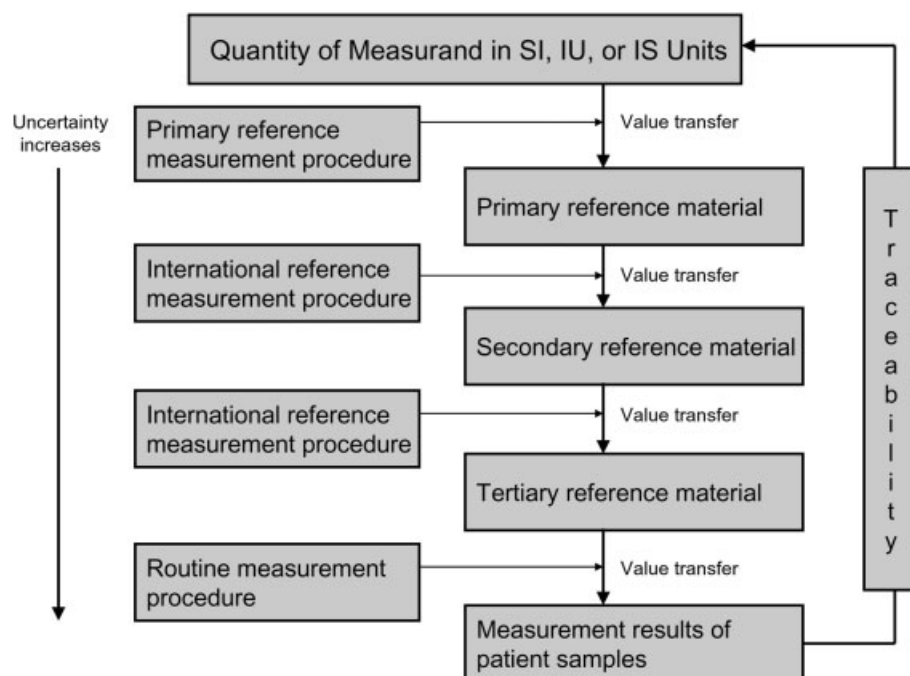
The aim of using a reference measurement system is to achieve trueness of measurement (Figs. 2 and 3). Reference measurement systems are based on a network of reference laboratories whose measurements are based on the national and international regulations and guidelines and that use external QA schemes, reference measurement procedures (reference methods) and certified reference materials [23, 26].

A reference measurement procedure is a thoroughly investigated measurement procedure that has shown to yield values that have an uncertainty of measurement commensurate with its intended use, especially in the assessment of the trueness of other measurement procedures for the same quantity and in characterizing reference materials. Often, it is not possible to use a reference measurement procedure for analysis directly in a routine laboratory, because of the high expenditure on apparatus and personnel and, in general, the low-throughput of a reference measurement procedure relative to a routine assay [12].

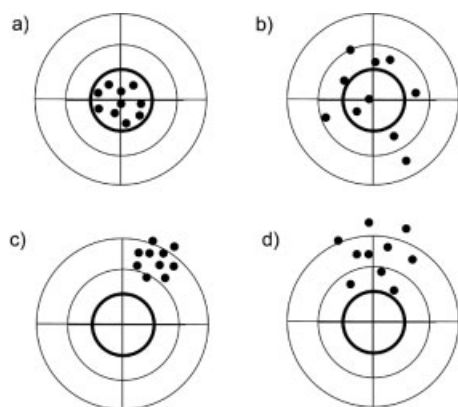
On the other hand, reference materials, whose values have been determined by a reference measurement procedure, can be used directly to calibrate or control the calibration of laboratory procedures, *i.e.*, the trueness of a routine procedure can be traced back to the reference procedure by means of the reference material. In practice, the traceability will be usually ensured *via* one or more intermediate stages, for example, by the transfer of values from a reference material to a set of calibrators that are provided by the manufacturer of a clinical assay.

For metrological correct measurements, the values assigned to the calibrator must be traceable to the system of units employed, *e.g.*, the *Système International d'Unités* (SI). The heterogeneous character of proteins and peptides aggravates the establishment of traceability to SI units and standardization (see below). For analytes that lack an





**Figure 2.** Main elements of a reference measurement system and traceability to SI, IU or IS units modified from [29, 31, 44].



**Figure 3.** A technology or method needs to provide measurement results that are accurate. This includes both, precision and trueness of measurement. Only method 'a' exhibits sufficient precision and trueness of measurement. Methods 'b', 'c' and 'd' are imprecise, biased, or both, biased and imprecise, respectively.

unequivocally recognized unique molecular entity, like many proteins and peptides, the international unit (IU) and IS concepts have been applied [9, 27–29].

## 6 Different classes of reference materials

There are different classes of reference materials, which include primary, secondary and tertiary reference materi-

als [30, 31]. Primary and secondary reference materials are developed by national and international organizations. Tertiary reference materials are industry or laboratory calibrators or controls. The hierarchy of reference materials and the differences between primary and secondary reference materials are shown in Fig. 2 and Table 1, respectively. A list of primary and secondary reference materials for plasma proteins and peptides is shown in Table 2.

### 6.1 Primary reference material

A primary reference material is a chemical of high purity, which is weighed out directly for the preparation of a solution with a specific concentration or for the calibration of the solution of unknown strength. The International Union of Pure and Applied Chemistry (IUPAC) proposed a degree of purity of 99.98%, although, most often this is not possible for the small organic molecules and generally not considered possible for proteins. Primary reference materials are supplied with a certificate of analysis.

According to the International Vocabulary of Basic and General Terms in Metrology [19] a primary reference material or primary measurement standard is a standard that is designed or widely acknowledged as having the highest metrological qualities and whose value is accepted without reference to other standards of the same quantity. The quantities of one or more properties of the material have been determined experimentally within stated uncertainties of measurement.

**Table 1.** Comparison of primary and secondary reference materials modified from [11, 44]

Characteristic	Primary reference material	Secondary reference material
Purified or recombinant protein or peptide	Biology, structure and/or functional activity exactly identified prime constituent or used for calibration	Biology, structure and/or functional activity exactly identified for calibration or overall QA
Matrix	Artificial, natural or comparable to patient specimen	Usually natural or comparable to patient specimen
Analytes	One or several	Preferentially several
Quantity	Certified value traceable to SI or IU; uncertainty statement for the assigned value	Certified value traceable to SI or IU; uncertainty statement for the assigned value
Value assignment	A reference measurement procedure or for pure substances a variety of methods based on different principles, especially if purity and the functional activity have to be determined	Either a reference measurement procedures in case of method defined measurands or a variety of methods based on different principles applied by a larger number of competent laboratories (proven by interlaboratory tests or accredited according to ISO17025) and selected methods
Certification Stability	Stable, internationally recognized organization ≥10 years, new batches as required	Stable, internationally recognized organization ≥10 years, new batches as required
Commutability with test sample	Not necessarily if matrix-independent value transfer methods from primary to secondary reference materials exist, otherwise commutability required	Absolute requirement; constant numerical relationship with different measurement procedures for all kinds of patient samples and reference materials
Batch size	2000–5000 vials	20 000 or more vials
Availability	Sometimes restricted	Universal

**Table 2.** Overview on the international certified reference materials for plasma proteins

Analyte	Matrix or form	Name of CRM	Producer
Acid phosphatase (human prostate)	Purified protein	BCR-410	IRMM <sup>a)</sup>
Alanine aminotransferase (pig)	Purified protein	ERM-AD454	IRMM
Albumin (bovine)	Aqueous solution	SRM 927d	NIST <sup>b)</sup>
Alkaline phosphatase (pig)	Purified protein <sup>c)</sup>	JC ERM 20327, Lot 003	HECTEF <sup>d)</sup>
Alpha-amylase	Purified protein	IRMM/IFCC-456	IRMM
Alpha-amylase	Purified protein <sup>c)</sup>	JC ERM 20327	HECTEF
Alphafetoprotein	Purified protein	BCR-486	IRMM
Apolipoprotein A-I	Purified protein	BCR-393	IRMM
Carbohydrate deficient transferrin	Human serum	ME 30890, ME 30891	LGC Promochem <sup>e)</sup>
Creatine kinase	Purified protein <sup>c)</sup>	JC ERM 20327	HECTEF
Creatine kinase	Purified protein	ERM-AD455	IRMM
Gamma-glutamyltransferase (pig)	Purified protein	ERM-AD452	IRMM
Glycated haemoglobin	Human hemolysate	BCR-405	IRMM
Glycated haemoglobin	Human hemolysate	JDS lot 2	HECTEF
Hemoglobinocyanide	Human hemolysate	BCR-522	IRMM
Lactate dehydrogenase	Purified protein	ERM-AD453	IRMM
Lactate dehydrogenase	Purified protein <sup>c)</sup>	JC ERM 20327 lot 003	HECTEF
Prostate specific antigen	Purified protein	BCR-613	IRMM
Serum proteins (15)	Human serum	ERM-DA470	IRMM
Thyroglobin	Purified protein	BCR-457	IRMM
Troponin complex (human cardiac)	Aqueous solution	SRM 2921	NIST

a) IRMM (Institute for Reference Materials and Measurements), EU; [www.irmm.jrc.be](http://www.irmm.jrc.be)b) NIST (National Institute of Standards and Technology), USA; [www.nist.gov](http://www.nist.gov)

c) Lyophilized with BSA

d) HECTEF (Health Care Technology Foundation), Japan; [www.hectef-src.or.jp](http://www.hectef-src.or.jp)e) LGC Promochem, UK; [www.lgcpromochem.com](http://www.lgcpromochem.com)

Ideally, primary reference materials should be used as a working calibrator (see below). This is, however, often impaired by lack of commutability. Producing primary reference materials can be extremely costly. A primary reference material is thus a precious and valuable resource that is difficult to replace. This is one reason why secondary reference materials calibrated against primary reference materials have been established to allow for universal availability. An alternative can be calibrators that are native samples which values have been assigned by a matrix-independent reference method, itself being calibrated with the primary reference material.

## 6.2 Secondary reference material

A secondary reference material is a standard whose value is assigned by a formal process of value transfer from a primary reference material of the same quantity. Usually, secondary reference materials contain one or more analytes in a matrix that reproduces or simulates the expected matrix. The matrix of a material comprises all components except the analyte.

## 6.3 Certified reference materials and standard reference material

Certified reference materials are primary or secondary reference materials that have one or more values certified by a technically valid procedure and that are accompanied by a certificate or to another document by a certifying body. Standard reference material is a trademark name for certified reference material that is certified and distributed by the National Institute of Standards and Technology (NIST). When a value is certified, the supplier or manufacturer of the reference material is responsible for assuring that the value is as close as possible to the true or conventional true value within the intervals of uncertainty based on the state-of-the-art [32]. Each certified value has to be accompanied by an uncertainty with a stated level of statistical confidence. The procedure for certification of secondary reference materials follows international guidelines [33–35]. To promote the use of certified reference materials it should be demonstrated that the use of the reference material improves the quality of measurement results in laboratories. This is done by studies with expert laboratories, which need to demonstrate the improvement of comparability, commutability and standardization (see below) [9, 31, 36–41].

## 6.4 Tertiary reference materials

Based on primary or secondary reference materials, tertiary reference materials like industry standards, master calibrators and derived working standards, working calibrators, *etc.* are produced to serve laboratories as calibrators or controls [31]. Such tertiary reference materials are used routinely to calibrate measuring instruments or as control materials. The main purpose of internationally certified reference

materials is usually the transfer of values to tertiary reference materials used by manufacturers. For a manufacturer's master and product calibrator, the most important features are reproducible production, sufficient stability and reasonable production costs.

## 7 Development and preparation of reference materials

In order to develop and supply international certified reference materials, networks of expert laboratories employing reference measurement procedures have been established. Such activities are conducted by international and regional organizations like the International Federation on Clinical Chemistry and Laboratory Medicine (IFCC), Bureau International des Poids et Mesures (BIPM), Institute for Reference Materials and Measurements (IRMM), International Standards Organization (ISO), International Society on Thrombosis and Haemostasis (ISTH), IUPAC, CLSI, World Health Organization (WHO), NIST, Health Care Technology Foundation (HECTEF), Centers for Disease Control and Prevention (CDC), United Kingdom's National Institute for Biological Standards and Control (NIBSC), *etc.* in collaboration with IVD laboratories, reference laboratories, analytical centres of competence, manufacturers and others. The broad application of certified reference materials ensures worldwide uniformity in most analytical measurements and provides users with reliable and comparable quantitative information based on true values or operationally defined 'conventional true values'. By calibrating measurement systems to a certified reference material or reference method, this traceability scheme allows for the standardization of assays. This scheme avoids bias introduced through the usage of different reference materials for a distinct measurement not commonly agreed upon [42].

Studies have shown that due to the introduction of the International Reference Preparation for Serum Igs G, A and M from the WHO in the late 1960s, WHO's International Reference Preparation of Six Human Serum Proteins for Immunoassay (WHO 6HSP) in the late 1970s, the United States National Reference Preparation for Specific Human Serum Proteins (USNRP) and the certified reference material ERM-DA470 for plasma proteins released in mid-1993, among others, the use of a single, international reference material by all manufacturers and laboratories has contributed to an overall improved analytical performance among laboratories and test kits [9, 31, 36–41].

ERM-DA470 was established by the committee for Plasma Protein Standardization of the IFCC [9, 43]. ERM-DA470 contains 15 proteins with certified concentrations, namely  $\alpha$ 1-acid glycoprotein (orosomucoid),  $\alpha$ 1-antitrypsin ( $\alpha$ 1-protease inhibitor),  $\alpha$ 2-macroglobulin, albumin, complement C3, complement C4, ceruloplasmin, C-reactive protein, haptoglobin, IgA, IgG, IgM, transferrin, transthyretin (prealbumin) and  $\alpha$ 1-antichymotrypsin. The procedures



used for the production of ERM-DA470 were tested extensively and were shown to account for the requirements to achieve the quality claims of ERM-DA470: (i) all serum samples for the pool to come only from healthy blood donors with complete anamnesis and established demographic constitution; (ii) certified absence of infectious diseases (human immunodeficiency virus, human T-cell leukaemia virus 1, hepatitis-B virus, hepatitis-C virus); (iii) serum pool suitable for as many plasma proteins as possible; (iv) physico-chemical characteristics of proteins clearly documented; (v) suitable for the majority of the common commercially available immunoassays with a very low method dependent bias result, (vi) long shelf-life; (vii) suitability as an official calibrator and (viii) availability in large quantities [9, 43]. ERM-DA470 fulfils the criteria essential for international certified reference materials used in laboratory medicine (Table 2) [44].

ERM-DA470 has been prepared according to a detailed protocol including sample collection and pooling, processing and handling procedures based on extensive testing, *e.g.*, accelerated degradation studies [9, 43]. In brief, ERM-DA470 was produced as a stabilized serum and processed to be offered freeze-dried in ampoules. Human serum obtained from spontaneously clotted blood of donors that fulfilled characteristics and requirements for 'healthy blood donors' was used (several hundred healthy individuals in five European cities). Frozen blood donations were transported to a central processing centre, thawed, pooled and further processed. Complement C3 was transformed into stable C3 fragments by treatment with inulin. Stabilization by delipidation was performed by treatment with Aerosil®, *i.e.*, fumed microparticulate silicon dioxide. Preservation of the final pool was done by adding sodium azide, benzamidinium chloride and aprotinin (Antagosan). Pure C-reactive protein was added, the material was buffered followed by sterile filtration, aliquoting, freezing, freeze drying and sealing. The influence of the treatments and storage on protein concentration and structure were investigated. Molecular characteristics of all the proteins in ERM-DA470 were established by electrophoretic and immunochemical techniques. Immunochemical techniques were also used for value assignment. The values of the 15 constituents are within the reference values of healthy people. Values have been assigned from existing international reference materials or from purified protein preparations. The latter aspects may be considered to turn ERM-DA470 into a primary reference material regarding these proteins, namely, transthyretin,  $\alpha$ 1-acid glycoprotein (orosomucoid),  $\alpha$ 1-antitrypsin,  $\alpha$ 1-antichymotrypsin and transferrin. A new lot of ERM-DA470 is currently under preparation following similar methodology. This new reference material will be certified for the concentration of 15 proteins with the exclusion of  $\alpha$ 1-antichymotrypsin and the addition of spiked  $\beta$ -microglobulin.

In conclusion, reference materials are used to allow for metrological correct measurements and QC, in order to provide reliable results. Before we discuss in more detail these

and further applications of reference materials, we need to outline the need for reliable results in greater depth to underline the benefit of the application of reference materials.

## 8 Reasons for the need to provide accurate measurements

The prioritization through which biomarker candidates should be verified is based on the information provided by discovery studies and experiments. The same is true for subsequent validation and transition to IVD applications. Consequently, it is crucial to assess the reliability of the information. For example, knowledge on measurement accuracy and adequate use of this information along with the potential need for improvement saves time, costs, and resources, and increases the likelihood of successful decisions and related developments.

Providing reliable information is crucial in IVD, as it is supposed to answer medical questions and thereby support decisions on interventions [7], *i.e.*, IVD should not only provide reliable but also meaningful information. Reliability of results in IVD is specified and required by regulations [45]. Sufficient reliability of measurements includes sufficient measurement accuracy, comparability of measurement results between laboratories on the regional, national or international levels and constancy over relatively long periods of time (often years). This is important to establish uniform reference intervals and decision limits (cut off values, threshold values), to harmonize and simplify clinical decision making to the benefit of the patient [12, 46]. With respect to the quote of Sir William Osler (1849–1919): 'Medicine is a science of uncertainty and an art of probability', the information provided by an IVD has to decrease uncertainty by increasing the probability to have the correct diagnosis or prognosis and consequently choose the intervention with the highest likelihood of success.

### 8.1 Predictive values of a test result indicate the reliability of the information it provides

The probability of a test result providing the correct diagnosis or prognosis is determined by its positive and negative predictive values. A positive predictive value indicates what proportion of positive tests correctly indicates the presence of a disease; a negative predictive value indicates the proportion of negative tests correctly indicating the absence of a disease. Predictive values are a function of the frequency of a disease in a respective population and the diagnostic accuracy of the test, including both, diagnostic sensitivity and diagnostic specificity [7]. Diagnostic accuracy describes the ability of a measure to discriminate between two groups. The area under receiver operating characteristic curves is often used to determine diagnostic accuracy. A measure can be a single analyte, multiple ones, patterns and information from the

patient's history or the exam of the physician among others. Qualitative discriminative measures are not common, *e.g.*, proteins or peptides, which are present in one group of subjects and absent in another. In most cases there is a quantitative dependence.

As an aside, in conjunction with the aspects outlined above, this explains the advantage of applying semi-quantitative or quantitative proteomics for discovery, and underlines the need for the application of proteomics techniques allowing for absolute quantitation or the transition to other quantitative techniques like functional tests or immunoassays for the verification, validation and eventually application of respective discoveries in IVD.

Quantitative dependencies usually do not allow for a clear-cut differentiation or separation of groups. In most cases, there is an overlap due to biological variations both, intra- and inter-individual ones. In addition, preanalytical, analytical and postanalytical variation and error further aggravate differentiation. Preanalytical processes have been reported to be a major source of variation and error in the IVD laboratory [47–52]. Preanalytical processes include study design, compliance of the subjects investigated and the technical staff in adherence to protocols, choice of specimens utilized and sample collection as well as sample processing. In clinical proteomics the challenge at this point in time seems to be more on the analytical side, *e.g.*, analytical sensitivity and accuracy of measurement.

## 8.2 Sufficient measurement accuracy increases potential success of discovery, verification and validation studies and is required for IVD applications

Sufficient measurement accuracy is necessary to achieve reproducible and comparable results [53]. It is mandatory to determine whether a measured concentration change of an analyte has a certain level of significance or not. Measurement accuracy includes both, precision, and trueness of a measurement (www.iso.org: ISO/DIS 5725-1) (Fig. 3). The imprecision of a measurement is usually displayed as CV. The trueness of a measurement is the systemic error component. It is commonly denoted as bias, *i.e.*, the deviation of the average value from a (conventional) true value. As shown in Fig. 4, imprecision and bias alter the dependence of a quantitative measure on its frequency in (a) given group(s) [7]. Imprecision can skew and broaden curves, whereas bias can lead to a shift or offset. Thus, the lack of accuracy of a measurement can significantly hamper the discrimination between different groups of subjects, and consequently affect diagnostic sensitivity and specificity as well as predictive values.

In view of the generally accepted guidelines and standards for precision and trueness of measurements [54, 55], and reported performance characteristics of distinct technologies applied in proteomics [50, 51, 56–60], further developments and improvements seem to be particularly

needed for the application of some proteomics technologies for verification and validation studies and eventually for IVD purposes.

The interlaboratory imprecision of IVD measurements should be below  $0.5 \times CV_i$  and bias should be below  $0.25 \times (CV_i^2 + CV_g^2)^{1/2}$ , whereas  $CV_i$  is the within-subject and  $CV_g$  is the between-subject biological variation [54]. Obviously, knowledge about the trueness and precision of the measurement is important.

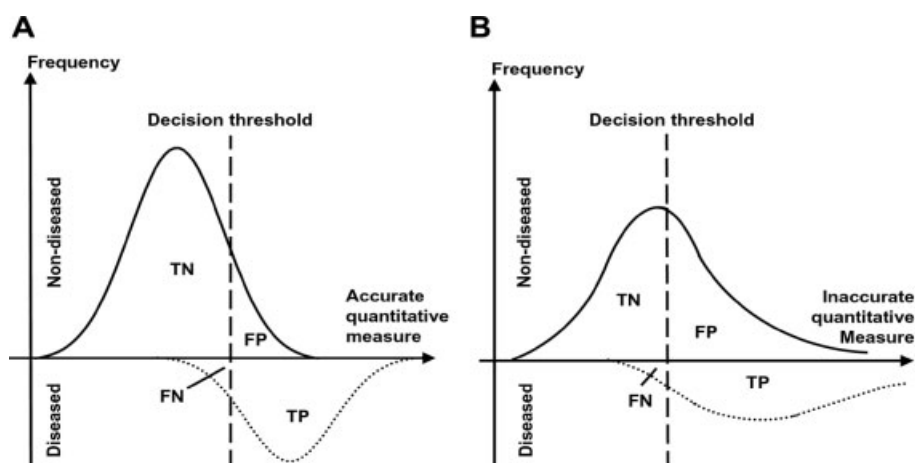
## 9 Use and benefits of reference materials beyond calibration and QC

Apart from achieving trueness of measurement, reference materials can also help to assess the precision of a measurement. For example, Zaninotto *et al.* [61] evaluated the analytical characteristics, including the precision, of a quantitative and fully automated assay that measures heart fatty acid binding protein and compared its clinical performance with respect to a myoglobin assay by testing three levels of QC materials and two in-house pool samples. Bons *et al.* [50] used a commercial QC sample that contained independent certified standards to determine the accuracy of SELDI-TOF-MS. Based on the determination of such performance characteristics, it is possible to compare different technologies and methods and thus eventually optimize and improve measurement procedures or even the development of new ones.

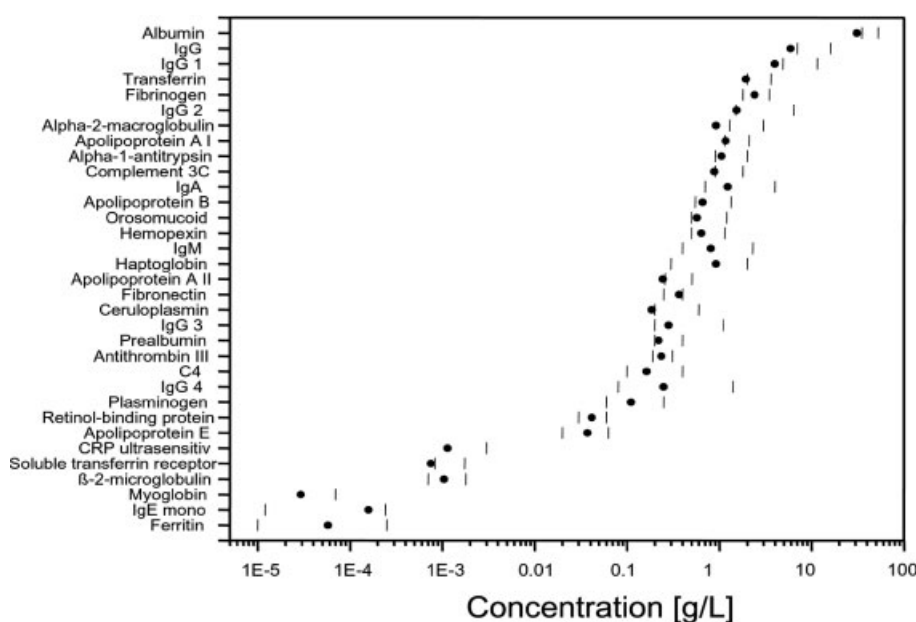
Beside these applications, reference materials or reference samples can aid in the evaluation of preanalytical influences. For example, Favaloro *et al.* [62] used reference samples to determine the preanalytical influences, which can have a significant impact on the results of a study [8]. It was shown that refrigerated storage of blood samples can have an effect on the measurement of Factor VIII and von Willebrand Factor and potentially result in the misdiagnosis of von Willebrand disorder or haemophilia A.

In addition, reference materials or specimens are useful to perform proficiency testing to make cross-laboratory and cross-technology comparisons. The use of certified reference materials instead of in-house reference materials allows for open and direct comparison of methods and technologies and therefore leads to a better understanding of their relative strengths and weaknesses.

For example, a charter of the pilot phase of the Plasma Proteome Project of the HUPO (HUPO PPP) was the assessment of the advantages and disadvantages of various technologies [8, 63]. For this purpose reference specimens and a coagulation standard, a lyophilized citrate-anticoagulated plasma of the NIBSC, were distributed to participating laboratories for analyses [63]. Reference specimens were pooled specimens prepared according to a distinct protocol [63, 64]. The concentration of distinct proteins of the reference specimens was determined by certified tests run on automated clinical analyzers that were calibrated and controlled by tertiary reference materials [8, 64–66] (Fig. 5).



**Figure 4.** The correct, original dependence of frequency and an accurate quantitative measure (A) is altered due to the impact of imprecision and bias of an inaccurate measurement (B). Imprecision skews and broadens curves, whereas bias leads to a shift [7]. The inaccurate measurement significantly hampers the discrimination between the two groups of nondiseased and diseased. Consequently, the diagnostic sensitivity ( $TP/(TP + FN)$ ) and the diagnostic specificity ( $TN/(TN + FP)$ ) as well as the positive predictive value ( $TP/(TP + FP)$ ) and the negative predictive value ( $TN/(TN + FN)$ ) are impacted markedly (TP: true positive; FN: false negative; TN: true negative; FP: false positive).



**Figure 5.** Concentration (●) and reference values (I) of distinct proteins of a coagulation standard (lyophilized citrate-anticoagulated plasma of the NIBSC that was distributed by the HUPO PPP as a reference specimen). The concentrations of distinct analytes were determined by certified clinical analysers, Behring Nephelometer (BN) II® and BN ProSpec® [8, 64–66]. The nephelometres used calibrators and controls with certified amounts of analytes some of which are traceable to primary or secondary reference materials. Traceability of the analytical values of the calibrators is as follows: N Protein Standard SL (Dade Behring, product code number OQIM13, Lot: 083642) ERM-DA470: IgG, IgA, IgM, C3c, C4, transferrin, albumin, alpha-1-antitrypsin (alpha1-proteinase-inhibitor), alpha-2-macroglobulin, haptoglobin, orosomucoid, acid-alpha-1-glycoprotein, transthyretin (prealbumin), ceruloplasmin SRP WHO 67/97: IgG 1, IgG 2, IgG 3, IgG 4 SRP WHO 80/578: Ferritin Sec. IRP Code 75/502: IgE Highly purified proteins: hemopexin, retinol-binding protein,  $\beta$ 2-microglobulin, soluble transferrin receptor N apolipoprotein standard serum (Dade Behring, product code number OUPG07, Lot: 063531) BCR – CRM 394: Apolipoprotein A-II (Of note, reference material has been withdrawn) de'DaIICHI-standard-serum: Apolipoprotein E. IFCC reference preparations: apolipoprotein A-I, apolipoprotein B N Protein Standard PY (Dade Behring, product number OUID13, Lot: 068048) Antithrombin III, fibrinogen, fibronectin, plasminogen (concentrations of proteins were calibrated against N Protein Standard preparations of Dade Behring Marburg; the concentration of human fibrinogen was calibrated by reference to an internal Master Calibrator from Dade Behring Marburg [95]) N myoglobin standard (Dade Behring, product number OWIP, Lot: 194944) Highly purified myoglobin N Rheuma Standard SL (Dade Behring, product number OQKZ13, Lot: 183826) ERM-DA470: CRP

Therefore, certain values were traceable to common reference materials including ERM-DA470, SRP WHO 67/97 and SRP WHO 80/578. The use of this common NIBSC pooled plasma material was the basis of a comprehensive comparison of the performance of distinct proteomics approaches used to decipher the serum or plasma proteome by various proteomics technologies and methods [65] and to assess distinct correlations and characteristics of technologies [66].

Furthermore, having the option to refer to traceable test results may be considered as an initial step to harmonizing and standardization of distinct measurement results, which is important to allow for adequate comparability between laboratories results. This may be helpful in preparation for multicentric, population-based verification and validations studies in clinical proteomics as is the case in clinical chemistry, *e.g.*, in the assessment of the clinical value of emerging markers [13].

## 10 Limitations of reference materials in general and for clinical proteomics in particular

### 10.1 Different scopes of IVD and clinical proteomics

The descriptions above underline the need and benefit of the use of reference materials, but compromises are involved and reference materials may fall short of an ideal solution. The different scopes of IVD and clinical proteomics create limitations for the application of IVD reference materials in clinical proteomics. In IVD, the goal is usually to measure a distinct peptide or protein analyte or a family thereof as specifically and accurately as possible with minimal labour and costs. Generally, a sample is analysed using a highly or fully automated instrument, such as a clinical chemistry analyser, that performs a specific analysis autonomously. In contrast, the clinical proteomics approach is *per se* a holistic one. The focus is on the analyses of a multitude of proteins and peptides simultaneously. The reference materials available only cover a limited number of plasma proteins or peptides and only contain a comparatively small number of assigned values. There is even a lack of matrix-based certified reference materials for proteins routinely tested in IVD, like cardiac troponins.

### 10.2 Complexity of the serum and plasma matrices

The complexity and potentially limited stability of serum and plasma and its components hinders the establishment of a set of reference materials for clinical proteomics covering a comprehensive range of proteins and peptides with certified values. To generate stable reference materials, serum or plasma pools must be processed (see above), which impacts their composition. As a consequence, reference materials do usually not reflect a perfect mirror of a 'normal' serum or plasma composition. On the other hand, it appears difficult

to exactly define the 'normal' serum or plasma composition, given all the variations regarding blood acquisition and processing [3]. As long as commutability is achieved, differences between the matrices of the reference material and the specimen to be analysed are not an issue for functional tests or immunoassays. The same should apply to clinical proteomics approaches that aim to detect a distinct set or subset of proteins, like protein chips used for multiplexing or mass spectrometric analysis of distinct proteins and peptides and their profiling (see below).

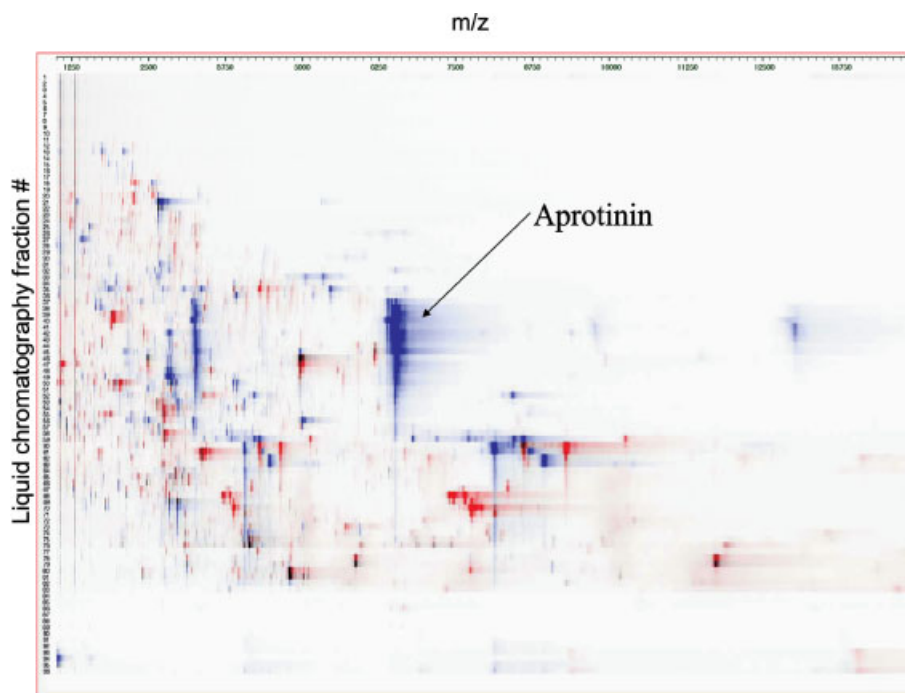
It will be more difficult to establish and apply reference materials for pattern or fingerprinting analyses or to account for calibration or QC needs for the multitude of fractionation techniques applied in clinical proteomics. These fractionation techniques are core to discovery proteomics approaches. The breadth of the concentration range and complexity of the serum and plasma proteomes need to be reduced dramatically and interfering substances have to be removed to account for the limitations of detection techniques.

For example, differential peptide display [67] of human plasma and a plasma-based tertiary reference material, *i.e.*, N/T Protein Control SL Level L (Dade Behring, product code number OQIN) revealed different patterns (Fig. 6). The peptide display of the N/T Protein Control SL Level L showed that many high abundance peptides were detectable. Aprotinin was particularly dominant. Such high abundance peptides eluted over several fractions. However, the median CV of around 29% of the peptide display of the N/T Protein Control SL Level L was in the expected range.

2-DE of N/T Protein Control SL Level L also showed a pattern different from a typical plasma pattern (data not shown). Again, this is due to the processes required to generate stable reference materials, in particular the depletion of distinct components and the addition of protease inhibitors that alter the composition and therefore the pattern or display of serum or plasma (see above).

### 10.3 Complexity of proteins and peptides

In addition to the complexity of serum and plasma, the complexity of the analytes themselves hamper the generation of respective reference materials. Proteins and peptides are usually heterogeneous in their molecular characteristics due to PTMs, alternative splicing, *etc.* Thus, proteins and peptides often lack an unequivocally recognized chemical entity. If the relative molecular mass is not known, concentration determinations of large molecules can no longer be reduced to the unit 'mol'. In such cases the definition of special 'arbitrary units' becomes necessary [27–29]. Instead of traceability to SI units (*e.g.*, mol/L), the heterogeneous character of proteins lead to the expression of concentrations in mass *per* volume rather than mol *per* volume, as molecular weight is usually variable according to the biological state of the protein. Consequently, the dry mass should be used to assign values to a primary reference material. On the other hand, the drying process may alter the reactivity of the protein.



**Figure 6.** Overlay of mean differential peptide display of human plasma (red) and a plasma-based tertiary reference material, N/T Protein Control SL Level L (blue) revealed different patterns. Differential peptide display has been extensively outlined elsewhere [67]. Briefly, after peptide extraction and protein removal, samples were separated by LC into 96 fractions. Each single fraction was analysed by MALDI-TOF MS. LC and mass spectra of four replicates of one individual sample were combined into a multidimensional diagram, a mean peptide display. Each signal's position is characterized by the coordinates of chromatographic fraction, molecular mass and signal intensity. The mean peptide displays of the individual samples were combined to generate a differential peptide display.

Thus, significant efforts are necessary for adequate value assignment [9]. An IS material is prepared with state-of-the-art purification and identification techniques and its function is tested by a bioassay, *i.e.*, by the response of a biological system. The IU is then assigned by convention.

In more detail, a given protein may be the product of more than one gene in a population or in an individual. It may be subject to PTMs and interactions and reactions with other substances present in the material, specimen or test components. Different forms of the same protein may behave differently and further metrological challenges arise with the measurement of 'free' analytes, in particular in view of the complex interactions and reactions of substances in serum or plasma [29]. Purified and recombinant proteins can exhibit different characteristics and heterogeneity than present in human serum and plasma. Yet another obstacle is potential protein alterations in abnormal patient samples. Different reactivity in distinct assays is thus not surprising. Reference and control materials that contain a different spectrum of heterogeneity of the analyte may react differently, if compared to respective samples. In addition, these differences may also depend on the measurement system applied, *e.g.*, based on the epitope specificity of the antibodies applied in immunoassays. In consequence, commutability may be significantly impaired.

A typical example is complement C3, which has a number of antigenic epitopes that segregate into different fragments upon activation by physiological conversion or *in vitro* proteolysis by proteases such as plasmin. In order to avoid changes during the lifetime of a reference material it is desirable to have complement C3 in a stable, fully degraded form [11, 43].

Another interesting example involves the cardiac troponins [68–70]. Cardiac troponins I and T are used for the diagnosis of acute myocardial infarction and prognosis. Troponin I (TnI) is part of the contractile apparatus of muscle and is mainly noncovalently associated with two additional nonstructurally related proteins, troponin C (TnC) and troponin T (TnT). Three structurally related isoforms of TnI exist, one found in cardiac muscle (the one of interest: cTnI), slow-twitch skeletal muscle (sskTnI), and fast-twitch skeletal muscle (fskTnI). In addition to the structural homology between the different isoforms, PTMs occur and are significant for accurately measuring the cTnI concentration in serum and plasma. For example, a stable 'core region' remains after numerous N- and C-terminal proteolytic cleavages and serine residues are differentially phosphorylated. cTnI also specifically interacts with TnC at several regions and interacts nonspecifically with heparin, likely due the overall positive charge of the TnI molecule. Among others, these aspects must be considered when generating a respective reference material, which is extremely challenging.

## 11 Use of technologies applied in proteomics as reference methods and to establish reference materials and immunoassays

The establishment of immunoassays, reference materials and reference methods for the determination of heterogeneous proteins and peptides, *i.e.*, a family, class or group of analytes, can be supported or performed by technologies



applied in proteomics, in particular by MS. MS can be applied to surmount metrological difficulties that arise when different analytes of one family that are traceable to SI units are measured together [29]. According to the International Centre for Metrology, ID-MS is a primary method, which yields results in SI units (mols) without requiring reference to a reference material or method [20], at least when applied for the measurements of exactly defined analytes, usually low molecular weight analytes like elements or metabolites, for which stable isotope-labelled internal standards can be obtained. Of note, a reference system based on trueness of measurement does not necessarily require reference materials, if the physical and chemical properties of these materials have been accurately defined, but it does require reference methods. ID-MS involves the addition of known amounts of an enriched isotope of an element or molecule to be determined to a sample. This addition is to be made prior to sample preparation. By measuring the isotope ratio of the sample and the sample spiked with the isotope as well as knowing the isotopic ratio, the sample concentration can be calculated. The measurement is based upon ratio measurements of one isotope to another.

For example, ID-MS is used as a definitive method for the quantification of a variety of compounds with low molecular weight, e.g., cholesterol [71, 72], cortisol [73] and dioxin [74]. It is also used for the measurement of distinct metabolites and metabolite profiles for newborn screening [75–77]. The quantification of low molecular weight compounds includes peptides. The quantification of peptide cleavage products of proteins consequently may allow for the quantification of the respective protein by ID-MS, if a stoichiometric relationship between the analyte protein and peptide measurand can be accurately and precisely maintained during the cleavage process.

### 11.1 Stable ID-MS for the quantification of proteins

ID-MS has been shown to be useful to quantify unique polypeptides formed by enzymatic hydrolysis of a specific protein for the quantification of this specific protein [78]. Briefly, quantification of proteins by ID-MS is usually performed by (i) development, synthesis and application of suitable internal protein or peptide standards, (ii) (restricted) proteolysis of the sample, (iii) chromatographic isolation of selected peptides that are characteristic of the protein(s) to be quantitated and (iv) quantification of the peptides by ID-MS.

For example, Barr *et al.* [79] developed an ID-MS method for the quantification of apolipoprotein A-I. To assess the performance of the method a reference material was analysed. The concentration of apolipoprotein A-I was determined in the CRM 393 of the European Community Bureau of Reference (BCR). The total CV was 3.95%. Fierens *et al.* pushed this approach further by establishing an ID-MS method suitable for measuring insulin C-peptide in urine [80], and applying this method as a basis for comparing the performance of existing IVD immunoassays; i.e., using ID-

MS as the gold-standard. These approaches make use of tandem-MS instruments (generally triple-quadrupole MS) to perform 'selected reaction monitoring' (SRM, plural MRM) measurements of a selected peptide against a stable-isotope labelled internal standard peptide of the same structure. While triple-quadrupole mass spectrometers are not typical in proteomics research, their superior specific analyte sensitivity and wide linear dynamic range (4–5 orders of magnitude) have made them the work-horse instruments of small molecule quantitation in drug measurement, inborn error screening and environmental testing. Many reference measurement procedures for the quantification of clinical organic analytes by ID-MS also use triple quadrupole mass spectrometers to achieve high precision and measurement sensitivity. Recent advances enable these systems to multiplex hundreds of specific analyte measurements in one run. ID-MS assays for tryptic peptides representing a large set of high-to-medium abundance plasma proteins have recently been proposed [5], with within-run CV's of 4–20%. The required stable-isotope labelled internal standard peptides can be made directly by conventional chemical synthesis (incorporating commercially available stable-isotope containing amino acids), or through expression in *Escherichia coli* or a cell-free labelling system of novel proteins containing a series of concatenated tryptic sequences [81, 82]. The sensitivity of peptide MRM measurements, currently a limitation at levels below  $\sim 1 \mu\text{g/mL}$ , can be further increased by 2–3 orders of magnitude by selective enrichment of analyte peptides by specific antibodies, as in the SISCAPA technique [82].

In a further refinement of this methodology, the biological specimen of interest can be utilized as internal reference. For example, larger amounts of entire human blood plasma are labelled with one version of a tandem mass tag (see isobaric mass tags such as Tandem Mass Tags TMT, Proteome Sciences; ITRAQ, Applied Biosystems; IPROT, Perkin Elmer) to generate a biological internal control reference. Since all proteins in the sample are labelled, the entire proteome of such sample will now be used as a reference against all proteins of study samples. All individual samples of a blood plasma study are labelled with one different version of this tandem mass tag. By spiking the biological internal control reference into study samples, virtually all MS/MS experiments performed with this material will result in pairs of reporter-ions. The reporter ion deriving from the biological internal control reference now serves as the basis for relative quantification of this analyte. If all plasma samples of the study are spiked with the identical amount of the biological internal control reference, relative quantification across all study samples becomes possible. Since the biological internal control reference can be used by multiple labs, cross-study and cross-lab comparisons are possible (manuscript in preparation, personal communication by Peter Schulz-Knappe). The quantification of multiple analytes by ID-MS approaches could be of special interest to quantitatively measure all members of a family of analytes individu-

ally with high specificity [29]. Such profiling is attractive, because measurements can be made on SI-traceable measurands, *e.g.*, peptides, and related to individual members within the family of analytes, which allows for measurement traceability to SI units (mol). It may aid in the determination of whether the relative concentrations of the members of the family of analytes are constant and/or whether there is a respective relationship to a surrogate measurand, *e.g.*, one that can be generated from the members of a family of analytes. In such case a surrogate could serve as the actual biomarker and as a standard for a respective reference material. Such information obtained by clinical proteomics approaches on the correlation of the individual members of a family of analytes and potential surrogate measurands with a distinct state of health or disease could be very helpful for both, the establishment of reference materials and of respective immunoassays [83].

In addition, preanalytical influences could be investigated to choose the most stable analyte measurable in a variety of specimens. Knowing which form(s) of a protein or peptide or protein and peptide complexes should be detected provides helpful information on the feasibility of technology transfer, *i.e.*, which test formats, in particular immunoassay formats, could be used to establish, develop and manufacture a respective test. In particular, valuable information on which the epitopes should be recognized by an immunoassay and what antibody pairs should be chosen, could be provided [84].

Proteomic techniques may also be helpful to establish and qualify reference materials, *e.g.*, by identifying changes in the reference material, both of the analyte(s) and the matrix. Questions like the choice of a purified protein, a recombinant protein, a peptide and the stability of the required epitopes under different conditions may be investigated. Physical changes could then be correlated to performance changes. The use of different technologies to analyse the characteristics of reference materials is helpful to add an additional level of assurance that the material is useful as a reference material and as a stable anchor for an assay [84].

## 12 Use of current IVD reference materials and the development of new ones for clinical proteomics

IVD reference materials are mainly thought to be stable anchors for assay calibration to allow for trueness of measurement and as QCs to verify calibration. The use of reference materials will be helpful in proteomics research for the assessment, comparison and improvement of performance characteristics of technologies and methods. The application of reference materials will be crucial for applying proteomics in IVD. For some applications in proteomics, reference materials currently used in IVD may be applied. In addition, new reference materials have to be developed to account for special needs of clinical proteomics.

### 12.1 Use of IVD reference materials in clinical proteomics

For some applications, it does not appear necessary to establish reference materials specifically for clinical proteomics. For the determination, comparison and improvement of performance characteristics of distinct technologies cost-effective reference materials that are universally available may fully suffice. As mentioned above, Barr *et al.* [79] assessed the performance characteristics of their ID-MS method for the quantification of apolipoprotein A-I by analysing BCR-CRM 393.

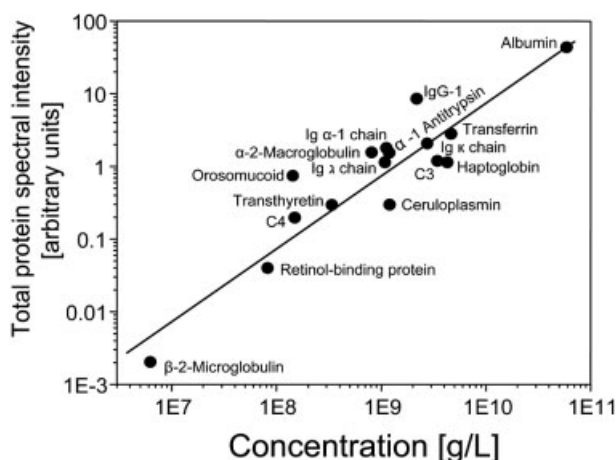
Even though matrix issues may impair the use of certain reference materials for distinct applications as shown by the differential peptide display analysis shown in Fig. 6, other reference materials may be suitable. For pattern or fingerprinting approaches or the assessment of fractionation technologies one may have to develop a reference material for the specific abnormal pattern or fingerprint. On the other hand, it may be worthwhile to assess the performance of standard human plasma preparations or other respective preparations as a reference material for the normal pattern or fingerprint. For example, standard human plasma may be obtained from pooled citrated plasma collected from selected healthy blood donors, stabilized with HEPES buffer (it does not contain preservatives), and subsequently lyophilized [22]. To avoid contact activation of the coagulation system preparations can be supplied in siliconized vials.

In contrast to the potential application of standard human plasma or serum preparations for pattern or fingerprinting approaches, reference materials with assigned values appear to be helpful to assess the performance characteristics of distinct MS approaches. Analysis of a tertiary reference material based on fractionation and MS analysis revealed a correlation of the total protein spectral intensities and the assigned values of distinct proteins of this reference material (Fig. 7). The dynamic dependence and correlation indicate that it should be possible to use such reference materials to determine, compare and improve performance characteristics of respective methods. This may not only be of value for MS approaches, but also for clinical proteomics technologies that aim to quantify specific proteins, like protein arrays.

A challenge that needs to be considered and addressed is commutability, though. Measurement results may be strongly dependent on the measurement procedure. For example, ambiguity in protein entries of databases, variations in ionization efficiencies, individual detection of the distinct members of a family of analytes instead of a surrogate analyte, *etc.* should be considered when correlating data of a MS analysis of a reference material to the assigned values.

### 12.2 Development of reference materials for clinical proteomics

As the number of assigned values of a reference material is limited it would be desirable to have several or ideally a single reference material for each plasma type or serum with a



**Figure 7.** Correlation of total protein spectral intensity of an MS analysis of N/T Protein Control SL/M (Dade Behring, product code number: OQIN) and the assigned values of distinct proteins of this reference material. Linear regression revealed a correlation coefficient of 0.97. Briefly, 150 µg N/T Protein Control SL/M was fractionated by OFFGEL electrophoresis (Agilent Application Note 5989-5814EN, [www.agilent.com/chem/offgel](http://www.agilent.com/chem/offgel)) into 24 pI fractions in a pH range between 4 and 7. Individual fractions were desalted, digested with trypsin and analysed by C18-RP-MS/MS as described [96].

comprehensive number of assigned values. However, this appears to be an unachievable goal, based on the considerations related to the establishment of reference materials outlined above and the remaining challenge to even establish a comprehensive catalogue of all human blood proteins, or even normal and abnormal variability [85]. It appears more realistic to establish standard plasma or serum preparations as matrix-based reference materials and internal standards.

### 12.3 Standard plasma or serum preparations as matrix-based reference materials

A standardized preparation of pooled serum or plasma may address the majority of the needs of the clinical proteomics community [85]. Instead of the implementation of a special preparation procedure for the clinical proteomics community, the preparation should be performed according to accepted standards of the clinical chemistry community to allow for comparability [3, 11, 43, 86, 87]. Such preparation would need to have sufficient stability and universal access over a sufficient period of time, *e.g.*, 3–5 years would be needed [85]. Similar to the reference specimens distributed by HUPO PPP during its pilot phase, the concentration of several proteins and peptides should be determined by reference measurement procedure or certified techniques, to generate traceable values [8, 64–66] (Fig. 5). If necessary, users could spike or enrich such a standard with various concentrations of proteins or peptides that are of special interest to them [85]. The reference material ERM-DA470 for serum proteins could be particularly useful for this purpose.

### 12.4 Purified proteins or peptides as internal standards

Spiking a sample with an internal standard is an attractive approach for calibration, QC and for the determination of performance characteristics [88]. The Association of Biomolecular Research Facilities (ABRF) ([www.abrf.org](http://www.abrf.org)) has developed protein collections for MS [89]. ABRF's Proteomics Standards Research Group supports proteomic standards, including materials, data and procedures, and is currently involved in a collaborative project for the development of validated protein standards that contain defined proteins, supplying standard mixtures as test sets for member laboratories, and making ABRF standards available to the proteomics community. For example, in collaboration with NIST, ABRF is currently developing a 3-peptide standard reference material, in which C-terminal arginines mimic tryptic peptides commonly amenable to LC-MS [85]. This standard reference material is designed as a standard for MALDI MS, electrospray MS, amino acid analysis, HPLC and CE.

As already outlined above, internal standards based on stable isotopes are of special value for quantitative MS approaches to achieve metrological correct measurements traceable to SI-units [20, 29, 78, 79, 90]. Of note, the impact of proteolysis and fractionation of the analytical serum or plasma sample before the actual MS analysis is performed, requires the addition of internal standards to the sample, before it is processed. In particular, to account for the completeness and variability of proteolysis, the use of protein standards with stable isotopes appears to be preferential over the use of peptides standards that will not be cleaved proteolytically.

Even though ID-MS is surely the preferred approach for quantitative MS analysis, the development of stable isotope standards and the associated costs restrict application. Therefore, one should consider that quantitative MS does not always depend upon the use of stable isotopes, but may also be performed based on the use of commutable reference materials

An example of such an approach is the determination of haemoglobin (Hb) A1c by HPLC/ESI MS (ESI-MS), which is even used as a reference method with interlaboratory CVs of 1.4 to 2.3% [91]. HbA1c is used for the long-term control of the glycaemic state in diabetic patients. Briefly, haemoglobin is cleaved into peptides by endoproteinase Glu-C. The non-glycated N-terminal hexapeptide cleavage products of the beta-chain are separated and quantified by HPLC/ESI-MS. Alternatively, quantification may be performed by 2-D approach using HPLC and CE with UV-detection. HbA1c is measured as ratio between the glycated and nonglycated hexapeptides. Consequently, there is no need to apply costly stable isotope peptides as internal standards. Instead, calibrators consisting of mixtures of highly purified HbA1c and HbA0 are used.

Currently, the US National Cancer Institute's CPTAC programme (<http://proteomics.cancer.gov/>) is investigating

the use of an equimolar mixture of up to 100 stable-isotope labelled recombinant proteins (to be produced at the Argonne National Laboratory) to compare detection sensitivity and quantitative performance of proteomics platforms. This mixture will be spiked at varying levels into plasma or other appropriate matrices to provide a suite of samples that may allow routine characterization of the depth and breadth of proteome coverage.

Whether reference materials or internal standards are to be used depends on the technologies applied and the goals of a study. However, in contrast to IVD it appears preferential at this point in time to focus more on technological aspects than clinical ones for clinical proteomics applications. Nevertheless, the generation of reference materials for clinical proteomics should aim towards the fulfilment of the criteria essential for international certified reference materials used in IVD (see above) (Table 2) [44]. Other measures to account for include distinct characteristics of purified or recombinant proteins or peptides that are used for reference materials [11]. These are: (i) contaminants of purified proteins should be at a low and defined level (even though extremely difficult to reach, ideally the degree of purity of 99.98% for primary reference materials proposed by IUPAC should be used as a guide), (ii) recombinant proteins should be prepared from the gene representing the predominant form found in human plasma, (iii) the protein or peptide should be as close as possible to the native state, (iv) molecular heterogeneity should mirror the one in patient samples, (v) molecular characteristics and stability should be defined and (vi) commutability should be shown by evaluating the performance of the reference materials in the proteomic measurement relative to either reference measurement procedures or existing IVD assays. Obviously, these characteristics are difficult to meet and therefore compromises are usually necessary. It appears reasonable to consider state-of-the-art and clinical needs to determine the appropriate level of compromise.

### 13 Summary and discussion

Reliability of results is crucial for the assessment of discoveries, the right choice of the most promising candidates for verification and validation studies, and ultimately for transition for usage in health care. This applies not only to biomarkers for IVD purposes. The same is true for the search for drugs, drug targets and biomarkers used in drug development or other proteomics research. Reliability depends on reproducibility, comparability and constancy of results. Important measures to assess and improve reliability are standardization and TQMS. In IVD standardization efforts and TQMS encompass the complete laboratory process, *e.g.*, organization and management, personnel, premises and environment, equipment, material and reagents, *etc.* Preanalytical, analytical and postanalytical processes are included.

If the goal of clinical proteomics studies is the generation of biomarkers useful for IVD applications, it appears helpful to consider and eventually adapt or adopt relevant guidelines and standard processes performed and generally accepted in IVD. This may be particularly helpful for preanalytical processes. Preanalytical processes for IVD biomarker investigations in clinical proteomics should be the same or at least comparable to the ones in IVD to allow for a smooth and swift transition to clinical application.

Standard procedures that have been implemented, broadly accepted and applied successfully over a comparatively long period of time in IVD for preanalytical processes, like the acquisition and preparation of analytical samples, *e.g.*, serum and plasma [86, 87, 92, 93], should be applied by clinical proteomics unless specific requirements demand for specific procedures. Apart from facilitating a smooth and swift transition to clinical application, the simple and straightforward adoption of existing standards is a measure to reduce preanalytical variation and error in proteomics investigations.

In contrast, standardization and QA for analytic and postanalytic processes specific to clinical proteomics are needed. Activities on standardization of certain post-analytical processes are undergoing under the auspices of the HUPO Protein Standards Initiative [94]. The situation on the analytical part has hardly been addressed so far [3]. Processing of an analytical sample, like fractionation of serum or plasma and fragmentation of proteins for proteomic analyses, is part of the analytical phase and contributes to the performance characteristics of a measurement. Any variations and errors associated with these processes impact measurement accuracy [3].

The implementation of general, binding SOPs for analytical processes in clinical proteomics to improve measurement accuracy does not currently appear feasible in view of the plethora of procedures and their combination. In fact, such regulations are not helpful in research, especially in nascent fields such as clinical proteomics, where flexibility is needed to ensure progress. An exception may be a consensus on defined serum and plasma fractions and standard procedures for their generation. In analogy to the investigation of the proteomes of organelles of a cell, serum and plasma fraction could be defined through distinct fractionation procedures and the determination of characteristic, fraction-specific proteins or peptides. As an aside, for mining the plasma or serum proteome it may be helpful to have a central institution distribute reference fractions. Even though the implementation of authoritative guidelines is not warranted in research, the establishment of general guidelines or recommendations on what should be considered in SOPs seems to be helpful, though, to support clinical proteomics laboratories to establish their own SOPs to account for analytical needs.

Based on the aforesaid, the best measure to account for the obvious and immediate analytical needs in clinical proteomics at this point in time seems to be the use of reference



materials. Instead of wasting precious and limited patient samples to determine, compare and improve the performance characteristics of analyses, like analytical sensitivity and precision of measurements, cost-effective and preferentially universally available reference materials can be applied. In-house reference materials or specimens may be helpful for such purposes, but interlaboratory comparison has to be encouraged and facilitated by common reference materials. Consensus master sample panels could be an excellent tool for meaningful comparisons [88], on the other hand access to such precious master sample panels will be limited and costly. When using reference materials to establish trueness of measurement common reference materials with certified assigned values are required anyhow. Clearly, there will not be a single reference material that will account for all needs. In some cases, reference materials currently applied in IVD may be applied readily in clinical proteomics. In other cases, there will be the need to establish reference materials or specimens to account for the specific needs of distinct applications in clinical proteomics. Internal standards, in particular internal standards based on stable isotope for quantitative ID-MS, are an excellent measure for metrological correct measurements and to allow for interlaboratory data comparison [88]. On the other hand, availability of equipment and reagents, and associated costs are likely to restrict the application of this approach. If commutability is established, common reference materials may suffice for quantitative MS, depending on the analytical setting and the requirements [91].

In conclusion, the use of reference materials and derived standards is crucial for IVD and promises to be beneficial for clinical proteomics, because it allows for calibration, control, traceability of values and the evaluation, comparison, and improvement of the performance of measurement procedures. Clinical proteomics studies should display the performance characteristics of the procedures applied and how they were determined, to provide information on reproducibility and to assess the reliability of the provided data. Standardization, implementation and use of reference materials depend upon interaction and trust among scientists in the same and different disciplines, industry, institutions and government organizations. There is a need for consensus, more multidisciplinary activity, interaction and dialogue between national and international organizations in establishing reference materials for clinical proteomics. Collaborations in the development of reference materials and procedures between the HUPO and other institutions active in the field of metrology and IVD have been initiated [85]. It would be beneficial, if further collaborations on an international level are initiated and pursued, e.g., to establish a network of reference laboratories, a directory of reference materials and procedures useful for distinct clinical proteomics applications, and to leverage the potential of clinical proteomics for the development of tests and reference materials to the benefit of the patient, health care and ultimately society.

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