

Protein Quantification by Mass Spectrometry: Is It Ready for Prime Time?

The successful interface of liquid chromatography with tandem mass spectrometry (LC-MS/MS)² in the 1980s opened new avenues for measuring low and high molecular weight analytes with exceptional analytical specificity and sensitivity. As discussed in the previous Q&A article, mass spectrometry (MS) is now a routine tool for measuring steroid hormones, drugs, vitamins, amino acids, biogenic amines, and many other classes of small molecules. We are now entering an era of protein quantification by MS for diagnostic purposes. The challenges for measuring proteins vs small molecules with MS are well recognized. In this Q&A article, 4 leaders in the field have been asked to comment on current and future capabilities of MS to quantify proteins (single or multiple) without the need for antibodies or other labeling reagents.



Why do you think MS-based methods for measuring proteins are not yet in widespread use in clinical laboratories?

Samir Hanash³: The instrumentation available in clinical laboratories generally has features particularly designed to meet the work flow and performance requirements applicable to a

clinical laboratory, together with standard operating procedures. Proteomic analysis by MS is currently applied primarily for discovery and does not meet these requirements for routine clinical assays. At best, it would have to be considered a “specialized assay platform,” available at a limited number of laboratories.

Mary Lopez⁴: There exists the misconception that MS-based assays are difficult and require very experienced



operators. The rapid evolution of this technology has made its operation no more complicated than the operation of clinical analyzers. There is also a perception that MS-based assays are expensive. With higher throughput, and the ability to multiplex assays, the cost per assay is not much different than for

ELISAs or other routine assays. Lastly, there is a natural reluctance of users to adopt new methods that are as yet perceived to be “unproven.” Related to this point is the misconception that MS-based assays are not robust or reproducible.



Steven Carr⁵: There are many reasons, including that ELISA and other immunoassays with excellent limits of quantification and assay performance are already in place for measuring most, if not all, proteins of current interest to clinicians. These assays run on a huge deployed base of highly automated clinical

analyzers and require little if any plasma or serum preparation before analysis. In contrast, MS-based assays require a substantial amount of biochemical sample manipulation that is not yet standardized, packaged, or automated. In addition, the LC-MS/MS instruments used require advanced operator knowledge to get good results and to know when things are

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² Nonstandard abbreviations: LC-MS/MS, liquid chromatography–tandem mass spectrometry; MS, mass spectrometry; HUPO, Human Proteome Organization; SRM, single reaction monitoring; hGH, human growth hormone; MRM, multiple reaction monitoring; FDA, Food and Drug Administration; CRO, contract research organization; LOD, limit of detection, LLOQ, lower limit of quantification;

QA, quality assurance; CAP, College of American Pathologists; PMA, Premarket Approval; Ab, antibody; SID, stable-isotope dilution.

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not working correctly. Robust, reliable “black-box” instruments for processing samples, running the quantitative protein assays by MS, and analyzing the resulting data are not yet available, but could be produced by vendors, given the right incentives.



Emanuel F. Petricoin III⁶:

Even the most sophisticated MS technology is limited by relatively poor analytical sensitivity, compared to most clinical immunoassays. Indeed, the Human Proteome Organization's (HUPO) own plasma proteome efforts have failed to demonstrate the ability to routinely

measure analytes in the dynamic range that most clinically useful analytes are found. Until we can employ rapid up-front concentrating and fractionating techniques for MS measurements, this analytical sensitivity barrier will likely not be overcome whereby hundreds to thousands of biological samples can be analyzed by MS every day for a given protein analyte.

What analytes are most suited for such applications?

Mary Lopez: Small molecules have been monitored in single reaction monitoring (SRM) assays in the clinical environment for years. Proteins and peptides are now increasingly becoming molecules of interest.

Steven Carr: The utility of targeted, quantitative MS-based assays for small molecules is widely appreciated. MS is heavily used (particularly in Pharma) for monitoring drug metabolism and pharmacokinetics and to assay hormones, drugs, and their metabolites. The application of MS to proteins is more recent. In principle, any protein can be assayed using MS-based approaches. However, at present, the best-performing MS assays have been configured for proteins in blood that are present in the low ng/mL concentration (so proteins at lower concentrations in blood are currently inaccessible). Achieving these concentrations requires either abundant protein depletion coupled with some limited fractionation or high-affinity peptide or protein antibody reagents for initial immunoprecipitation of the analyte before measurement by MS.

Emanuel F. Petricoin III: We have always proposed that a low molecular weight peptidome is an attractive analyte for routine clinical laboratory use because the abundance of these entities may be higher than the parental isoform, and the diagnostic value is in the fragment isoform concentrations, not in the parental analyte expression. Moreover, it may be very difficult for an ELISA to be manufactured that can distinguish protein isoforms from one another. If one needs to measure a specific protein isoform that is more diagnostic than other isoforms from the same protein, routine immunoassays fall apart. A real-world example of this is human growth hormone (hGH) testing, where total-hormone measurement is not informative of doping, but the isoform distribution is the smoking gun. MS may be the only way to rapidly distinguish these isoforms from each other.

Samir Hanash: Analytes whose concentrations are not affected by variable modifications, such as single nucleotide polymorphisms, posttranslational modifications, alternative splicing, or compounds with great dependence on particular sample collection and processing procedures or a propensity to degradation.

Do you think that “multiplexing” various analytes will make these methods more attractive?

Samir Hanash: Yes, definitely, given the likelihood that panels of analytes will emerge and will need to be analyzed under healthy and disease conditions.

Steven Carr: Yes, of course. The ease with which these assays can be multiplexed is a tremendous advantage of methods based on multiple reaction monitoring (MRM) MS over traditional immunoassays. Using a single 10–100 μ L aliquot of patient plasma, we can assay tens of protein analytes, provided these analytes are within the detection and quantification range of the combined processing method and instrument used. Current immunoassays have very limited multiplexing capability before specificity and assay performance are unduly affected.

Mary Lopez: Absolutely. It has been common knowledge that monitoring only one biomarker at a time for complex diseases in most cases does not deliver the specificity required. Multiplexing analytes enhances the efficiency, throughput, and cost of most assays.

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Emanuel F. Petricoin III: The days are over for the vision that some new single analyte will have the magical qualities of high clinical sensitivity and specificity in just one measurement. Indeed, the future belongs to multiplexed signature tests, and the ability to multiplex will be a necessary requirement not only for MS, but for any clinical test. MRM, specifically, has embedded attributes that make it highly attractive for multiplexed assays of defined isoform variants, especially those analytes that are not distinguishable by immunoassay and as long as they are of sufficient abundance to detect.

Do you think that ELISA assays will give way to MS-based protein assays anytime soon?

Emanuel F. Petricoin III: Not anytime soon because of the analytical sensitivity issue, but as new technologies are developed for rapid protein concentration combined with fractionation, ELISA assays will lose popularity. Immuno-MS approaches, nanoharvesting particle fractionation, etc. are lead embodiments in work flows that use the MS as the readout and could replace ELISA-based assays.

Steven Carr: I think that ELISA assays are here to stay, but MS-based protein assays will complement immunoassays in the clinical laboratory in the next 2–3 years. Adoption of MS-based methods as laboratory tests is most likely to occur for proteins where interferences in the immunoassay are known, such as the recent case of thyroglobulin reported in the November 2008 issue of *Clinical Chemistry*, where Hoofnagle and coworkers developed an MRM assay that sidesteps issues with the current clinical assay. On a longer timescale, it will also occur for new panels of protein markers, where the immuno-based reagents do not exist or are not of sufficient quality. Replacement of current ELISA assays will likely require Food and Drug Administration (FDA) approval, which, in turn, will require instruments that are far more automated, traceable, and fool-proof than today's systems, as well as more intelligent software for robust data analysis without expert oversight. This will require major cooperation from the MS vendors, who will need to see the opportunity clearly before they will be willing to make the investments. It will also require some change on part of the regulatory agencies to help facilitate development of new tests using new devices.

Mary Lopez: There will invariably be instances where an ELISA is the most cost-effective way to monitor an analyte. However, in instances where the analyte may be proteolytically cleaved or exist in several forms because of posttranslational modifications, MS provides the best way to monitor the specific sequences. This enhances the specificity of the assay. Therefore, it is

likely that many assays that are currently measured by ELISA will indeed give way to MS-based assays.

Your view on the future of assays for protein analysis in the routine laboratory seems much more optimistic than that of the other panelists. What is the basis for your optimism?

Mary Lopez: It is likely that MS will soon be the detector of choice for clinical research and, eventually, for many routine clinical assays. This opinion is based on a number of factors. First, immunoassay-based methods cannot provide the requisite specificity for the detection of multiple forms of target biomarkers. In many cases, diseases are linked to proteolytically cleaved as well as intact forms of proteins. Posttranslational modifications also add heterogeneity. Often, it is the ratio of the cleaved or posttranslationally modified to intact forms that can be diagnostic, or even prognostic. It is impractical and often impossible to differentiate and accurately quantify these forms with antibodies. MS provides a very accurate and precise way to measure these subtle differences. Second, the ability to multiplex lowers the cost and increases the throughput of MS-based assays, therefore, bringing them more in line with the economics of the clinical laboratory environment. Indeed, many large contract research organizations (CROs) are already converting their traditional ELISA assays to MS-based assays. Another area where MS is widely used in a clinical environment is neonatal screening. As these assays become more routine, the costs will drop even further.

Third, improvements in MS technology and sample preparation are rapidly increasing the analytical sensitivity, simplicity, and robustness of MS-based methods. A combination of antibody-based enrichment with triple quadrupole single reaction monitoring assays can currently deliver limits of quantification equivalent to ELISAs for some clinically relevant markers, yet with higher specificity. These factors are pushing MS deep into the clinical environment, but much work still needs to be done to standardize and lock down methods across laboratories. In addition, regulatory issues will, of course, need to be addressed. However, a real clinical and market need will drive the development of FDA-approved software and hardware rapidly. We are currently in a period of rapid developments in these areas, and I expect that within the next few years most clinical laboratories will have adopted at least one or two MS-based assays as part of the standard menu.

Do you have any additional comments?

Emanuel F. Petricoin III: I have been giving thought to one question. If MRM MS methods are to become the bases of clinical diagnostic assays, what are the more

important criteria that must be met to realize this vision? I think that there are several barriers that prevent any technology from entering into the clinical laboratory. Even if a technology provides aspects of scientific and/or analytical superiority (superior limit of detection (LOD), lower limit of quantification (LLOQ), etc.), if the costs per assay, start-up costs, or costs for the ease of use (clinical laboratory technician) are too high, then MRM assays may never be adopted. Moreover, if sample and reagents for quality assurance (QA)/QC, labeled standards required for true quantification, etc., are not scalable and widely distributed, then routine clinical use will not take hold. Routine clinical use of MRM-based protein assays will never progress beyond esoteric testing if measures of reproducibility, quantification, and inter- and intralaboratory validation do not proceed in a stepwise fashion. Since different MS platforms are currently being used by different investigators, it is not clear how and when this could happen, as methods developed on one platform may not be usable for another platform. Lastly, given the current environment for FDA oversight for protein biomarker assays, including multivariate assays, MRM-derived assays, or any MS-based assay, will require formal evaluation in a College of American Pathologists (CAP)/CLIA approved laboratory and will require a Premarket Approval (PMA) or 510(k) submission.

Steven Carr: The main rationale for developing such assays today is not to replace ELISA or other immunoassays for proteins that are already being measured with sufficient specificity, sensitivity, and absence of known interferences. Rather, it is to address the great need for developing *new* protein biomarkers for early detection and prognosis of disease where the necessary antibody (Ab) reagents currently do not exist. Discovery “omics” experiments have produced a surfeit of new protein biomarker candidates. Unfortunately, few, if any, of these candidates will ever become useful biomarkers because of a lack of precise and specific quantitative immunoassays to measure the levels of these proteins in large numbers of patient case and appropriate control samples. This process, referred to as *verification*, is essential to demonstrate that the candidate or panel of candidates has sufficient discriminatory power to be useful as a biomarker. The catalog of immunoaffinity reagents suitable for configuration quantitative assays is simply far too small to tackle this problem. Targeted, MS-based methods with quantifi-

cation based on stable-isotopically labeled peptides or proteins hold great promise as the technology capable of bridging the yawning gulf between discovery “omics” experiments and clinical validation. The main methods that we and others are exploring include stable-isotope dilution (SID)-MRM-MS alone, peptide immunoaffinity coupled to SID-MRM-MS, and protein immunoaffinity coupled to SID-MRM-MS. Today, my view is that these MS-based assays are the drafting tool used before committing the time and resources required to create clinical-grade immunoassays that can run on the current deployed base of instrumentation. I also believe that ELISA and other immunoassays are here to stay, but that true clinical implementation of MS-based protein assays (501(k) completed; FDA approved), while a few years away, will happen in specific cases. Its adoption will be driven by the ability to highly multiplex these MS-based assays while maintaining suitable assay performance. Other factors favoring some level of adoption is a rapid development timescale relative to clinical ELISA tests, as well as the higher degree of molecular specificity and ability to detect interferences.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures of Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:*

Employment or Leadership: None declared.

Consultant or Advisory Role: E.F. Petricoin III, Ceres Nanosciences.

Stock Ownership: E.F. Petricoin III, Ceres Nanosciences.

Honoraria: None declared.

Research Funding: E.F. Petricoin III, Ceres Nanosciences.

Expert Testimony: None declared.

Role of Sponsor: The sponsor played a direct role in the design of the study, the review and interpretation of data, and the preparation and final approval of the manuscript.

Acknowledgments: E.F. Petricoin III would like to acknowledge the many helpful discussions with Lance Liotta, Mark Ross, and Paul Russo at George Mason University.

Previously published online at DOI: 10.1373/clinchem.2009.128058
