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Special Report

Protein Biomarker Quantification by Immunoaffinity Liquid Chromatography–Tandem Mass Spectrometry: Current State and Future Vision

Hendrik Neubert,[a,*] Christopher M. Shuford,[b] Timothy V. Olah,[c] Fabio Garofolo,[d] Gary A. Schultz,[e] Barry R. Jones,[f] Lakshmi Amaravadi,[g] Omar F. Laterza,[h] Keyang Xu,[i] and Bradley L. Ackermann[j]

Immunoaffinity–mass spectrometry (IA-MS) is an emerging analytical genre with several advantages for profiling and determination of protein biomarkers. Because IA-MS combines affinity capture, analogous to ligand binding assays (LBAs), with mass spectrometry (MS) detection, this platform is often described using the term *hybrid* methods. The purpose of this report is to provide an overview of the principles of IA-MS and to demonstrate, through application, the unique power and potential of this technology. By combining target immunoaffinity enrichment with the use of stable isotope-labeled internal standards and MS detection, IA-MS achieves high sensitivity while providing unparalleled specificity for the quantification of protein biomarkers in fluids and tissues. In recent years, significant uptake of IA-MS has occurred in the pharmaceutical industry, particularly in the early stages of clinical development, enabling biomarker measurement previously considered unattainable. By comparison, IA-MS adoption by CLIA laboratories has occurred more slowly. Current barriers to IA-MS use and opportunities for expanded adoption are discussed. The path forward involves identifying applications for which IA-MS is the best option compared with LBA or MS technologies alone. IA-MS will continue to benefit from advances in reagent generation, more sensitive and higher throughput MS technologies, and continued growth in use by the broader analytical community. Collectively, the pursuit of these opportunities will secure expanded long-term use of IA-MS for clinical applications.

Introduction

Protein biomarkers are essential to clinical diagnosis and disease treatment in the practice of medicine. Protein biomarkers are also applied at all stages of pharmaceutical research and development to provide information about drug efficacy, mechanism of action, target engagement, safety, and patient selection *(1–3)*. A commonality to both areas is that LBAs have traditionally been the default method of analysis. Several practical advantages, including cost and ease of implementation, underscore the mainstay status of LBA in the clinical laboratory. At the same time, LBA methods are not without challenges including limited specificity, difficulty when analyzing matrices such as tissue, and interference from heterophilic and autoantibodies *(4)*.

Mass spectrometry has several unique attributes that make it an important complement to LBA for protein analysis. In addition to unparalleled specificity, MS can achieve high precision in complex matrices using stable isotope-labeled (SIL) internal standards *(5)*. Furthermore, by coupling MS to liquid chromatography (LC; LC-MS), peptide and protein mixtures can be separated for improved quantification of single or multiple proteins from a single sample. To maximize sensitivity, LC-MS typically occurs after proteolytic digestion to yield peptides acting as surrogates for the protein of interest (bottom-up analysis).

LC-MS has made important inroads into protein biomarker analysis including clinical applications. Table 1 contains a partial list of published clinical protein biomarker methods, both from clinical diagnostics (Table 1, A) and pharmaceutical or academic laboratories (Table 1, B). A big segment is bottom-up methods with conventional extraction. Although most methods come from academic or pharmaceutical research, several examples show that LC-MS assays have been adopted in CLIA laboratories *(6–9)*.

A current disadvantage to MS without the use of enrichment techniques is analytical sensitivity. When

[a] Pfizer Worldwide Research and Development, Andover, MA [b] Laboratory Corporation of America Holdings, Burlington, NC [c] Bristol-Myers Squibb Company, Princeton, NJ [d] Angelini Pharma, Pomezia, Rome, Italy [e] Munson Technology LLC, Ithaca, NY [f] Q Squared Solutions, Ithaca, NY [g] Shire plc, a Takeda Company, Cambridge, MA [h] Merck and Co., Inc., Kenilworth, NJ [i] Genentech, CA South San Francisco [j] Eli Lilly and Company, Lilly Research Laboratories, Indianapolis, IN

* Address correspondence to this author at: Biomedicine Design, Pfizer Worldwide Research and Development, 1 Burtt Road, Andover, MA 01810; E-mail hendrik.neubert@pfizer.com.

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Table 1. Quantitative human protein mass spectrometry assays representing applications in clinical diagnostics (A) and pharmaceutical and academic research (B).

Analyte(s)	Extraction	LC	Ionization	Detection	References
(A) Representative clinical diagnostics applications					
C-peptide, insulin	IA-protein	Yes	ESI	QqQ	(6)
Thyroglobulin	IA-peptide	Yes	ESI	QqQ	(10, 11)
Angiotensin-1 (renin activity)	SPE	Yes	ESI	QqQ	(12)
Insulin-like growth factor-1	SPE	Yes	ESI	Q-ToF	(7, 13)
ADAMTS13 activity	IMAC	No	MALDI	ToF	(14)
Lp-PLA_2	IA-peptide	Yes	ESI	QqQ	(8)
Parathyroid hormone-related peptide (PTHrP)	IA-protein	Yes	ESI	QqQ	(15)
Immunoglobulin light and heavy chain (M-proteins)	IA-protein	No	MALDI	ToF	(16)
IgG subclasses	None	Yes	ESI	QqQ	(17, 18)
Vitamin D binding globulin	None	Yes	ESI	QqQ	(19)
Serum apolipoprotein panel (apoAI, B, CI, CII, CIII, E)	None	Yes	ESI	QqQ	(9)
Galectin-3-binding protein, scavenger receptor cysteine-rich type 1 protein M130	Depletion	Yes	ESI	QqQ	(20, 21)
High-density lipoprotein particle panel (Apo AI, CI, CII, CIII)	Coaffinity purification	Yes	ESI	QqQ	(22, 23)
β-Amyloid (Aβ-42)	SPE	Yes	ESI	Orbitrap	(24)
Hemoglobin A_{1c}	None	Yes	ESI	QqQ	(25)
(B) Representative pharmaceutical and academic applications					
β-Amyloid peptides (Aβ)	SPE	Yes	ESI	QqQ	(26)
Glucagon	SPE	Yes	ESI	QqQ	(27)
Hepcidin	SPE	No	MALDI	ToF	(28)
Hepcidin	PPT	Yes	ESI	QqQ	(29)
Insulin and therapeutic analogs	SPE	Yes	ESI	QqQ	(30)
Insulin growth factor-1 (IGF-1) and leucine-rich α-2-glycoprotein (LRG)	PPT	Yes	ESI	QqQ	(31)
Hemoglobin variants	Liq-liq	Yes	ESI	FT-MS	(32)
Proprotein convertase subtilisin/kexin type 9 (PCSK9)	SPE	Yes	ESI	QqQ	(33)
Insulin and therapeutic analogs	IA-protein	No	MALDI	ToF	(34)
Insulin growth factor-1 (IGF-1)	IA-protein	No	MALDI	ToF	(35)
Protein C inhibitor	IA-peptide	No	MALDI	ToF	(36)
β-2-Microglobulin	IA-protein	No	MALDI	ToF	(37)
Retinol binding protein	IA-protein	No	MALDI	ToF	(38)
Apolipoprotein CI, CII, CIII	IA-protein	No	MALDI	ToF	(39)
Brain natriuretic factor (BNP)	IA-peptide	No	MALDI	ToF	(40)
Macrophage migration inhibitory factor (MIF)	IA-protein	No	MALDI	ToF	(41)
Serum transferrin receptor	IA-peptide	Yes	ESI	QqQ	(42)
Clinical protein panel	IA-peptide	Yes	ESI	QqQ	(43)
Total salivary pepsin/pepsinogen	IA-peptide	Yes	ESI	QqQ	(44)

(continued)

Table 1. (continued)

Analyte(s)	Extraction	LC	Ionization	Detection	References
Clinical protein panel	IA-protein	Yes	ESI	QqQ	(45)
β-Amyloid peptides (Aβ)	IA-protein	Yes	ESI	ITD	(46)
Tau-protein	IA-protein	Yes	ESI	QqQ	(47)
Brain natriuretic factor (BNP)	IA-protein	Yes	ESI	QqQ	(48)
Glucagon-like peptide-1 (GLP-1), glucagon, oxyntomodulin (OXM)	IA-protein	Yes	ESI	QqQ	(49)
Oxyntomodulin (OXM)	IA-protein	Yes	ESI	Orbitrap	(50)
Interferon γ-induced protein-10 (IP-10)	IA-protein	Yes	ESI	QqQ	(51)
Proprotein convertase subtilisin/kexin type 9 (PCSK9)	IA-protein	Yes	ESI	QqQ	(52)
Progastrin releasing peptide (ProGRP) and neuron-specific enolase (NSE)	IA-protein	Yes	ESI	QqQ	(53)
Carbonic anhydrase II	IA-protein	Yes	ESI	QqQ	(54)
Angiotensin 1 (renin activity)	IA-protein	Yes	ESI	QqQ	(55)
Platelet frataxin	IA-protein	Yes	ESI	Orbitrap	(56)
High-mobility group box 1 (HMGB1)	IA-protein	Yes	ESI	Orbitrap	(57)
Fibroblast growth factor 21 (FGF21)	IA-protein	Yes	ESI	QqQ	(58)
β-Nerve growth factor (β-NGF)	IA-sequential	Yes	ESI	QqQ	(59)
Interleukin 21	IA-sequential	Yes	ESI	QqQ	(60)

FT-MS = Fourier transform MS; IA = immunoaffinity;IMAC = immobilized metal affinity chromatography; ITD = ion trap detector; Liq-Liq = liquid/liquid extraction; PPT = protein precipitation; Q-ToF = quadrupole time-of-flight; SPE = solid phase extraction; ToF = time-of-flight.

using conventional extraction techniques, LC-MS analysis allows for only low quantification (ng/mL) without resorting to exaggerated sample volumes (0.1–1 mL) or extensive sample preparation *(61)*. To this end, immunoaffinity (IA) enrichment has been successfully applied to MS analysis to improve sensitivity. The original IA-MS format, MS-immunoassay, was introduced by Nelson et al. *(62)* and incorporates MS analysis by matrix-assisted laser desorption ionization (MALDI). However, because it utilizes a top-down format (no digestion) without chromatography, it is largely restricted to higher abundance proteins.

During the past decade, there has been immense growth in the number of IA-MS methods for proteins using bottom-up analysis by LC-MS with electrospray ionization (ESI) *(46, 47, 60, 63)*. These assays, often referred to as *hybrid* methods because they combine elements of LBA and MS, have become quite highly adopted for pharmaceutical applications *(64)*. Importantly, this combination provides maximum sensitivity for MS-based protein biomarker methods, with several assays reported with detection limits in the range of 1–10 pg/mL *(50, 59, 60)*. Despite uptake in the pharmaceutical industry, adoption by CLIA laboratories has occurred at a slower pace. Thyroglobulin *(10)* and parathyroid hormone-related peptide *(15)* are a few noteworthy examples (Table 1, A).

This report aims to document the current status of IA-MS and offers guidance intended to stimulate awareness to influence broader adoption by both pharmaceutical and CLIA laboratories. The greater adoption seen thus far by pharmaceutical laboratories is likely a reflection of the greater potential for exploratory work. Regardless of this and other factors, both areas must place emphasis on high-profile applications that cannot be addressed reliably using either LBA or MS alone. Finally, an assessment of current gaps and opportunities is offered to stimulate work on current challenges and to accelerate broader adoption of the IA-MS technology.

Modes of IA-MS Workflow

IA-MS methods are generally classified by the method used for affinity capture. The 3 most common IA-MS flow schemes are depicted in Fig. 1, A–C, which illustrates the experimental steps involved in *(1)* protein affinity capture, *(2)* peptide affinity capture, and *(3)* sequential affinity capture, respectively. Although the incorporation of LC occurs in all applications involving

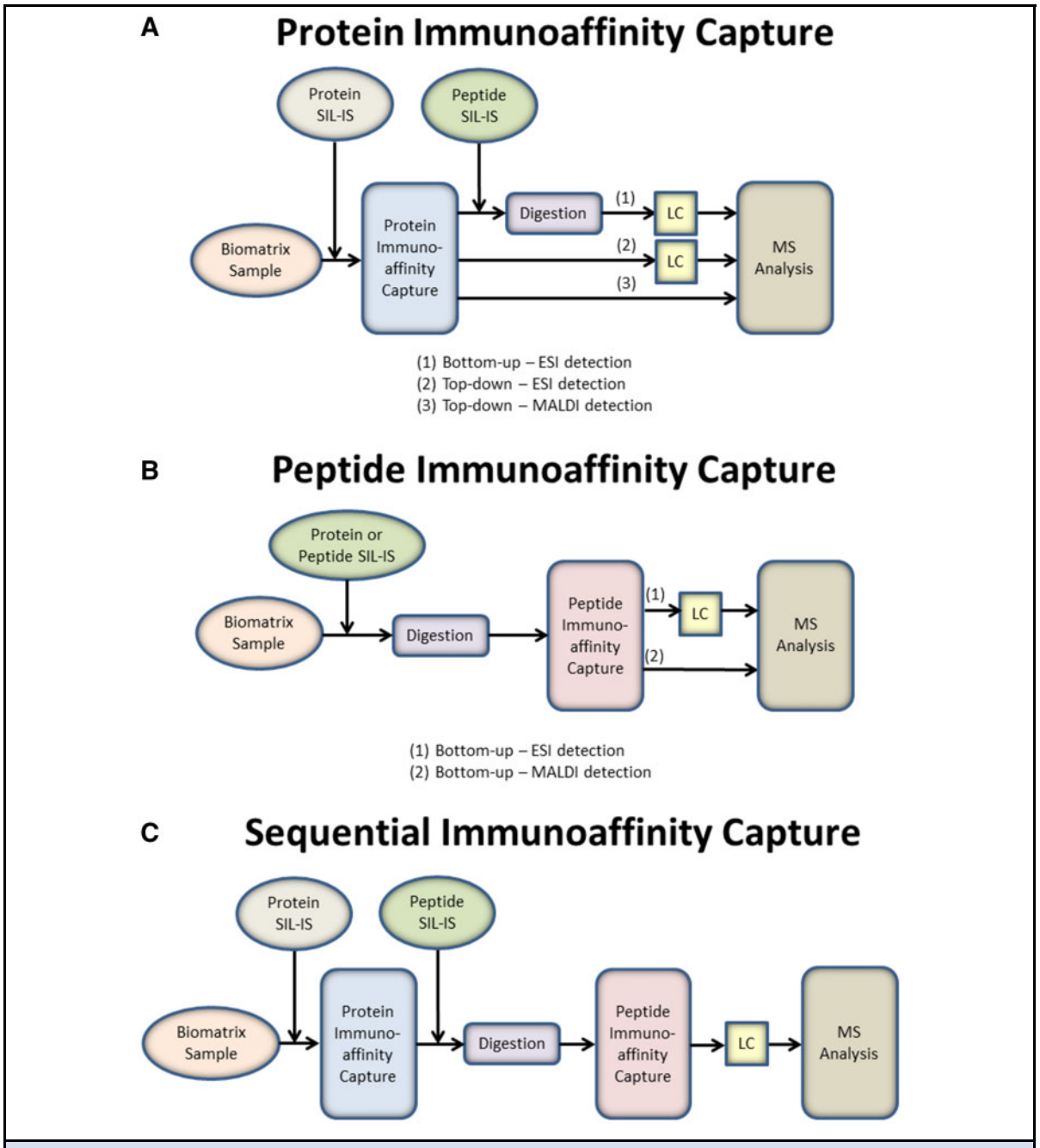


Fig. 1. Experimental modes of IA-MS.

(A) Protein IA capture occurs in 3 formats: (1) bottom-up and (2) top-down with LC-ESI and (3) top-down using MALDI. (B) Peptide IA capture has been used with 2 bottom-up formats: (1) LC-ESI and (2) MALDI. (C) Sequential IA capture involves 2 stages of IA capture, before and after enzymatic digestion, and has been used only with LC-ESI. Two types of SIL-IS may be used with all modes to track recovery and matrix effects: target protein and surrogate peptide.

ESI, methods based on MALDI do not utilize LC. Elimination of LC increases the overall sample throughput of MALDI, an advantage that has been exploited for specific clinical applications (*35*, *36*). Despite such applications and the inherent instrumental sensitivity of MALDI, LC-MS using ESI is the more selective technique and consequently dominates most IA-MS applications. The incorporation of SIL peptides, and in some

cases SIL proteins, as internal standards offers improved precision and ruggedness by normalizing for differences in extraction and ionization.

PROTEIN IA CAPTURE

Protein IA capture (Fig. 1, A) is the most common IA-MS format. Conceptually, it is similar to LBA in that it uses an antibody for selective capture but relies on MS for detection rather than a secondary antibody. The wide availability of antiprotein antibodies increases the utilization of this method, which is often undertaken to achieve increased specificity and/or rapid method development compared with western blotting or other methods. Although antibodies are the primary capture reagent used, alternative capture formats exist.

IA-MS methods are further differentiated by post-capture processing and, specifically, by whether proteolytic digestion is used to create surrogate peptides for measurement. As shown in Fig. 1, A, a captured target protein is processed by 1 of 2 options: bottom-up or top-down detection methods. Bottom-up, which involves enzymatic digestion to yield surrogate peptides, is the more common approach owing to greater sensitivity for MS detection. Considerations for surrogate peptide selection have been widely described *(65)* and include uniqueness to the target protein, sensitivity, stability, and the likelihood for genetic variation or post- or cotranslational modification within the target sequence. Top-down methods involve the direct analysis of an intact protein (i.e., no digestion) and commonly use high-resolution MS (HRMS) to achieve specificity. Despite being less sensitive, top-down methods reduce the time needed for sample preparation and allow specific detection of discrete proteoforms. Currently, top-down methods by LC with ESI (Fig. 1, A, format 2) are ideally applied to smaller proteins of up to 10–20 kDa depending on the sensitivity requirements of the assay *(66)*, but larger proteins may also be possible.

PEPTIDE IA CAPTURE

Figure 1, B depicts IA-MS for affinity capture of a surrogate peptide. This method is commonly referred to by the acronym SISCAPA (stable isotope standards and capture by antipeptide antibodies) *(67)*. A peptide IA workflow most commonly occurs using LC-MS, although clinical applications involving MALDI detection have been reported *(36, 68)*. Peptide IA methods can be used either with protein calibrators introduced at the beginning of the workflow or peptide calibrators for relative measurement.

Whole-sample proteolytic digestion, as commonly deployed by bottom-up proteomic methods, is the first step. The peptide IA approach largely evolved from targeted proteomics experiments and the desire to achieve greater sensitivity to support biomarker qualification experiments *(69)*. Accordingly, these methods are frequently applied for analysis of protein biomarker panels (e.g., >10 analytes). An important advantage of the peptide IA method is that proteins may be measured following harsh upfront conditions that often denature native protein epitopes. This advantage has led to new applications to protein targets inaccessible to standard LBA methods. Examples include quantification of membrane proteins in tissue homogenates *(70, 71)* or quantification of plasma biomarkers in the presence of autoantibodies *(10, 11)*. A disadvantage to peptide IA methods is the lack of broad availability of commercial antipeptide antibodies making upfront investment in antibody production a necessary part of this approach. It should also be noted that, because of upfront digestion, peptide IA methods are capable only of total determination of the protein biomarker, the specific sequence, or modification.

SEQUENTIAL IA CAPTURE

A third IA-MS configuration, depicted in Fig. 1, C, is an approach combining both protein and peptide IA capture. This technique, referred to as *sequential affinity capture*, maximizes the enrichment and selectivity of both IA-MS methods and provides tangible signal-to-noise benefits. Consequently, sequential IA-MS should be considered for applications in which maximum sensitivity is desired. Typical gains in sensitivity versus single protein capture are estimated to be in the range of at least 2- to 5-fold *(60)*. The initial enrichment step involves immunoprecipitation of the target protein. After trypsin digestion, the surrogate peptide is extracted using an antipeptide antibody either in an online column format or using a bead-based off-line configuration. Subsequently, peptides are quantified by LC-MS. To date, several applications of sequential IA-MS have been published, including clinical and preclinical analyses of nerve growth factor *(59, 72, 73)* and analysis of cytokines such as interleukin 21 *(60)*.

Approaches to IA-MS Quantification

Given the broad range of IA-MS applications in drug discovery, development, and clinical diagnostics, the subject of method validation is beyond the scope of this communication. As with any bioanalytical method, it is reasonable to expect that core attributes including precision, relative accuracy, sensitivity, selectivity, stability, and robustness will be tested in a fit-for-purpose manner.

An issue facing all protein biomarker methods, regardless of technology, is the difficulty establishing definitive accuracy using recombinant reference materials

to mimic endogenous proteins. A recent white paper provides more specific information on this subject (74). Despite these limitations, we recommend that relative accuracy be generated using the best available protein calibrator whenever possible (75), including the use of recombinant protein calibrators to estimate and track recovery for antipeptide IA methods. Because of acknowledged limitations associated with spiking recombinant protein calibrators into biological matrices, value-assigned pools (9) or a method known as *admixing* has been adopted in which pools having inherently different concentrations (e.g., disease vs. normal) are mixed in various ratios to avoid spiking (76). Regardless of the approach taken to calibration, quality-control (QC) samples used in both pre- and in-study validation experiments should be prepared in authentic biological matrix through spiking, admixing, or diluting with a surrogate matrix for which parallelism to the biological matrix has been established. The approach taken for validation should be aligned with the intended context of use of the assay, along with any regulatory guidance governing the work (75, 77, 78).

An inherent challenge to biomarker methods is that control matrices used to prepare calibrators and QC samples contain the target analyte(s) of interest. In certain cases, it is possible to prepare calibrators using control matrix having a target analyte concentration sufficiently below the intended lower limit of quantification (LLOQ) to avoid interference by the endogenous analyte. Because this is not always possible, 2 general strategies are routinely used to address this fundamental issue: surrogate matrix and surrogate analyte (79). Surrogate matrix methods utilize a substitute matrix devoid of the target analyte to prepare calibration standards and QCs. Surrogate matrices may sometimes be supplied from an alternative species possessing a different protein/peptide sequence or by immunodepletion of the target protein. Alternatively, a buffer solution possibly containing protein such as albumin can also serve as a surrogate matrix. In all cases, a key part of method development and validation is to qualify the surrogate matrix through an appropriate assessment of parallelism using experiments such as dilutional linearity and spike recovery (80–83).

For surrogate analyte methods, a SIL version of the analyte is used as the calibrant to avoid interference from the endogenous molecule. Although this practice has been successfully applied to small molecule biomarkers (84–86), it is not commonly used for protein quantification and will not be discussed further (24).

A central concept to all quantitative biomarker methods is parallelism. *Parallelism* is used to describe results from experiments that evaluate whether observed changes in response when altering the analyte concentration are equivalent between the surrogate and the authentic biological matrix across the range of analysis. Deviations from this concordance undermine the relative accuracy of the assay. The well-documented influence of matrix effects on LC-MS presents a concern about assay parallelism given differences between the surrogate and authentic matrix. Fortunately, these differences are largely normalized using SIL internal standards. A bigger issue for protein quantification is that recombinant protein calibrators do not typically resemble the endogenous form of the target analyte in terms of structure, folding, posttranslational variations, access to binding partners, digestion recovery and other behaviors, including immunorecognition. Importantly, because these differences are difficult to quantify and cannot be readily compensated for by SIL standards, IA-MS protein assays can provide only relative and not definitive accuracy (80) in the absence of metrological traceability (87). In a sense, protein calibrators are themselves surrogate analytes and may not be commutable with native patient specimens (88). Given the use of immunorecognition (and enzymatic recognition), IA-MS assays will likely face the same challenges as LBAs regarding interlaboratory discordance (74) until commutable, protein-level reference materials become available.

Calibrators, Internal Standards, and Affinity Capture Reagents

Reagents are critical for overall IA-MS assay performance and important for troubleshooting and issue resolution. Certain reagents, such as proteins used for calibration and affinity capture reagents, are termed *critical* reagents because they have a direct influence on the readout of the assay. Although several papers have discussed this subject for LBA (89, 90), Santockyte and coworkers recently discussed critical reagent characterization for hybrid LC-MS biomarker assays (91). SIL internal standards represent another important reagent category.

PROTEIN AND PEPTIDE REFERENCE CALIBRATORS

The inability for protein reference materials, typically derived by recombinant methods, to serve as legitimate surrogates for endogenous proteins is an acknowledged limitation of protein biomarker methods (74, 80). Despite these limitations, the best available protein calibrator should be used to track assay performance over time and, whenever possible, to calculate relative accuracy. Furthermore, because most IA-MS assays utilize a bottom-up strategy, use of the most appropriate protein calibrators to understand digestion efficiency is of paramount importance. This point was corroborated by Shuford et al. (88), who performed a detailed assessment of sources of bias in the determination of thyroglobulin

in human serum. Their work suggests that incomplete and variable digestion recovery is a significant source of error in LC-MS protein assays. Shuford and colleagues also provided a detailed comparison of human-derived and recombinant thyroglobulin and showed that the 2 were not commutable under a variety of digestion conditions. Lastly appropriate analytical characterization of protein and surrogate peptide calibrators is an important expectation for all assays *(88, 92)*.

INTERNAL STANDARDS

SIL standards are vital to track recovery and matrix effects of LC-MS assays and provide a distinct advantage over LBAs. SIL versions of surrogate peptides are readily prepared by solid-phase synthesis and may be made with extended sequences at both termini to track digestion recovery. Although such cleavable versions are assumed to be superior, results often vary, and their performance needs to be tested on a case-by-case basis *(88, 93)*. Although less frequently available, recombinant SIL protein standards have been shown to deliver superior assay performance *(88, 93)*, particularly in the presence of patient-specific matrix effects *(94)*, and are recommended for those clinical assays requiring a higher degree of validation.

AFFINITY CAPTURE REAGENTS

IA-MS assays utilize affinity capture to deliver selective enrichment of analytes of interest and the removal of background. Together, these factors account for the sensitivity advantage over conventional LC-MS methods for protein biomarkers. Monoclonal and polyclonal antibodies remain the standard and most abundantly used reagents for affinity capture; other options include antibody fragments such fragment antigen-binding *(95)* or single-chain variable fragments, recombinant targets, protein A/G, aptamers *(52, 96)*, and endogenous ligands. Chemical probes *(97)*, small-molecule drugs *(98)*, and immobilized metals *(99, 100)* can also be used to enrich a protein biomarker. Several benefits but also potential risks exist for each reagent type when applied in quantitative methods, including potential lot-to-lot variability and inadequate specificity and affinity. Although several commercial sources for antibodies exist, de novo generation requires a time commitment (multiple months) without a guarantee of success. These factors must be considered and the risks mitigated, ideally, early in the development of IA-MS methods. In fact, inadequate reagent quality and long-term supply can prompt the choice of alternative assay strategies.

Characterization of affinity reagents typically involves surface plasmon resonance or biolayer interferometry to screen antibodies for their relative binding affinity and kinetics *(101)*. High-affinity antibodies (low Kd/dissociation constant) are desired for maximal target enrichment, along with slow off-rates to allow for more stringent wash steps. Importantly, because of the use of MS detection, capture reagents for IA-MS can adopt a broader range of specificity than allowed for LBAs. However, detailed assessment of selectivity, in terms of which other antigens bind to a specific reagent, is not typically performed. Consequently, reagents not suitable for LBA may still be used with IA-MS, provided enrichment of the analyte(s) of interest is sufficient. For cases in which reagent binding specificity is uncertain, additional experiments may be undertaken to understand epitope recognition by one of several techniques *(102)*. This information is often critical to understanding competitive binding interactions. Moreover, capture performance in biological matrix needs to be interrogated. If available, the influence of suspected competitive binding proteins may be spiked to assess their impact on recovery. Immunocapture efficiency may also be estimated by spiking a protein analyte before and after IA capture and comparing differences in observed signal *(103)*. Although this process is routinely performed for biotherapeutics, caution must be exercised when applying this approach to biomarkers given potential binding differences between a spiked protein calibrator and its endogenous counterpart and complications brought about by the presence of endogenous targets in the matrix. Finally, unlike the peptide IA format, protein-based capture assays could be affected by the presence of heterophilic antibodies or human anti-mouse antibodies when a mouse monoclonal capture antibody is used. Although these antibodies are not expected to result in a false-positive signal, they might reduce capture efficiency. This issue needs to be considered and conditions optimized during method development.

Sample Preparation

Sample preparation plays a key role in determining the overall performance of IA-MS assays. In general, IA-MS assays can achieve high sensitivity by adopting a large sample volume and, if needed, an adjustable capacity of the capture reagent. Various forms of a protein analyte (i.e., free, bound, or total) can be specifically captured. For example, to quantify the free form of a biomarker, caution should be taken to minimize any potential disruption of the existing equilibrium with an endogenous binding protein or biotherapeutic. In addition, various factors such as binding capacity, incubation time and temperature, sample dilution, and pH of wash buffer should be carefully optimized to best preserve the complex *(104, 105)*. In contrast, for the measurement of total concentration, it is critical to ensure that all forms of the biomarker, free and bound, will be fully captured. Sample dilution, possibly with a high salt buffer or addition of acid or chaotropic agents (e.g., guanidine or urea) or acid dissociation *(106)* if necessary, may help liberate the protein before IA capture *(60, 107)*. Alternatively, digestion may be used to break down any

preexisting complexes to produce surrogate peptides for subsequent antipeptide IA-MS *(10)*. A significant advantage afforded by IA-MS is multiplexing, which can be achieved by using several capture antibodies to allow simultaneous enrichment of multiple biomarkers before selective LC-MS detection *(49, 108, 109)*.

Magnetic beads are the most commonly used IA capture media, although agarose beads are used in some cases. Other formats of solid supports may include columns, pipette tips, or cartridges *(110, 111)*; plates are used less frequently because of limited loading capacity. These solid supports can have protein A/G for binding to the Fc of the capture antibody; can carry streptavidin, avidin, or their derivatives for attaching to biotinylated capture reagents; or use an active chemical moiety for covalent coupling to antibody capture reagents. However, potential interference by excess biotin to IA should be evaluated when the biotin/streptavidin approach is used *(112)*. Furthermore, antibody binding should be confirmed after covalent attachment because unintended reaction of residues in the complementarity-determining regions of the antibody may compromise binding affinity.

The loading capacity of the capture phase also needs to be evaluated. A large excess in capture phase may lead to unnecessary nonspecific binding of matrix components contributing to elevated background noise or interference in LC-MS, whereas insufficient capture phase leads to reduced recovery. Both off-line and online IA approaches have been implemented. The off-line approach offers parallel processing for multiple samples and is amenable to automation on liquid or bead-handling platforms. Advantages also include an adjustable capacity and no well-to-well carryover. Online IA using a column format may be performed for peptide enrichment and has shown robustness for protein biomarker analyses *(44, 113, 114)*. However, the potential for carryover should be carefully evaluated.

As depicted in Fig. 1, SIL standards can be introduced at different sample preparation steps to compensate for potential variability occurring in IA capture and digestion and for other known factors such as injection, LC separation, and MS detection *(65)*. For protein IA methods, SIL peptides are typically introduced after IA capture but before digestion. In contrast, in a peptide IA method, the SIL peptide is spiked before IA capture.

Although trypsin is the most commonly used proteolytic enzyme to generate suitable surrogate peptides in IA-MS, other proteases including chymotrypsin, LysC, LysN, AspN, GluC, and ArgC have also been used individually or in combination to obtain peptides at suitable lengths and with unique properties to allow selective detection by MS *(115)*. Digestion can be performed either after or before enrichment of the analyte. Postenrichment digestion can be performed either directly on the solid support for example, on-bead *(116)* or after analyte elution from the solid support. On-line digestion using a trypsin column has also been documented *(117)*. Preenrichment digestion is the typical method of choice for antipeptide capture. Strategies and general rules for the selection of appropriate surrogate peptides have been well documented *(118, 119)*.

Chromatography and MS

The key consideration in selecting a chromatography format (Table 2) is the required LLOQ for an assay. In general, chromatographic sensitivity is improved with the use of smaller diameter columns while maintaining sample aliquot and injection volumes. However, the IA enrichment approach has a fundamental impact on the achievable LLOQ in combination with the appropriate LC-MS configuration. Several options for IA-MS LC configurations exist (Fig. 2).Several options for IA-MS LC configurations exist (Fig. 2). Matching sample cleanliness with the column-loading capacities of the LC-MS configuration provides optimal assay sensitivity while balancing analysis time, column longevity, and matrix suppression. LC gradients having a high slope are often used but may not provide adequate time to separate analytes from coeluting matrix species when using smaller diameter columns, causing a reduction in sensitivity due to isobaric interferences and ion suppression. Ultra–high-performance LC provides improved sensitivity, selectivity, and reduced analysis times compared with HPLC by providing higher resolution separations and less matrix coelution with analytes. Therefore, implementing ultra–high-performance LC separations for low-flow LC-MS can offset the typically longer chromatographic run times associated with the technique. Most often, a trap column is used for analyte preconcentration in combination with capillary flow and nanoflow analytical columns. Generally, the trap column diameter is larger than the analytical separation column to enhance sample-loading capacities. The use of a trap column allows for larger sample-injection volumes while minimizing injection times with the use of higher flow rates. An illustration of the above concepts is given in Fig. 3 which shows online antipeptide IA integrated with trap and nano flow analytical columns for high sensitivity application.

The sequential affinity capture workflow (Fig. 1, C) provides the most sensitive approach for IA-MS because it allows for maximal analyte enrichment. The use of an antipeptide antibody enables specific enrichment of analytes that provides a less complex sample and more optimal mass load for smaller diameter columns. The reduced sample complexity also ensures that low-flow columns and the MS ion source are less susceptible to contamination. A less complex sample reduces ion suppression and improves overall assay robustness while optimally balancing sensitivity and analysis time by enabling the use of steeper separation gradients.

Table 2. Flow rate schemes used for IA-LC-MS/MS.

Chromatography	Column Diameter (mm)	Flow Rate (μl/min)	References
Nano LC	< 0.1	< 1	(44, 59, 60, 70, 72, 120)
Capillary LC	0.1–0.3	1–10	(10, 47)
Micro LC	0.5–1.0	20–100	(108)
Analytical LC	2.1–4.6	300–1000	(11, 15, 46)

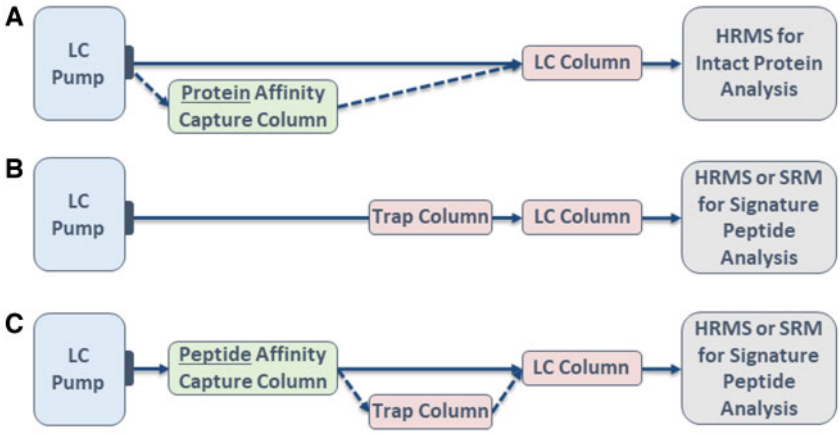


Fig. 2. Protein and peptide affinity capture LC-MS workflows.

Workflow (A) is used for intact protein MS analysis using HRMS with the protein antibody column optionally used to increase selectivity and column loading capacity. Workflow (B) is used for peptide MS analysis using either HRMS or QqQ-SRM-MS. Workflow (C) is used for sequential affinity capture in combination with microflow to nanoflow LC-MS to achieve the highest selectivity, sensitivity, and robustness by incorporating selective surrogate peptide capture using an antipeptide antibody column in line with LC-MS. A trap column is used with capillary flow and nanoflow LC-MS to preconcentrate the eluate from the antibody column before elution to the analytical column.

The triple-quadrupole (QqQ) mass spectrometer remains the primary MS system for targeted quantification operating in selected reaction monitoring (SRM) mode, sometimes also referred to as *multiple reaction monitoring* when multiple product ions from one or more precursor ions are measured. Most often, multiple SRMs are acquired for each target analyte where typically one of the SRMs is used to quantify the analyte while others are used for qualitative confirmation. Monitoring of the response ratios of the SRMs within and between samples may be used to identify potential interference caused by coeluting components that produce an overlapping SRM response.

QqQ instruments are predominately operated at nominal mass resolution. However, nominal mass measurement of either precursor or product ions in complex sample matrices can be susceptible to interference (121, 122). Increasing quadrupole resolution can be considered to reduce any interference. Furthermore, ion mobility can be combined with SRM detection to enhance selectivity for target analytes (123–125).

HRMS is the default approach for proteomics and is finding increased application for IA-MS protein biomarker assays. Two instrumental configurations are used to provide HRMS analysis. Quadrupole time-of-flight (Q-ToF) instruments typically provide mass resolution in the range of 20 000 to 50 000. A second instrument type, known as the *orbitrap*, can provide mass resolution as high as 10^6; however, typical operating resolutions for quantification are 20 000 to 120 000. QqQ is thought to have higher sensitivity than HRMS for peptide quantification owing to a greater duty cycle for SRM detection (126), which refers to the fraction of analyte ions entering the mass analyzer optics that are detected. However, protein biomarker IA-MS methods based on SRM can be "selectivity challenged", owing to the

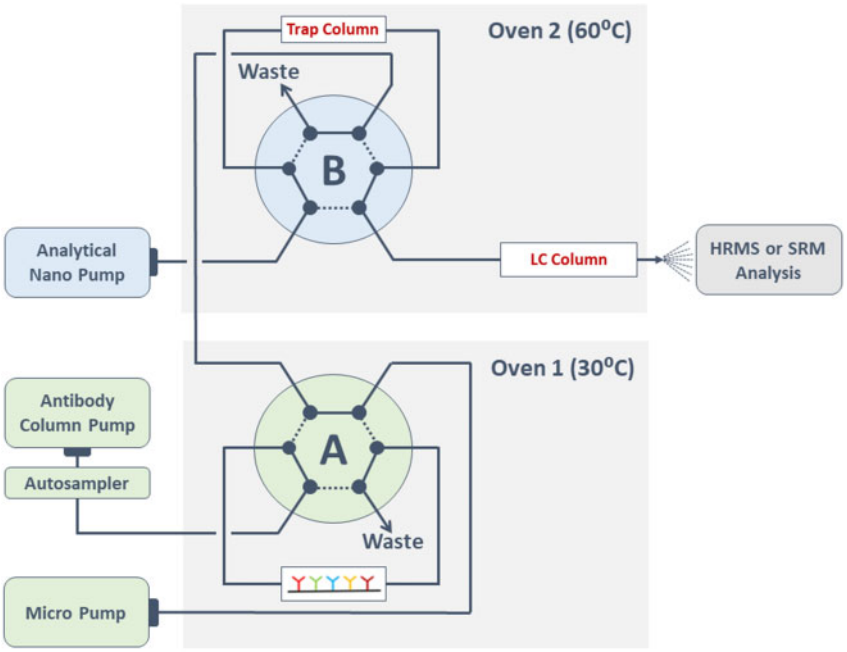


Fig. 3. An example fluidic configuration used for an online immunoaffinity capture LC-MS workflow.

This workflow includes 3 pumps, 3 columns, and two 10-port valves in ovens. A loading pump connected with an autosampler delivers a sample volume to an antibody column containing ≥ 1 antipeptide antibodies, which are covalently bound to a solid support bead. The oven temperature is optimized for peptide-antibody capture efficiency. After appropriate washing of the sample on the antibody column, Valve A changes states to elute the captured peptide(s) using a micropump to a trap column. After appropriate washing of the sample on the trap column, Valve B changes states, which places the trap column in line with an analytical column (either capillary or nano) for LC-MS analysis. The temperature of column Oven 1 is optimized for maximized peptide affinity capture, typically performed at room temperature or 30 °C, whereas Oven 2 is optimized for chromatographic selectivity and analysis speed (typically 60 °C).

remaining complexity of sample extracts. HRMS scan modes provide significant selectivity improvements that can offset any QqQ sensitivity benefits with the potential to have a simpler sample-preparation method. To increase the fraction of ions detected, orbitraps incorporate an ion trap between the ion source and the orbitrap analyzer. This ion trap accumulates and stores ions while previously trapped ions are being mass analyzed in the orbitrap analyzer. This approach improves both duty cycle and sensitivity. For orbitraps, the dominant MS/MS scan mode used for quantification is parallel reaction monitoring, a technique that records full product ion spectra for a targeted precursor ion. Parallel reaction monitoring allows the most sensitive and selective transitions to be chosen for quantification after acquisition *(127)*. Furthermore, with access to high mass resolution, investigators have recently sought to gain high selectivity with mass resolution alone (i.e., without MS/MS). This proposition, shown in a recent article by Cox and coworkers *(50)*, demonstrated the utility of IA-MS with orbitrap analysis (70 000 mass resolution) to quantify oxyntomodulin in human plasma (LLOQ, 7.8 pg/mL). A recent article on hemoglobinopathy detection utilized Fourier transform ion cyclotron resonance MS of red blood cell proteins *(32)*. The top-down MS/MS methods used both precursor and product ion scans to correctly identify all hemoglobin variants.

Coupling ion mobility spectrometry with HRMS can further enhance analyte selectivity and sensitivity. A promising recent development based on a Q-ToF platform uses trapped ion mobility spectrometry to perform parallel accumulation–serial fragmentation scans with high sensitivity due to high efficiency of ion utilization *(128)*. Although targeted quantitative protein biomarker applications using the timsTOF Pro instrument have not yet been published, signal-to-noise gains are anticipated to translate to improved sensitivity for protein biomarker assays.

Finally, top-down analysis is increasingly applied by detecting the intact peptide/protein without

digestion (50). Several examples of top-down quantification for biomarkers have now been reported using LC-MS (30, 46, 129–131). A review of intact protein methods by LC-MS puts the current molecular weight ceiling at roughly 20 kDa, depending on the sensitivity needed for analysis (66). An even larger number of top-down IA-MS applications for biomarkers have been reported for MALDI, in which this format was originally pioneered (35, 40, 132, 133).

Implications for Method Development

Optimization of IA-MS assays largely follows the same principles used in traditional IA purification to optimize recovery of the target antigen (protein or peptide) (134) while reducing or eliminating contaminants that can result in matrix effects and isobaric interferences. Generally, conditions in each phase of IA purification are modulated to increase or decrease the stringency (i.e., selectivity of binding/washing), with higher stringency conditions, often resulting in lower recovery but greater selectivity. Although not unique to IA-MS, optimal conditions for proteolytic digestion, which can vary widely between and within proteins, must be determined empirically (107); however, consideration should be given to the downstream effects of digestion on IA enrichment and vice versa. In addition, proteins and peptides are notorious for adsorbing to surfaces, particularly in the absence of sample matrix, which can have deleterious effects during preparation of calibrators and result in apparent loss in analytical sensitivity. As such, it is crucial to evaluate adsorptive loss of the analyte through a contact study, which has been described recently for handling of tryptic peptide calibrators (92) and applies likewise to proteins.

A facile way to evaluate and optimize recovery and matrix effects resulting from IA enrichment is to perform the same processing step in 2 parallel experiments in which the same amount of surrogate analyte (protein or peptide) is either spiked into the sample prior to or just after the IA step (103, 135, 136). This "spike-order" experiment can be used to optimize a single enrichment step by comparing the recovery and matrix effects across a number of different experimental conditions (e.g., time, temperature, pH), although one should be cognizant that the surrogate analyte may not mimic the recovery of the endogenous analyte. Nonetheless, characterizing analyte recovery incurred during each step of an IA-MS workflow (137) can be particularly critical in isolating sources of variation/bias within a workflow, specifically in steps lacking the control of an internal standard. For example, comparing recovery and matrix effects of recombinant standard in surrogate and true matrices can help to assess suitability of the calibration matrix.

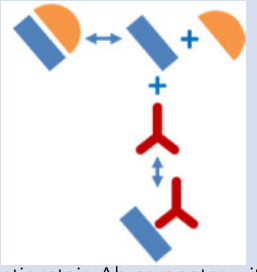
Even with the added selectivity afforded by IA-MS measurements over LBAs, which rely primarily on immunorecognition to infer selectivity, the prevalence of nonspecific binding during IA enrichment can still allow for significant interference (138), and absolute specificity is not guaranteed because isobaric or near-isobaric peptide sequences can interfere with MS/MS detection (139, 140). Consequently, the assay selectivity afforded by the combination of IA enrichment, LC separation, and MS/MS detection should be confirmed empirically. Specifically, the fundamental properties of a peptide's fragmentation pattern can be used in ion ratio (i.e., transition ratio) monitoring, in which the relative intensities of multiple product ions during SRM (or precursor ions during high-resolution mass analysis (7)) are compared between authentic standards and incurred samples to detect the presence of isobaric interferences (141, 142). If isobaric interferences are observed, it is often straightforward to select alternate, interference-free precursor/product ions to quantify the analyte. Alternatively, it may be necessary to reduce nonspecific binding by increasing the IA enrichment wash stringency, pretreating the solid support to block nonspecific binding sites, or adding multiple dimensions of chromatography or enrichment.

Current and Emerging Applications

The pressing need to improve protein biomarker measurement capabilities has led to the creative and innovative use of IA-MS. New IA-MS strategies now enable new protein biomarker applications, which only recently were considered impossible. IA-MS has become an established quantification method and the trend toward increasing utilization is expected to continue. The principal IA workflows described in Fig. 1 can be implemented in different ways (Table 3) depending on the analytical goal, reagent availability, and required assay characteristics such as sensitivity and throughput.

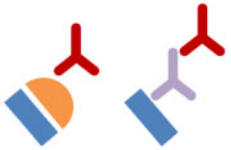
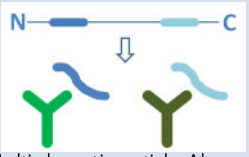
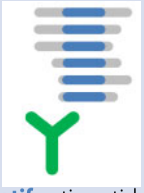
Frequently, a protein IA-MS format provides a good starting point. Such an assay can measure single or multiple peptides (Table 3, A) including peptides from variants or posttranslational modifications (Table 3, B). The IA strategy typically involves a reagent antibody that is insensitive to sequence variants, truncations or modifications but instead binds all proteoforms. Capture antibodies are selected to either purposefully avoid the competition with an endogenous interactor or a binding biotherapeutic (Table 3, C) or to seek it (Table 3, D) to yield either a total or free biomarker measurement (143). Likewise, various formats of co-IA can be established (Table 3, E). In this case, an antibody against a binding partner or a binding biotherapeutic can be used to pull down the protein complex, followed by analysis of peptides from the biomarker and its interactors. Digestion of

Table 3. Practical implementation of key IA-LC-MS/MS workflows enabling pharmaceutical and clinical applications.

	Immunoaffinity strategy	Protein Biomarker Analyte	Peptides measured (when using digestion)	Key Application	Reagent and Assay Considerations	Example References
Protein immunoaffinity capture						
A	 Antiprotein Ab	Total	One or serval proteotypic peptides	Quantify protein biomarker	Binding partners don't exist or antibody can outcompete potential binding partner	(15, 55, 144–146)
B	 Antiprotein Ab against region common to all/selected forms of a protein	All or selected isoforms	One or several peptides from common region and one or serval peptides representing modified regions	Quantify different forms of the protein biomarker	Capture reagent against modified region can also be used for verification or quantification of modified protein biomarker	(47, 48, 57, 147, 148)
C	 Antiprotein Ab does not compete with **binding protein** or **biotherapeutic**	Total (free and bound to binding partner)	One or several peptides from biomarker; may include peptides from binding protein or biologics	Total biomarker irrespective of protein binding; total target engagement assay for protein therapeutics	Co-IA assay can measure target and its binding partner or biologics; lack of competition needs to be confirmed experimentally; can incorporate dissociation step	(51, 149, 150)
D	 Antiprotein Ab competes with **binding protein** or **biotherapeutic (not shown)**	Unbound (free) from binding partner	One or several peptides from biomarker	Free protein biomarker assay; free form of biomarker may be the active form; measures of target suppression following administration of protein therapeutic	Reagent properties assessed and selected to avoid/minimize disruption of preexisting target/ binding partner equilibrium; effect of binding protein/ protein therapeutic needs to be demonstrated	(51, 151)

(continued)

Table 3. (continued)

	Immunoaffinity strategy	Protein Biomarker Analyte	Peptides measured (when using digestion)	Key Application	Reagent and Assay Considerations	Example References
E	 Co-IA; antiprotein Ab binds to **binding protein** or **biotherapeutic** and does not compete with target binding	Binding partner and protein biomarker complexed to binding partner	At least one peptide from binding partner and from protein biomarker	Targeted interaction studies; bound assay for PK/PD; i.e., direct information on target engagement or duplex PK/bound target assay	Likely relative measurement only of the bound protein; ensure washing conditions post capture don't dissociate protein biomarker	(22, 106)
Peptide Immunoaffinity Capture						
F	 Antipeptide Ab	Total	Peptide representing all forms of the protein biomarker, if multiple forms exist	Quantify protein biomarker	Requires complete digestion of sample; can be combined with pellet digestion	(11, 43, 44, 152)
G	 Multiple antipeptide Abs	Total; region specific	Peptides representing different regions of protein biomarker	Correlation of data from ≥ 2 (distant) regions increases confidence in structural integrity of quantified protein biomarker	Can also multiplex to quantify different protein biomarkers	(153)
H	 Antipeptide Ab against multiple forms of a peptide	One or multiple forms	One or multiple peptides including variants, neoepitopes, PTMs, or with covalent modifier	Quantify different forms of the protein biomarker	Good quality antipeptide Ab can be challenging to develop	(154)
I	 Anti-**motif** antipeptide Ab	Family or group of protein biomarker	Multiple peptides, at least one for each target protein	Quantify homologous proteins	Need to develop multispecific Ab	(71, 155, 156)

Ab = antibody; PTM = post-translational modification.

the entire sample followed by enrichment using antipeptide antibodies also provides a sensitive total biomarker assay. This format is insensitive to binding partners, and the biomarker can be measured with a single peptide (Table 3, F) or several peptides (Table 3, G). Antipeptide antibodies can also be used to bind to a constant sequence in a set of related peptides that are either variants or are from a truncated, chemically or posttranslationally modified protein (Table 3, H) or from a family of proteins that share a common motif (Table 3, I).

Recent IA-MS applications in the clinical diagnostics lab have mainly focused on total protein quantitation for established biomarkers as a response to deficiencies in the alternative methodologies (typically LBAs) such as lack of selectivity (C-peptide and insulin), interference from autoantibodies/binding partners (thyroglobulin), and/or intermethod variability (parathyroid hormone-related protein) that adversely affects clinical application *(6, 11, 15)*. In each case, IA enrichment has been necessitated by the combined requirements for high sensitivity and high-throughput measurements that would not be readily achievable with conventional extraction modalities (e.g., solid phase extraction). One exemplar application includes the use of antibody enrichment of IgG, IgA, and IgM heavy chains and immunoglobulin λ and κ light chains to identify and quantify monoclonal proteins found in multiple myeloma patients *(16)*. In the past, several analytical techniques were required for isotyping and quantification of monoclonal proteins; however, through the use of IA enrichment, this task is readily achievable in a MALDI-MS workflow.

Pharmaceutical applications of IA-MS address biomarker questions in early clinical drug development, drug discovery, and biology. Many examples have been published for pharmacodynamic or mechanism-of-action biomarkers, targets of biotherapeutics, and toxicity and disease biomarkers. For example, the multiplexed measurement of proglucagon-derived peptides including glucagon-like peptide (GLP) 1-(7–36), GLP-1-(9–36), glucagon, and oxyntomodulin was established using IA-MS *(49)*. These peptides are involved in regulating glucose homeostasis and are examined as pharmacodynamic biomarkers in the development of antidiabetic drugs. This assay provides subnanomolar sensitivity and uses 2 capture antibodies, each not necessarily specific to single analytes, to simultaneously bind unique and shared sequences in all 4 peptides (format shown in Table 3, H) *(49)*.

A key IA-MS application is the quantification of protein targets and target engagement biomarkers in drug development. Although IA-MS is a suitable tool for the measurement of protein targets of all sizes, it can be especially useful for quantifying smaller proteins with limited binding sites for drug, capture, and detection reagents, which may reduce the likelihood for successful LBA development. For example, the protein interferon γ-induced protein-10 (IP-10) is present at low abundance (approximately 30–60 pM), and after dosing with anti-IP-10 monoclonal antibodies, it was expected that free IP-10 would be significantly reduced *(51)*. Therefore, a highly sensitive method (LLOQ, 1 pM) was developed using a competing antibody (format shown in Table 3, D) to measure the magnitude and duration of IP-10 suppression in the presence of high drug concentrations with minimal disruption of the drug-target complex. The selection and careful optimization of reagent capture conditions is critical. Another example is the quantification of total β nerve growth factor (NGF) in human serum following treatment with an anti-NGF biotherapeutic using sequential protein and peptide IA-MS (Fig. 1, C; combined assay format shown in Tables 2, F, and 3, C) *(59)*. A nonneutralizing antibody avoiding competition with the biotherapeutic is used for NGF enrichment. The assay with single-digit picogram-per milliliter sensitivity was used to measure total NGF concentrations in nearly 20 000 samples from clinical studies of an anti-NGF biotherapeutic *(72)*. Such assays have since been developed for other low-abundance cytokines, such as interleukin-21 *(60)*.

The quantification of tissue proteins is an emerging IA-MS application and represents a unique advantage of IA-MS compared with other methods. Although all major IA workflows described in Fig. 1 can be used in principle, the antipeptide antibody approach (Fig. 1, B) can bypass possible limitations associated with the lack of capture efficiency of antiprotein antibodies in tissue lysate *(120)*. The antipeptide antibody method is compatible with harsh, denaturing conditions for extraction including the use of strong detergents to enable high recovery. Such an assay (format shown in Table 3, F) is reported for the quantification of neonatal Fc receptor in human and transgenic mouse tissues to aid with prediction of pharmacokinetic properties of therapeutic antibodies *(70, 157, 158)*.

Other emerging IA-MS applications include the analysis of protein kinetics *(146, 159–162)*, the quantification of protein biomarkers in exosomes *(163)*, and the measurements of transgene protein expression following gene therapy *(164)*.

Gaps and Opportunities

IA-MS technology, despite its power and potential, as well as increasing utilization, is far from mainstream adoption for protein biomarker quantification. Table 4 identifies various gaps and opportunities, both technical and nontechnical in nature, for increased adoption. Primary technical issues include sensitivity, instrumental complexity, and the need for higher throughput. The

Table 4. Gaps in the current state of IA-MS for protein biomarkers and opportunities for future development.

	Gaps	Opportunities
Sensitivity	Improvements in sensitivity needed to measure less abundant markers and/or to accommodate smaller sample volumes.	Biggest gains expected to come from improvements in MS ionization and detection, although sample prep and chromatography also have significant impact.
Selectivity	Matrix complexity limits LLOQ. Use of low-resolution triple quadrupole instrumentation with bottom-up sample prep is dominant and can require extensive method development to overcome background.	Increase use of HRMS and orthogonal separation techniques (ion mobility, multi-dimensional LC) to maximize resolution of complex mixtures.
Throughput	Matrix complexity mandates long LC cycle times and extensive sample preparation. LC cycle times can be particularly long when using capillary LC. Bottom-up methods require additional time for digestion.	Increase use of multiplexing for improved efficiency. IA sample enrichment enables use of shorter LC run times. Reduce LC run times further by using microflow over capillary LC, if sufficient sensitivity. Consider LC removal when possible with alternate ionization (e.g., MALDI). Investigate top-down methods. Employ options for faster digestion. Test shorter IA incubation times.
Robustness–low-flow chromatography	Capillary columns with nano-ESI are more prone to clogging. Extensive sample prep needed. Spray stability at low flow can be challenging.	Continue to improve ion source design to maximize signal at low microliter/min flow rates. Improve overall integration of low-flow LC systems with mass spectrometers across all vendors.
Reagents	Full length protein reference standards are not commutable; SIL proteins are not widely available. Insufficient availability of well characterized capture reagents with a continuous supply.	Reduce sources of inter-laboratory and inter-method error through use of commutable reference standards for calibration and full-length SIL protein for internal standardization.
Analyst availability/ organization	Limited number of scientists experienced in the hybrid science. Broad skill set required. Many pharmaceutical / clinical LBA and LC-MS labs are separated within institutions by both physical and organizational boundaries.	Reach across LBA, MS, proteomics disciplines, cross train workforce to become proficient in IA-MS science. Stronger collaboration of different disciplines to achieve shared learning.
Cost	Expensive instrumentation, relatively low throughput, high reagent costs.	Invest in technologies that reduce complexity. Increase multiplexing, improve throughput and use reusable reagent formats. Justify cost and create demand by delivering high value assays that require IA-MS.

most advanced IA-MS assays already provide sensitivities in single-digit picograms per milliliter. Sensitivity is typically scalable with the input sample volume, which accounts for the large sample volumes (>0.1 mL) often used by IA-MS methods. Although ranking the gaps is difficult with respect to their importance, sustained improvements in sensitivity are vital because they lead to smaller sample volumes, simplified LC methods, improved throughput, and robustness.

The primary nontechnical gaps are the need for more scientists who are proficient using IA-MS technology and the overall cost of implementation. The first issue is largely a function of the diverse skill set required: proteomics, ligand binding, LC-MS, and bioanalysis. Because few analysts are skilled in all of these areas, teams of scientists with complementary strengths are found in successful IA-MS laboratories. Unfortunately, organizational structures in the pharmaceutical industry, CLIA laboratories, and contract research organizations tend to segregate LBA and LC-MS laboratories, preventing facile adoption of hybrid methodology. Because most IA-MS implementation has been driven by LC-MS laboratories, a common gap occurs with respect to the proper use, characterization, and handling of antibody reagents and development of quantitative assays based on LBA principles. LC-MS practitioners would be

well served by approaching their LBA partners for specific training, and we endorse cultivating bioanalysts with fluency in both LBA and LC-MS methods. Other shared learning can be offered by proteomic scientists owing to their proficiency in protein chemistry and capillary LC-MS/MS techniques.

Perhaps the largest obstacle to broader IA-MS adoption is cost, primarily related to the acquisition and maintenance of MS instrumentation. A troubling paradox is that gains in sensitivity and other measures are often accompanied by more complex and costly instrumentation. Innovative solutions must be sought that take both performance and simplicity into account. This aspect is particularly important for CLIA laboratories given the high-throughput and regulated nature of the work. In recent years, QqQ systems have been introduced by multiple instrument vendors and are qualified by the US Food and Drug Administration for specific applications *(165)*. Although small molecule biomarker assays are more prevalent, we anticipate growth in protein applications and endorse increased growth in this trend. Ultimately, adoption of IA-MS protein quantification will be driven by a demonstrated need for performance for high-value applications that only IA-MS can provide. The applications highlighted earlier in this report were selected to illustrate the level of impact possible by IA-MS. Moreover, the routine ability for multiplexed analysis permits high versatility and decreases cost on a per-sample basis.

Conclusions

Increasing IA-MS adoption over the past decade has been driven by improved MS sensitivity, an unparalleled ability of LC to resolve and quantitate complex mixtures, new IA workflows, and improvements in high-resolution MS and in the robustness of low-flow LC methods. Despite increased use of IA-MS for discovery and early stage clinical applications in pharmaceutical development, adoption by CLIA laboratories has proceeded at a slower pace. This report provides an overview of IA-MS applications for protein biomarker analysis, with the aim of achieving greater awareness and increased adoption of IA-MS platforms.

Nonstandard abbreviations: LBA, ligand binding assay; MS, mass spectrometry; SIL, stable isotope-labeled; LC, liquid chromatography; IA, immunoaffinity; MALDI, matrix-assisted laser desorption/ionization; ESI, electrospray ionization; HRMS, high-resolution mass spectrometry; QC, quality control; LLOQ, lower limit of quantification; QqQ, triple quadrupole; SRM, selected reaction monitoring; NGF, nerve growth factor.

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References

1. FDA-NIH Biomarker Working Group. BEST (Biomarkers, EndpointS, and other Tools) Resource; 2016, Bethesda (MD), https://www.ncbi.nlm.nih.gov/books/NBK326791/ (accessed January 2020).
2. US Food and Drug Administration; Drug development tool (DDT) qualification programs. https://www.fda.gov/drugs/development-approval-process-drugs/drug-development-tool-qualification-programs (Accessed May 2017).
3. Biomarker qualification: evidentiary framework guidance for industry and FDA staff; draft guidance. Rockville (MD): US Department of Health and Human Services, Food and Drug Administration; 2018.
4. Hoofnagle AN, Wener MH. The fundamental flaws of immunoassays and potential solutions using tandem mass spectrometry. J Immunol Methods 2009;347:3–11.
5. Ciccimaro E, Blair IA. Stable-isotope dilution LC–MS for quantitative biomarker analysis. Bioanalysis 2010;2:311–41.
6. Taylor SW, Clarke NJ, Chen Z, McPhaul MJ. A high-throughput mass spectrometry assay to simultaneously measure intact insulin and C-peptide. Clin Chim Acta 2016;455:202–8.
7. Bystrom CE, Sheng S, Clarke NJ. Narrow mass extraction of time-of-flight data for quantitative analysis of proteins: determination of insulin-like growth factor-1. Anal Chem 2011;83:9005–10.
8. Topbas C, Swick A, Razavi M, Leigh Anderson N, Pearson TW, Bystrom C. Measurement of lipoprotein-associated phospholipase A2 by use of 3 different methods: exploration of discordance between ELISA and activity assays. Clin Chem 2018;64:697–704.
9. van den Broek I, Romijn F, Nouta J, Van Der Laarse A, Drijfhout JW, Smit NPM. Automated multiplex LC-MS/MS assay for quantifying serum apolipoproteins A-I, B, C-I, C-II, C-III, and E with qualitative apolipoprotein E phenotyping. Clin Chem 2016;62:188–97.
10. Hoofnagle AN, Becker JO, Wener MH, Heinecke JW. Quantification of thyroglobulin, a low-abundance serum protein, by immunoaffinity peptide enrichment and tandem mass spectrometry. Clin Chem 2008;54:1796–804.
11. Kushnir MM, Rockwood AL, Roberts WL, Abraham D, Hoofnagle AN, Meikle AW. Measurement of thyroglobulin by liquid chromatography-tandem mass spectrometry in serum and plasma in the presence of antithyroglobulin autoantibodies. Clin Chem 2013;59:982–90.

12. Bystrom CE, Salameh W, Reitz R, Clarke NJ. Plasma renin activity by LC-MS/MS: development of a prototypical clinical assay reveals a subpopulation of human plasma samples with substantial peptidase activity. Clin Chem 2010;56:1561–9.
13. Bystrom C, Sheng S, Zhang K, Caulfield M, Clarke NJ, Reitz R. Clinical utility of insulin-like growth factor 1 and 2; determination by high resolution mass spectrometry. PLoS One 2012;7:e43457–7.
14. Jin M, Cataland S, Bissell M, Wu HM. A rapid test for the diagnosis of thrombotic thrombocytopenic purpura using surface enhanced laser desorption/ionization time-of-flight (SELDI-TOF)-mass spectrometry. J Thromb Haemost 2006;4:333–8.
15. Kushnir MM, Rockwood AL, Strathmann FG, Frank EL, Straseski JA, Meikle AW. LC-MS/MS measurement of parathyroid hormone-related peptide. Clin Chem 2016;62:218–26.
16. Mills JR, Kohlhagen MC, Dasari S, Vanderboom PM, Kyle RA, Katzmann JA, et al. Comprehensive assessment of M-proteins using nanobody enrichment coupled to MALDI-TOF mass spectrometry. Clin Chem 2016;62:1334–44.
17. Ladwig PM, Barnidge DR, Snyder MR, Katzmann JA, Murray DL. Quantification of serum IgG subclasses by use of subclass-specific tryptic peptides and liquid chromatography-tandem mass spectrometry. Clin Chem 2014;60:1080–8.
18. van der Gugten G, DeMarco ML, Chen LYC, Chin A, Carruthers M, Holmes DT, et al. Resolution of spurious immunonephelometric IgG subclass measurement discrepancies by LC-MS/MS. Clin Chem 2018;64:735–42.
19. Henderson CM, Lutsey PL, Misialek JR, Laha TJ, Selvin E, Eckfeldt JH, et al. Measurement by a novel LC-MS/MS methodology reveals similar serum concentrations of vitamin D-binding protein in blacks and whites. Clin Chem 2016;62:179–87.
20. Li X-J, Hayward C, Fong P-Y, Dominguez M, Hunsucker SW, Lee LW, et al. A blood-based proteomic classifier for the molecular characterization of pulmonary nodules. Sci Transl Med 2013;5:207ra142.
21. Silvestri GA, Tanner NT, Kearney P, Vachani A, Massion PP, Porter A, et al. Assessment of plasma proteomics biomarker's ability to distinguish benign from malignant lung nodules. Chest 2018;154:491–500.
22. Collier TS, Jin Z, Topbas C, Bystrom C. Rapid affinity enrichment of human apolipoprotein A-I associated lipoproteins for proteome analysis. J Proteome Res 2018;17:1183–93.
23. Jin Z, Collier TS, Dai DLY, Chen V, Hollander Z, Ng RT, et al. Development and validation of apolipoprotein AI-associated lipoprotein proteome panel for the prediction of cholesterol efflux capacity and coronary artery disease. Clin Chem 2019;65:282–90.
24. Leinenbach A, Pannee J, Dülffer T, Huber A, Bittner T, Andreasson U, et al. Mass spectrometry-based candidate reference measurement procedure for quantification of amyloid-β in cerebrospinal fluid. Clin Chem 2014;60:987–94.
25. Jeppsson J-O, Kobold U, Barr J, Finke A, Hoelzel W, Hoshino T, et al. Approved IFCC reference method for the measurement of HbA_{1c} in human blood. Clin Chem Lab Med 2002;40:78–89.
26. Lame ME, Chambers EE, Blatnik M. Quantitation of amyloid beta peptides Aβ 1-38, Aβ 1-40, and Aβ 1-42 in human cerebrospinal fluid by ultra-performance liquid chromatography-tandem mass spectrometry. Anal Biochem 2011;419:133–9.
27. Howard JW, Kay RG, Tan T, Minnion J, Ghatei M, Bloom S, et al. Development of a high-throughput UHPLC–MS/MS (SRM) method for the quantitation of endogenous glucagon from human plasma. Bioanalysis 2014;6:3295–309.
28. Laarakkers CMM, Wiegerinck ET, Klaver S, Kolodziejczyk M, Gille H, Hohlbaum AM, et al. Improved mass spectrometry assay for plasma hepcidin: detection and characterization of a novel hepcidin isoform. PLoS One 2013;8:e75518.
29. Vialaret J, Picas A, Delaby C, Bros P, Lehmann S, Hirtz C. Nano-flow vs standard-flow: which is the more suitable LC/MS method for quantifying hepcidin-25 in human serum in routine clinical settings? J Chromatogr B 2018;1086:110–7.
30. Chambers EE, Fountain KJ, Smith N, Ashraf L, Karalliedde J, Cowan D, et al. Multidimensional LC-MS/MS enables simultaneous quantification of intact human insulin and five recombinant analogs in human plasma. Anal Chem 2014;86:694–702.
31. Kay R, Barton C, Ratcliffe L, Matharoo-Ball B, Brown P, Roberts J, et al. Enrichment of low molecular weight serum proteins using acetonitrile precipitation for mass spectrometry based proteomic analysis. Rapid Commun Mass Spectrom 2008;22:3255–60.
32. He L, Rockwood AL, Agarwal AM, Anderson LC, Weisbrod CR, Hendrickson CL, et al. Diagnosis of hemoglobinopathy and β-thalassemia by 21 tesla Fourier transform ion cyclotron resonance mass spectrometry and tandem mass spectrometry of hemoglobin from blood. Clin Chem 2019;65:986–94.
33. Croyal M, Fall F, Krempf M, Thédrez A, Ouguerram K, Ferchaud-Roucher V, et al. Plasma PCSK9 measurement by liquid chromatography–tandem mass spectrometry and comparison with conventional ELISA. J Chromatogr B 2017;1044–1045:24–9.
34. Nedelkov D, Niederkofler EE, Oran PE, Peterman S, Nelson RW. Top-down mass spectrometric immunoassay for human insulin and its therapeutic analogs. J Proteomics 2018;175:27–33.
35. Oran PE, Trenchevska O, Nedelkov D, Borges CR, Schaab MR, Rehder DS, et al. Parallel workflow for high-throughput (>1,000 samples/day) quantitative analysis of human insulin-like growth factor 1 using mass spectrometric immunoassay. PLoS One 2014;9:e92801.
36. Razavi M, Johnson LDS, Lum JJ, Kruppa G, Anderson NL, Pearson TW. Quantification of a proteotypic peptide from protein C inhibitor by liquid chromatography-free SISCAPA-MALDI mass spectrometry: application to identification of recurrence of prostate cancer. Clin Chem 2013;59:1514–22.
37. Niederkofler EE, Tubbs KA, Gruber K, Nedelkov D, Kiernan UA, Williams P, et al. Determination of β-2 microglobulin levels in plasma using a high-throughput mass spectrometric immunoassay system. Anal Chem 2001;73:3294–9.
38. Kiernan UA, Tubbs KA, Nedelkov D, Niederkofler EE, Nelson RW. Comparative phenotypic analyses of human plasma and urinary retinol binding protein using mass spectrometric immunoassay. Biochem Biophys Res Commun 2002;297:401–5.
39. Trenchevska O, Schaab MR, Nelson RW, Nedelkov D. Development of multiplex mass spectrometric immunoassay for detection and quantification of apolipoproteins C-I, C-II, C-III and their proteoforms. Methods 2015;81:86–92.
40. Niederkofler EE, Kiernan UA, O'Rear J, Menon S, Saghir S, Protter AA, et al. Detection of endogenous B-type natriuretic peptide at very low concentrations in patients with heart failure. Circ Heart Fail 2008;1:258–64.
41. Sherma ND, Borges CR, Trenchevska O, Jarvis JW, Rehder DS, Oran PE, et al. Mass spectrometric immunoassay for the qualitative and quantitative analysis of the cytokine macrophage migration inhibitory factor (MIF). Proteome Sci 2014;12:52.
42. Xu Q, Zhu M, Yang T, Xu F, Liu Y, Chen Y. Quantitative assessment of human serum transferrin receptor in breast cancer patients pre- and post-chemotherapy using peptide immunoaffinity enrichment coupled with targeted proteomics. Clin Chim Acta 2015;448:118–23.
43. Razavi M, Anderson NL, Yip R, Pope ME, Pearson TW. Multiplexed longitudinal measurement of protein biomarkers in DBS using an automated SISCAPA workflow. Bioanalysis 2016;8:1597–609.
44. Neubert H, Gale J, Muirhead D. Online high-flow peptide immunoaffinity enrichment and nanoflow LC-MS/MS: assay development for total salivary pepsin/pepsinogen. Clin Chem 2010;56:1413–23.
45. Krastins B, Prakash A, Sarracino DA, Nedelkov D, Niederkofler EE, Kiernan UA, et al. Rapid development of sensitive, high-throughput, quantitative and highly selective mass spectrometric targeted immunoassays for clinically important proteins in human plasma and serum. Clin Biochem 2013;46:399–410.
46. Oe T, Ackermann BL, Inoue K, Berna MJ, Garner CO, Gelfanova V, et al. Quantitative analysis of amyloidβ peptides in cerebrospinal fluid of Alzheimer's disease patients by immunoaffinity purification and stable isotope dilution liquid chromatography/negative electrospray ionization tandem mass spectrometry. Rapid Commun Mass Spectrom 2006;20:3723–35.
47. McAvoy T, Lassman ME, Spellman DS, Ke Z, Howell BJ, Wong O, et al. Quantification of tau in cerebrospinal fluid by immunoaffinity enrichment and tandem mass spectrometry. Clin Chem 2014;60:683–9.
48. Chappell DL, Lee AY, Bernstein HS, Lassman ME, Laterza OF. Development and validation of an IA-LC/MS method to quantitate active and total B-type natriuretic peptide in human plasma. Bioanalysis 2016;8:2341–9.
49. Lee AYH, Chappell DL, Bak MJ, Judo M, Liang L, Churakova T, et al. Multiplexed quantification of proglucagon-derived peptides by immunoaffinity enrichment and tandem mass spectrometry after a meal tolerance test. Clin Chem 2016;62:227–35.
50. Cox JM, Berna MJ, Jin Z, Cox AL, Sloop KW, Gutierrez JA, et al. Characterization and quantification of oxyntomodulin in human and rat plasma using high-resolution accurate mass LC–MS. Bioanalysis 2016;8:1579–95.
51. Zhang H, Gu H, Shipkova P, Ciccimaro E, Sun H, Zhao Q, et al. Immunoaffinity LC-MS/MS for quantitative determination of a free and total protein target as a target engagement biomarker. Bioanalysis 2017;9:1573–88.
52. Gupta V, Lassman ME, McAvoy T, Lee AY, Chappell DL, Laterza OF. An evaluation of an aptamer for use as an affinity reagent with MS: PCSK9 as an example protein. Bioanalysis 2016;8:1557–64.
53. Torsetnes SB, Levernæs MS, Broughton MN, Paus E, Halvorsen TG, Reubsaet L. Multiplexing determination of small cell lung cancer biomarkers and their isovariants in serum by immunocapture LC-MS/MS. Anal Chem 2014;86:6983–92.
54. Callipo L, Caruso G, Foglia P, Gubbiotti R, Samperi R, Laganà A. Immunoprecipitation on magnetic beads and liquid chromatography–tandem mass spectrometry for carbonic anhydrase II quantification in human serum. Anal Biochem 2010;400:195–202.
55. Chappell DL, McAvoy T, Weiss B, Weiner R, Laterza OF. Development and validation of an ultra-sensitive

method for the measurement of plasma renin activity in human plasma via LC–MS/MS. Bioanalysis 2012;4: 2843–50.
56. Guo L, Wang Q, Weng L, Hauser LA, Strawser CJ, Rocha AG, et al. Liquid chromatography-high resolution mass spectrometry analysis of platelet frataxin as a protein biomarker for the rare disease Friedreich's ataxia. Anal Chem 2018;90:2216–23.
57. Weng L, Guo L, Vachani A, Mesaros C, Blair IA. Quantification of serum high mobility group box 1 by liquid chromatography/high-resolution mass spectrometry: implications for its role in immunity, inflammation, and cancer. Anal Chem 2018;90:7552–60.
58. Zhao Y, Liu G, Kwok S, Jones BR, Liu J, Marchisin D, et al. Highly selective and sensitive measurement of active forms of FGF21 using novel immunocapture enrichment with LC–MS/MS. Bioanalysis 2018;10:23–33.
59. Neubert H, Muirhead D, Kabir M, Grace C, Cleton A, Arends R. Sequential protein and peptide immunoaffinity capture for mass spectrometry-based quantification of total human β-nerve growth factor. Anal Chem 2013;85:1719–26.
60. Palandra J, Finelli A, Zhu M, Masferrer J, Neubert H. Highly specific and sensitive measurements of human and monkey interleukin 21 using sequential protein and tryptic peptide immunoaffinity LC-MS/MS. Anal Chem 2013;85:5522–9.
61. Keshishian H, Addona T, Burgess M, Kuhn E, Carr SA. Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution. Mol Cell Proteomics 2007;6: 2212–29.
62. Nelson RW, Krone JR, Bieber AL, Williams P. Mass spectrometric immunoassay. Anal Chem 1995;67: 1153–8.
63. Ackermann BL, Berna MJ. Coupling immunoaffinity techniques with MS for quantitative analysis of low-abundance protein biomarkers. Expert Rev Proteomics 2007;4:175–86.
64. Stevenson L, Garofolo F, DeSilva B, Dumont I, Martinez S, Rocci M, et al. 2013 White paper on recent issues in bioanalysis: "hybrid"–the best of LBA and LCMS. Bioanalysis 2013;5:2903–18.
65. Jenkins R, Duggan JX, Aubry A-F, Zeng J, Lee JW, Cojocaru L, et al. Recommendations for validation of LC-MS/MS bioanalytical methods for protein biotherapeutics. AAPS J 2015;17:1–16.
66. van den Broek I, van Dongen WD. LC–MS-based quantification of intact proteins: perspective for clinical and bioanalytical applications. Bioanalysis 2015;7:1943–58.
67. Anderson NL, Anderson NG, Haines LR, Hardie DB, Olafson RW, Pearson TW. Mass spectrometric quantitation of peptides and proteins using stable isotope standards and capture by anti-peptide antibodies (SISCAPA). J Proteome Res 2004;3:235–44.
68. van den Broek I, Nouta J, Razavi M, Yip R, Bladergroen MR, Romijn F, et al. Quantification of serum apolipoproteins A-I and B-100 in clinical samples using an automated SISCAPA–MALDI-TOF-MS workflow. Methods 2015;81:74–85.
69. Rifai N, Gillette MA, Carr SA. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. Nat Biotechnol 2006;24:971–83.
70. Fan Y-Y, Neubert H. Quantitative analysis of human neonatal Fc receptor (FcRn) tissue expression in transgenic mice by online peptide immuno-affinity LC-HRMS. Anal Chem 2016;88:4239–47.
71. Weiß F, Hammer HS, Klein K, Planatscher H, Zanger UM, Norén A, et al. Direct quantification of cytochromes P450 and drug transporters–a rapid, targeted mass spectrometry-based immunoassay panel for tissues and cell culture lysates. Drug Metab Dispos 2018;46: 387–96.
72. Schultz GA, McCardle K, Neubert H. Large-scale implementation of sequential protein and peptide immunoaffinity enrichment LC/nanoLC–MS/MS for human β-nerve growth factor. Bioanalysis 2016;8:753–64.
73. Neubert H, Clark TH, Deng S, Hurst SI. Serum β-nerve growth factor concentrations in pregnant female, nonpregnant female, and male cynomolgus monkeys. Neuroreport 2014;25:829–32.
74. Cowan KJ, Amaravadi L, Cameron MJ, Fink D, Jani D, Kamat M, et al. Recommendations for selection and characterization of protein biomarker assay calibrator material. AAPS J 2017;19:1550–63.
75. Arnold ME, Booth B, King L, Ray C. Workshop report: crystal city VI–bioanalytical method validation for biomarkers. AAPS J 2016;18:1366–72.
76. Grant RP, Hoofnagle AN. From lost in translation to paradise found: enabling protein biomarker method transfer by mass spectrometry. Clin Chem 2014;60:941–4.
77. Bioanalytical method validation guidance for industry. Silver Springs (MD): US Department for Health and Human Services, Food and Drug Administration; 2018.
78. Critical Path Institute (C-Path); Piccoli SP, Sauer JM; Biomarker Assay Collaborative Evidentiary Considerations Writing Group; Points to consider document: scientific and regulatory considerations for the analytical validation of assays used in the qualification of biomarkers in biological matrices. https://c-path.org/wp-content/uploads/2019/06/EvidConsid-WhitePaper-AnalyticalSectionV20190621.pdf. Tucson, AZ (Accessed January 2020).
79. Jones BR, Schultz GA, Eckstein JA, Ackermann BL. Surrogate matrix and surrogate analyte approaches for definitive quantitation of endogenous biomolecules. Bioanalysis 2012;4:2343–56.
80. Lee JW, Devanarayan V, Barrett YC, Weiner R, Allinson J, Fountain S, et al. Fit-for-purpose method development and validation for successful biomarker measurement. Pharm Res 2006;23:312–28.
81. Stevenson LF, Purushothama S. Parallelism: considerations for the development, validation and implementation of PK and biomarker ligand-binding assays. Bioanalysis 2014;6:185–98.
82. King LE. Parallelism experiments in biomarker ligand-binding assays to assess immunological similarity. Bioanalysis 2016;8:2387–91.
83. Tu J, Bennett P. Parallelism experiments to evaluate matrix effects, selectivity and sensitivity in ligand-binding assay method development: pros and cons. Bioanalysis 2017;9:1107–22.
84. Jian W, Edom R, Weng N, Zannikos P, Zhang Z, Wang H. Validation and application of an LC-MS/MS method for quantitation of three fatty acid ethanolamides as biomarkers for fatty acid hydrolase inhibition in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci 2010;878:1687–99.
85. Liu L, Cui Z, Deng Y, Dean B, Hop C, Liang X. Surrogate analyte approach for quantitation of endogenous NAD+ in human acidified blood samples using liquid chromatography coupled with electrospray ionization tandem mass spectrometry. J Chromatogr B 2016; 1011:69–76.
86. Ongay S, Hendriks G, Hermans J, van den Berge M, ten Hacken NHT, van de Merbel NC, et al. Quantification of free and total desmosine and isodesmosine in human urine by liquid chromatography tandem mass spectrometry: a comparison of the surrogate-analyte and the surrogate-matrix approach for quantitation. J Chromatogr A 2014;1326:13–9.
87. Smit NPM, Romijn F, van den Broek I, Drijfhout JW, Haex M, van der Laarse A, et al. Metrological traceability in mass spectrometry-based targeted protein quantitation: a proof-of-principle study for serum apolipoproteins A-I and B100. J Proteomics 2014;109: 143–61.
88. Shuford CM, Walters JJ, Holland PM, Sreenivasan U, Askari N, Ray K, et al. Absolute protein quantification by mass spectrometry: not as simple as advertised. Anal Chem 2017;89:7406–15.
89. Geist BJ, Egan AC, Yang T-Y, Dong Y, Shankar G. Characterization of critical reagents in ligand-binding assays: enabling robust bioanalytical methods and lifecycle management. Bioanalysis 2013;5:227–44.
90. King LE, Farley E, Imazato M, Keefe J, Khan M, Ma M, et al. Ligand binding assay critical reagents and their stability: recommendations and best practices from the global bioanalysis consortium harmonization team. AAPS J 2014;16:504–15.
91. Santockyte R, Zeng J, Zheng N. Critical reagent screening and characterization: benefits and approaches for protein biomarker assays by hybrid LC–MS. Bioanalysis 2019;11:785–95.
92. Hoofnagle AN, Whiteaker JR, Carr SA, Kuhn E, Liu T, Massoni SA, et al. Recommendations for the generation, quantification, storage, and handling of peptides used for mass spectrometry-based assays. Clin Chem 2015;62:48–69.
93. Scott KB, Turko IV, Phinney KW. Quantitative performance of internal standard platforms for absolute protein quantification using multiple reaction monitoring-mass spectrometry. Anal Chem 2015;87:4429–35.
94. van den Broek I, Smit NPM, Romijn F, Van Der Laarse A, Deelder AM, Van Der Burgt YEM, et al. Evaluation of interspecimen trypsin digestion efficiency prior to multiple reaction monitoring-based absolute protein quantification with native protein calibrators. J Proteome Res 2013;12:5760–74.
95. Whiteaker JR, Zhao L, Frisch C, Ylera F, Harth S, Knappik A, et al. High-affinity recombinant antibody fragments (Fabs) can be applied in peptide enrichment immuno-MRM assays. J Proteome Res 2014;13: 2187–96.
96. Duo J, Chiriac C, Huang RYC, Mehl J, Chen G, Tymiak A, et al. Slow off-rate modified aptamer (SOMAmer) as a novel reagent in immunoassay development for accurate soluble glypican-3 quantification in clinical samples. Anal Chem 2018;90:5162–70.
97. Zhang H, Yan W, Aebersold R. Chemical probes and tandem mass spectrometry: a strategy for the quantitative analysis of proteomes and subproteomes. Curr Opin Chem Biol 2004;8:66–75.
98. Discenza LN, Cornelius G, Gan J, Szapiel N, Talbott RL, Chaudhry C, et al. Ultrasensitive quantitative LC–MS/MS of an inhibitor of apoptosis protein's antagonist in plasma using protein target affinity extraction. Bioanalysis 2016;8:265–74.
99. de Graaf EL, Giansanti P, Altelaar AFM, Heck A. Single-step enrichment by Ti4+-IMAC and label-free quantitation enables in-depth monitoring of phosphorylation dynamics with high reproducibility and temporal resolution. Mol Cell Proteomics 2014;13:2426–34.
100. Villén J, Gygi SP. The SCX/IMAC enrichment approach for global phosphorylation analysis by mass spectrometry. Nat Protoc 2008;3:1630.

101. He X, Dembo M, Goldstein B, Myszka DG, Morton TA. Extending the range of rate constants available from BIACORE: interpreting mass transport-influenced binding data. Biophys J 2009;75:583–94.

102. Li J, Wei H, Krystek SR, Bond D, Brender TM, Cohen D, et al. Mapping the energetic epitope of an antibody/interleukin-23 interaction with hydrogen/deuterium exchange, fast photochemical oxidation of proteins mass spectrometry, and alanine shave mutagenesis. Anal Chem 2017;89:2250–8.

103. Duggan J, Ren B, Mao Y, Chen L-Z, Philip E. LC–MS quantification of protein drugs: validating protein LC–MS methods with predigestion immunocapture. Bioanalysis 2016;8:1951–64.

104. Lee JW. Method validation and application of protein biomarkers: basic similarities and differences from biotherapeutics. Bioanalysis 2009;1:1461–74.

105. Lee JW, Kelley M, King LE, Yang J, Salimi-Moosavi H, Tang MT, et al. Bioanalytical approaches to quantify "total" and "free" therapeutic antibodies and their targets: technical challenges and PK/PD applications over the course of drug development. AAPS J 2011;13:99–110.

106. Palandra J, Quazi A, Fitz L, Rong H, Morris C, Neubert H. Quantitative measurements of GDF-8 using immunoaffinity LC-MS/MS. Proteomics Clin Appl 2016;10:597–604.

107. Proc JL, Kuzyk MA, Hardie DB, Yang J, Smith DS, Jackson AM, et al. A quantitative study of the effects of chaotropic agents, surfactants, and solvents on the digestion efficiency of human plasma proteins by trypsin. J Proteome Res 2010;9:5422–37.

108. Ippoliti PJ, Kuhn E, Mani DR, Fagbami L, Keshishian H, Burgess MW, et al. Automated microchromatography enables multiplexing of immunoaffinity enrichment of peptides to greater than 150 for targeted MS-based assays. Anal Chem 2016;88:7548–55.

109. Ciccimaro E, Zhu Y, Ostanin D, Suchard S, Macguire J, Xiao Q, et al. Antibody drug-target engagement measurement in tissue using quantitative affinity extraction liquid chromatography-mass spectrometry: method development and qualification. Anal Chem 2017;89:5115–23.

110. Yassine H, Borges CR, Schaab MR, Billheimer D, Stump C, Reaven P, et al. Mass spectrometric immunoassay and MRM as targeted MS-based quantitative approaches in biomarker development: potential applications to cardiovascular disease and diabetes. Proteomics Clin Appl 2013;7:528–40.

111. Russell J. As sample prep improves, so does mass spec. Genet Eng Biotechnol News 2012;32:18–22.

112. Samarasinghe S, Meah F, Singh V, Basit A, Emanuele N, Emanuele MA, et al. Biotin interference with routine clinical immunoassays: understand the causes and mitigate the risks. Endocr Pract 2017;23:989–98.

113. Berna M, Schmalz C, Duffin K, Mitchell P, Chambers M, Ackermann B. Online immunoaffinity liquid chromatography/tandem mass spectrometry determination of a type II collagen peptide biomarker in rat urine: Investigation of the impact of collision-induced dissociation fluctuation on peptide quantitation. Anal Biochem 2006;356:235–43.

114. Nemirovskiy OV, Dufield DR, Sunyer T, Aggarwal P, Welsch DJ, Mathews WR. Discovery and development of a type II collagen neoepitope (TIINE) biomarker for matrix metalloproteinase activity: from in vitro to in vivo. Anal Biochem 2007;361:93–101.

115. Giansanti P, Tsiatsiani L, Low TY, Heck A. Six alternative proteases for mass spectrometry–based proteomics beyond trypsin. Nat Protoc 2016;11:993–1006.

116. Levernæs MCS, Broughton MN, Reubsaet L, Halvorsen TG. To elute or not to elute in immunocapture bottom-up LC–MS. J Chromatogr B Analyt Technol Biomed Life Sci 2017;1055–1056:51–60.

117. Toth CA, Kuklenyik Z, Jones JI, Parks BA, Gardner MS, Schieltz DM, et al. On-column trypsin digestion coupled with LC-MS/MS for quantification of apolipoproteins. J Proteomics 2017;150:258–67.

118. Kuzyk MA, Smith D, Yang J, Cross TJ, Jackson AM, Hardie DB, et al. Multiple reaction monitoring-based, multiplexed, absolute quantitation of 45 proteins in human plasma. Mol Cell Proteomics 2009;8:1860–77.

119. Parker CE, Borchers CH. Mass spectrometry based biomarker discovery, verification, and validation: quality assurance and control of protein biomarker assays. Mol Oncol 2014;8:840–58.

120. Neubert H, Fan Y-Y, Fernandez Ocaña M. Quantification of protein biomarkers in tissues: new capabilities with pellet digestion peptide immunoaffinity LC–MS/MS. Bioanalysis 2016;8:1551–5.

121. Duan X, Dai L, Chen S-C, Balthasar JP, Qu J. Nano-scale liquid chromatography/mass spectrometry and on-the-fly orthogonal array optimization for quantification of therapeutic monoclonal antibodies and the application in preclinical analysis. J Chromatogr A 2012;1251:63–73.

122. Duan X, Abuqayyas L, Dai L, Balthasar JP, Qu J. High-throughput method development for sensitive, accurate, and reproducible quantification of therapeutic monoclonal antibodies in tissues using orthogonal array optimization and nano liquid chromatography/selected reaction monitoring mass spectrometry. Anal Chem 2012;84:4373–82.

123. Burnum-Johnson KE, Nie S, Casey CP, Monroe ME, Orton DJ, Ibrahim YM, et al. Simultaneous proteomic discovery and targeted monitoring using liquid chromatography, ion mobility spectrometry, and mass spectrometry. Mol Cell Proteomics 2016;15:3694–705.

124. Pfammatter S, Bonneil E, McManus FP, Prasad S, Bailey DJ, Belford M, et al. A novel differential ion mobility device expands the depth of proteome coverage and the sensitivity of multiplex proteomic measurements. Mol Cell Proteomics 2018;17:2051–67.

125. Hebert AS, Prasad S, Belford MW, Bailey DJ, McAlister GC, Abbatiello SE, et al. Comprehensive single-shot proteomics with FAIMS on a hybrid orbitrap mass spectrometer. Anal Chem 2018;90:9529–37.

126. Plumb RS, Fujimoto G, Mather J, Potts WB, Rainville PD, Ellor NJ, et al. Comparison of the quantification of a therapeutic protein using nominal and accurate mass MS/MS. Bioanalysis 2012;4:605–15.

127. Gallien S, Domon B. Quantitative proteomics using the high resolution accurate mass capabilities of the quadrupole-orbitrap mass spectrometer. Bioanalysis 2014;6:2159–70.

128. Meier F, Brunner A-D, Koch S, Koch H, Lubeck M, Krause M, et al. Online parallel accumulation–serial fragmentation (PASEF) with a novel trapped ion mobility mass spectrometer. Mol Cell Proteomics 2018;17:2534–45.

129. Bredehöft M, Schänzer W, Thevis M. Quantification of human insulin-like growth factor-1 and qualitative detection of its analogues in plasma using liquid chromatography/electrospray ionisation tandem mass spectrometry. Rapid Commun Mass Spectrom 2008;22:477–85.

130. Abbas IM, Hoffmann H, Montes-Bayón M, Weller MG. Improved LC-MS/MS method for the quantification of hepcidin-25 in clinical samples. Anal Bioanal Chem 2018;410:3835–46.

131. Chambers EE, Lame ME, Bardsley J, Hannam S, Legido-Quigley C, Smith N, et al. High sensitivity LC–MS/MS method for direct quantification of human parathyroid 1–34 (teriparatide) in human plasma. J Chromatogr B 2013;938:96–104.

132. Gao J, Meyer K, Borucki K, Ueland PM. Multiplex immuno-MALDI-TOF MS for targeted quantification of protein biomarkers and their proteoforms related to inflammation and renal dysfunction. Anal Chem 2018;90:3366–73.

133. Duncan MW, Nedelkov D, Walsh R, Hattan SJ. Applications of MALDI mass spectrometry in clinical chemistry. Clin Chem 2016;62:134–43.

134. ELV Mayes. Immunoaffinity purification of protein antigens. In: JM Walker, editor. Totowa (NJ): Humana Press; 1984. p. 13–20.

135. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC—MS/MS. Anal Chem 2003;75:3019–30.

136. Houghton R, Horro Pita C, Ward I, Macarthur R. Generic approach to validation of small-molecule LC–MS/MS biomarker assays. Bioanalysis 2009;1:1365–74.

137. Jiang H, Zeng J, Titsch C, Voronin K, Akinsanya B, Luo L, et al. Fully validated LC-MS/MS assay for the simultaneous quantitation of coadministered therapeutic antibodies in cynomolgus monkey serum. Anal Chem 2013;85:9859–67.

138. Mellacheruvu D, Wright Z, Couzens AL, Lambert J-P, St-Denis NA, Li T, et al. The CRAPome: a contaminant repository for affinity purification-mass spectrometry data. Nat Methods 2013;10:730–6.

139. Sherman J, McKay MJ, Ashman K, Molloy MP. How specific is my SRM?: the issue of precursor and product ion redundancy. Proteomics 2009;9:1120–3.

140. Sauvage FL, Gaulier JM, Lachâtre G, Marquet P. Pitfalls and prevention strategies for liquid chromatography-tandem mass spectrometry in the selected reaction-monitoring mode for drug analysis. Clin Chem 2008;54:1519–27.

141. Kushnir MM, Rockwood AL, Nelson GJ, Yue B, Urry FM. Assessing analytical specificity in quantitative analysis using tandem mass spectrometry. Clin Biochem 2005;38:319–27.

142. Abbatiello SE, Mani DR, Keshishian H, Carr SA. Automated detection of inaccurate and imprecise transitions in peptide quantification by multiple reaction monitoring mass spectrometry. Clin Chem 2010;56:291–305.

143. Zheng S, McIntosh T, Wang W. Utility of free and total target measurements as target engagement and efficacy biomarkers in biotherapeutic development-opportunities and challenges. J Clin Pharmacol 2015;55:S75–84.

144. Ocaña MF, Neubert H. An immunoaffinity liquid chromatography–tandem mass spectrometry assay for the quantitation of matrix metalloproteinase 9 in mouse serum. Anal Biochem 2010;399:202–10.

145. Gordon ED, Palandra J, Wesolowska-Andersen A, Ringel L, Rios CL, Lachowicz-Scroggins ME, et al. IL1RL1 asthma risk variants regulate airway type 2 inflammation. JCI Insight 2016;1.

146. Chen X, Farrokhi V, Singh P, Ocana MF, Patel J, Lin L-L, et al. Biomeasures and mechanistic modeling highlight PK/PD risks for a monoclonal antibody targeting Fn14 in kidney disease. MAbs 2018;10:62–70.

147. Woldemariam GA, Butch AW. Immunoextraction-tandem mass spectrometry method for measuring intact human chorionic gonadotropin, free β-subunit, and β-subunit core fragment in urine. Clin Chem 2014;60:1089–97.

148. Chen Y-T, Tuan L-P, Chen H-W, Wei I-A, Chou M-Y, Chen H-M, et al. Quantitative analysis of prostate specific antigen isoforms using immunoprecipitation and stable isotope labeling mass spectrometry. Anal Chem 2015;87: 545–53.

149. Gao X, Lin H, Krantz C, Garnier A, Flarakos J, Tse FL, et al. Quantitative analysis of factor P (properdin) in monkey serum using immunoaffinity capturing in combination with LC–MS/MS. Bioanalysis 2016;8:425–38.

150. Parng C, Singh P, Pittman DD, Wright K, Leary B, Patel-Hett S, et al. Translational pharmacokinetic/pharmacodynamic characterization and target-mediated drug disposition modeling of an anti–tissue factor pathway inhibitor antibody, PF-06741086. J Pharm Sci 2018; 107:1995–2004.

151. Hassan-Zahraee M, Banerjee A, Cheng JB, Zhang W, Ahmad A, Page K, et al. Anti-MAdCAM antibody increases ß7+ T Cells and CCR9 gene expression in the peripheral blood of patients with Crohn's disease. J Crohn's Colitis 2018;12:77–86.

152. Razavi M, Leigh Anderson N, Pope ME, Yip R, Pearson TW. High precision quantification of human plasma proteins using the automated SISCAPA immuno-MS workflow. N Biotechnol 2016;33:494–502.

153. Kuhn E, Carr SA. Multiplexed immunoaffinity enrichment of peptides with anti-peptide antibodies and quantification by stable isotope dilution multiple reaction monitoring mass spectrometry. In: S Sechi, editor. Quantitative proteomics by mass spectrometry. New York (NY): Springer; 2016. p. 135–67.

154. Whiteaker JR, Zhao L, Schoenherr RM, Kennedy JJ, Ivey RG, Paulovich AG. Peptide immunoaffinity enrichment with targeted mass spectrometry: application to quantification of ATM kinase phosphosignaling. In: SV Kozlov, editor. Methods in molecular biology. New York (NY): Springer; 2017. p. 197–213.

155. Weiß F, Schnabel A, Planatscher H, van den Berg BHJ, Serschnitzki B, Nuessler AK, et al. Indirect protein quantification of drug-transforming enzymes using peptide group-specific immunoaffinity enrichment and mass spectrometry. Sci Rep 2015;5:8759.

156. Bustos D, Bakalarski CE, Yang Y, Peng J, Kirkpatrick DS. Characterizing ubiquitination sites by peptide-based immunoaffinity enrichment. Mol Cell Proteomics 2012;11:1529–40.

157. Fan Y-Y, Avery LB, Wang M, O'Hara DM, Leung S, Neubert H. Tissue expression profile of human neonatal Fc receptor (FcRn) in Tg32 transgenic mice. MAbs 2016;8:848–53.

158. Fan Y-Y, Farrokhi V, Caiazzo T, Wang M, O'Hara DM, Neubert H. Human FcRn tissue expression profile and half-life in PBMCs. Biomolecules 2019; 9:373.

159. Farrokhi V, Chen X, Neubert H. Protein turnover measurements in human serum by serial immunoaffinity LC-MS/MS. Clin Chem 2018;64:279–88.

160. Farrokhi V, Chabot JR, Neubert H, Yang Z. Assessing the feasibility of neutralizing osteopontin with various therapeutic antibody modalities. Sci Rep 2018; 8:7781.

161. Razavi M, Farrokhi V, Yip R, Anderson NL, Pearson TW, Neubert H. Measuring the turnover rate of clinically important plasma proteins using an automated SISCAPA workflow. Clin Chem 2019;65:492–4.

162. Lassman ME, McAvoy T, Lee AYH, Chappell D, Wong O, Zhou H, et al. Practical immunoaffinity-enrichment LC-MS for measuring protein kinetics of low-abundance proteins. Clin Chem 2014;60:1217–24.

163. Greening DW, Simpson RJ. Understanding extracellular vesicle diversity: current status. Expert Rev Proteomics 2018;15:887–910.

164. Aartsma-Rus A, Morgan J, Lonkar P, Neubert H, Owens J, Binks M, et al. Report of a TREAT-NMD/World Duchenne Organisation meeting on dystrophin quantification methodology. JND 2019;6:147–59.

165. Tolan NV. Development of a 25-hydroxyvitamin d liquid chromatography–tandem mass spectrometry assay, cleared by the Food and Drug Administration, via the de novo pathway. Clin Lab Med 2018;38: 553–64.