Clinical Chemistry 56:2
000–000 (2010)

Reviews

The Bottleneck in the Cancer Biomarker Pipeline and Protein Quantification through Mass Spectrometry–Based Approaches: Current Strategies for Candidate Verification

Shalini Makawita[1] and Eleftherios P. Diamandis[1,2,3*]

BACKGROUND: Although robust discovery-phase platforms have resulted in the generation of large numbers of candidate cancer biomarkers, a comparable system for subsequent quantitative assessment and verification of all candidates is lacking. Established immunoassays and available antibodies permit analysis of small subsets of candidates; however, the lack of commercially available reagents, coupled with high costs and lengthy production and purification times, have rendered the large majority of candidates untestable.

CONTENT: Mass spectrometry (MS), and in particular multiple reaction monitoring (MRM)-MS, has emerged as an alternative technology to immunoassays for quantification of target proteins. Novel biomarkers are expected to be present in serum in the low (μg/L–ng/L) range, but analysis of complex serum or plasma digests by MS has yielded milligram per liter limits of detection at best. The coupling of prior sample purification strategies such as enrichment of target analytes, depletion of high-abundance proteins, and prefractionation, has enabled reliable penetration into the low microgram per liter range. This review highlights prospects for candidate verification through MS-based methods. We first outline the biomarker discovery pipeline and its existing bottleneck; we then discuss various MRM-based strategies for targeted protein quantification, the applicability of such methods for candidate verification, and points of concern.

SUMMARY: Although it is unlikely that MS-based protein quantification will replace immunoassays in the near future, with the expected improvements in limits of detection and specificity in instrumentation, MRM-based approaches show great promise for alleviating the existing bottleneck to discovery.

Despite the recent progress toward understanding of cancer etiology and the implementation of preventative measures and novel therapeutic modalities, cancer remains a major disease burden. According to the International Agency for Research on Cancer, in 2008 there were approximately 12.4 million new cancer cases and 7.6 million cancer-related deaths worldwide *(1)*. Within the US, this translates to approximately 1.5 million new cases and half a million deaths expected for 2009 *(2)*. Although recent trends show a slight decline in incidence and mortality rates for cancers affecting some of the more prevalent cancer sites, such as lung, prostate, colon, and breast, the overall burden of cancer will likely increase in the near future as an increasing percentage of the world's population reaches old age *(2)*. To alleviate both the economic and social costs posed by cancer, there is an urgent need to discover and validate novel cancer biomarkers suitable for early disease diagnosis and optimal patient management.

biomarkers are generally quantifiable molecules or processes indicative of a certain biological state or condition, and in the context of cancer, various molecular analytes (such as DNA, mRNA, microRNA, and proteins) and physiological processes (e.g., angiogenesis and proliferation) have proven useful for cancer detection and management *(3–6)*. All of these genetic and molecular alterations, however, tend to ultimately culminate in the aberrant (increased or decreased) expression of protein products. As a result, proteins and the study of cancer proteomics are a potential gold mine for discovery of novel biomarkers. In this review we outline a widely used pipeline for discovery of novel cancer biomarkers, the existing "bottleneck" in moving markers from discovery phases to the clinical arena,

[1] Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada; [2] Department of Clinical Biochemistry, University Health Network, Toronto, ON, Canada; [3] Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, ON, Canada.
* Address correspondence to this author at: Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 6th Floor, Room 6-201, Box 32, 60 Murray Street, Toronto, Ontario, Canada, M5T 3L9. Fax +416-619-5521; e-mail ediamandis@mtsinai.on.ca.
Received August 11, 2009; accepted November 13, 2009.
Previously published online at DOI: 10.1373/clinchem.2009.127019

and strategies based on mass spectrometry (MS)[4] as a means for alleviating the bottleneck.

The Discovery Pipeline

Although a concrete model or standard roadmap for biomarker discovery through unbiased proteomic approaches does not necessarily exist, a widely used theoretical model described by Rifai et al. *(7)* and others *(8–11)* serves as an efficient platform for the discovery of novel cancer biomarkers. This model has 3 primary phases, discovery, verification, and validation. Discovery entails the proteomic profiling of various biological sources such as tissue, proximal biological fluids, cell culture supernatants, and serum, resulting in the identification of several thousands of proteins *(7–12)*. The generated lists of proteins are then mined by applying a set of criteria (usually arbitrary and defined by the study group), involving semiquantitative assessments, multiple bioinformatic analyses, and literature searches, yielding a shortened list of putative biomarkers (approximately 50–100 proteins). These candidates are then moved along to verification phases, during which their ability to enable differentiation of cases from controls for purposes of cancer diagnosis, prognosis, therapeutic stratification, detection recurrence, or other measurable outcomes is assessed through quantitative analysis in a moderate number of samples (50 to several hundred per patient group). Of the candidate biomarkers assessed, the majority are usually rejected because of poor discriminatory potential between cases and controls, or because they are outperformed by markers currently in clinical use. A handful of potentially useful proteins (usually 2–5 proteins) are moved forward into the final phases of clinical validation, in which they are tested by means of established quantitative assays with high analytical sensitivity and specificity in a large cohort of clinically relevant samples (several hundred to thousands of samples per group), collected either retrospectively or prospectively. This 3-phase process and slight variations thereof comprise a "pipeline" for biomarker discovery that has been reviewed in detail *(7–11)*.

Existing Challenges in the Pipeline

As reflected by the increasing number of publications detailing such biomarker studies, it can be safely said that robust discovery-phase platforms have been established in many laboratories, and large numbers of putative biomarkers have been generated *(12)*. As yet, this process has failed to materialize into markers with adequate diagnostic sensitivity for use in the clinic *(13, 14)*. This bottleneck in the biomarker discovery pipeline is due in large part to the inability to quantitatively verify the majority of novel candidates generated in discovery-phase studies *(7, 11, 15–17)*.

At present, established immunoassay platforms, in particular ELISA *(18)*, are the paragon for quantitative analysis of protein analytes in sera. Immunoassays require the use of antibodies for protein capture, and in the case of sandwich ELISAs a second antibody is required to aid in detection. With good quality antibodies, ELISAs allow quantification in the nanogram per liter range, and their 96- or 384-microtiter plate designs facilitate high throughput of samples *(18)*. Although some biomarker candidates identified in discovery phases have commercially available antibodies or ELISA kits for reliable quantification in serum, the majority of novel candidates lack available reagents. In addition, owing to the high costs ($50 000–$100 000 or more for development of a research-grade ELISA sufficient for verification purposes for a single analyte) and lengthy development time (approximately 1 year) for production of novel ELISAs *(7, 11, 18)*, a large number of candidates are left untested. In fact, the availability of antibodies or ELISA kits is often the final filtering criterion when generating lists of putative candidates *(19)*. It is highly likely, however, that hidden within the lists of untested candidates are markers that, when used alone or in combination, could have the potential to contribute substantially to cancer management. As a result, the development and improvement of assays for protein quantification that do not require antibodies, and can be rapidly optimized for any protein of interest at low cost, is an important priority. With the increasing ability to penetrate deeper into the proteome and identify larger numbers of proteins (>5000) in single studies *(20–23)*, the need for novel quantification technologies will be further underscored as initial discovery-phase studies result in the generation of longer lists of putative candidates for verification.

MS methods, and in particular multiple reaction monitoring (MRM)-based approaches, are a favorable alternative to immunoassays for quantitative measurement of proteins because they are not necessarily dependent on the use of antibodies and can therefore be rapidly and cost-efficiently developed in comparison to traditional ELISAs *(16–18)*. In addition, MRM offers superior multiplexing capabilities, allowing for the simultaneous quantification of numerous proteins in parallel. In theory, MRM can also be developed and optimized for all novel candidates of interest (permit-

[4] Nonstandard abbreviations: MS, mass spectrometry; MRM, multiple reaction monitoring; PSA, prostate-specific antigen; LOQ, limit of quantification; LOD, limit of detection; immuno-MS, coupling of immunoextraction to MS; SISCAPA, stable isotope standards with capture by antipeptide antibodies.

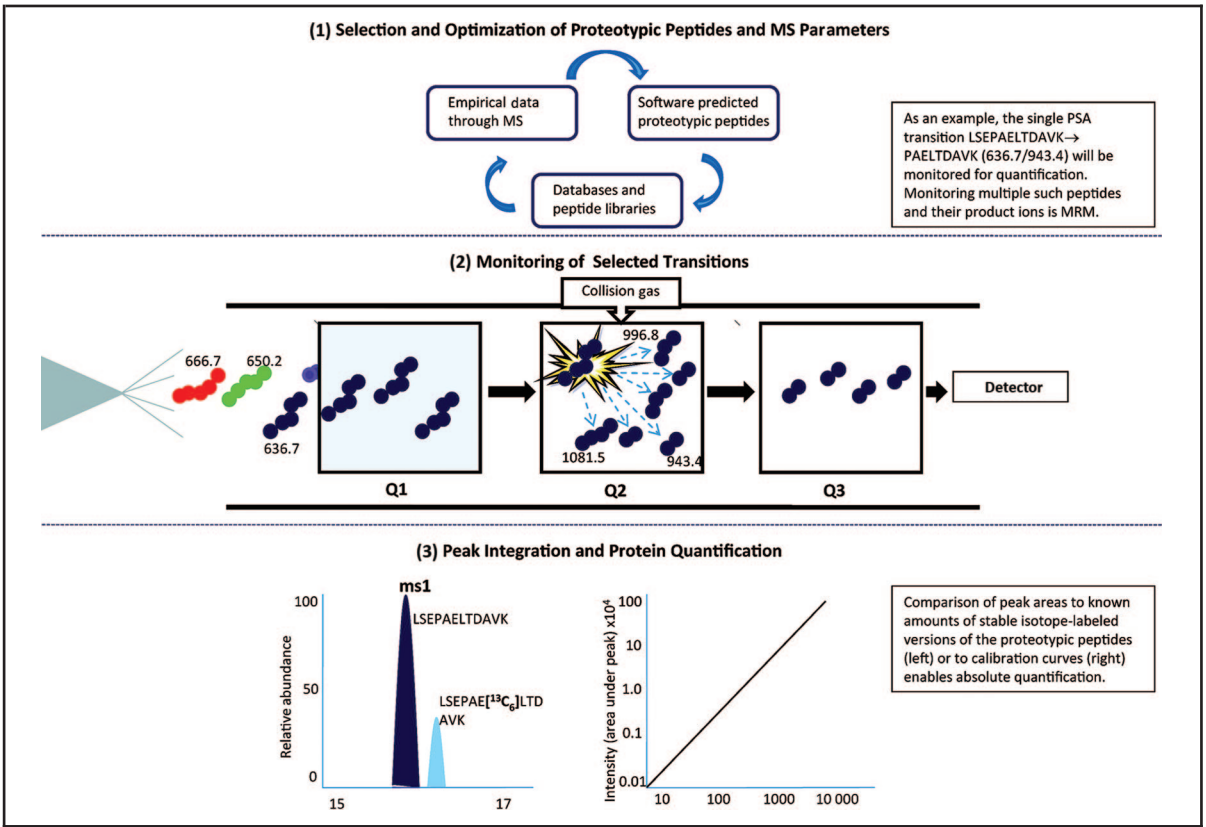

Fig. 1. MS-based MRM targeted protein quantification.

ting they undergo ionization) *(24–26)*. Furthermore, execution of both discovery and verification phases on the same MS platform allows for an easier transition between the 2 phases. In the following sections we review current and emerging MS-based protein quantification strategies relevant for candidate verification.

Multiple Reaction Monitoring–Mass Spectrometry

MS-based quantification relies on the monitoring of selected ionized products of target analytes, integrating the resulting extracted ion chromatograms, and comparing peak areas between 2 experimental conditions for relative quantification or to reference areas generated by standards of known concentration for absolute quantification *(24–26)*. One of the most widely used forms of MS-based quantification is MRM performed on triple quadrupole or linear ion-trap mass spectrometers *(24–28)*. These instruments have the ability to select parent or MS1 peptide ions of target proteins based on mass-to-charge ratio (*m/z*), subject them to collision-induced dissociation, and monitor select fragments or MS2 product ions, again based on *m/z*. The monitoring of a single MS1 ion fragmenting to 1 or more product ions is referred to as single-reaction monitoring, whereas monitoring multiple such reactions is MRM *(24–26)*. The peptides chosen for analysis are proteotypic and serve as representations of the intact protein of interest. These peptides are chosen primarily based on their high abundance and repeated identification in MS runs, as well as sequence specificity for the protein of interest *(24, 29)*. Other criteria such as peptide length and absence of missed cleavage sites are also considered. MRM assays are used in the majority of studies described below. A typical MRM workflow is depicted in Fig. 1.

MRM for Protein Quantification in Serum or Plasma

MRM-like approaches have been used since the late 1970s in clinical laboratories for quantification of low molecular weight substances and metabolites *(30, 31)*. In the late 1990s enzymatically cleaved peptide products were used for quantification of a target protein analyte through MS, as demonstrated by Barr et al. *(32)* for apolipoprotein A-I. To date, numerous reported studies have used MS for the quantification of targeted

Table 1. Summary of quantified proteins and purification strategy coupled to MS in discussed reports.

Reference	Proteins quantified[a]	Purification strategy (enrichment, depletion or fractionation)[b]
(33) 2004	PSA	None
(34) 2004	C-reactive protein	Depletion, fractionation
(35) 2002	Human growth hormone	Fractionation
(36) 2006	C-reactive protein; serum amyloid P component; retinol binding protein	Enrichment
(37) 2007	Liver alcohol dehydrogenase (ADHC1) isoenzyme	None
(38) 2007	Somatropin (recombinant form of human growth hormone)	Fractionation
(39) 2009	PSA	Depletion, fractionation
(40) 2009	C-reactive protein	None
(41) 2007[c]	Aprotinin,[d] leptin,[d] myoglobin,[d] myelin basic protein,[d] PSA, peroxidase[d]	Depletion and/or fractionation
(42) 2009[c]	C-reactive protein, myeloid-related protein 14, myeloperoxidase, cardiac troponin I, cardiac tropinin T, N-terminal prohormone B-type natriuretic	Depletion, fractionation
(43) 2006[c]	47[a]	None or with depletion
(44) 2009[c]	45[a]	None
(46) 2008	PSA	Enrichment
(49) 2008	Carcinoembryonic antigen, secretory leukocyte peptidase inhibitor,[c] tissue factor pathway inhibitor 2,[c] tissue factor pathway inhibitor,[c] metalloproteinase inhibitor 1[c]	Enrichment
(50) 2009	My13, N-terminal prohormone B-type natriuretic	Enrichment
(53) 2004	Hemopexin, α_1 antichymotrypsin, interleukin 6,[e] tumor necrosis factor α[e]	Enrichment (SISCAPA)
(54) 2009[f]	α_1 antichymotrypsin, lipopolysaccharide-binding protein, serum albumin	Enrichment (SISCAPA)
(55) 2009	Troponin I; interleukin 33	Enrichment (SISCAPA)
(56) 2008	Thyroglobulin	Enrichment

[a] For studies in which more than 10 proteins were quantified, only the number is given.
[b] Experiments without a prior purification may have also been conducted in these studies in addition to the indicated strategy. Enrichment can mean at the protein or peptide level.
[c] Multiplexed at the level of MRM.
[d] Indicates nonhuman protein.
[e] Peptide standards were detected but not endogenous forms.
[f] Other peptides may have also been monitored through MRM.

proteins in both single *(33–40)* and multiplexed assay formats *(41–44)*. The proteins quantified in these studies are summarized in Table 1. In general, direct plasma or serum digests without prior attempts to minimize sample complexity have resulted in low milligram per liter quantification ranges at best *(33, 43, 45)*. For instance, a number of different studies that have quantified prostate-specific antigen (PSA) through coupling of various sample purification strategies have generally shown that quantification improves from the low milligram per liter range in untreated plasma analysis *(33)* to the low microgram per liter range with prior depletion and fractionation *(39, 41)* and the low to sub–microgram per liter range with prior immunoextration *(46)* (Table 2). Although various confounding factors such as variations in instrumentation used in the different laboratories undoubtedly contributed to some of the differences observed, this trend can also be seen within the same study (Table 2) *(41, 46)*.

Serum is a highly complex biological fluid in which approximately 20 proteins comprise 99% of the total protein content, and the concentrations of all proteins span 12 orders of magnitude, ranging from albumin and immunoglobulins (g/L range) to interleukins and cytokines (ng/L range) and other analytes at lower concentrations *(47, 48)*. For quantitative analysis of proteins in serum or plasma, direct digests are ideal because they do not require extensive sample preparation. This characteristic enables higher throughput of samples, reduces chances for sample loss, and decreases variability that may be introduced during sample processing. Because of matrix effects and interference from other more highly abundant proteins, however, the

Table 2. Summary of PSA quantified through different pretreatment approaches coupled to MS-based quantification.

Protein	Signature peptide(s) monitored[a] (Q1/Q3[b])	Sample type/pretreatment	Assay LOD, LOQ[c]	% CV	Reference
PSA	IVGGWECEK (539.5/866)	Serum/no pretreatment	LOD = 4.5 mg/L	13% run to run and 5.7% within run	2004 (33)
PSA[c]	IVGGWECEK (539.3/865.3) LSEPAELTDAVK[d] (636.7/943.4)	Plasma/Depletion of 7 high-abundant proteins	LOQ = 124 μg/L	19.7	2007 (41)
		Depletion of 12 high-abundant proteins	LOQ = 46.11 μg/L	8.5	
		Depletion of 12 high-abundant proteins + SCX	LOQ = 2.2 μg/L	2.8	
PSA	LSEPAELTDAVK (637/943)	Serum/immunoextraction of protein by antibody	LOD <0.1 μg/L LOQ = 1 μg/L	<20	2008 (46)
PSA	LSEPAELTDAVK[e] (636.8/943.5 636.8/312.2)	Serum from PC and BPH/depletion of albumin and solid-phase extraction fractionation	LOD = 1.5 μg/L; LOQ = 5 μg/L	<10	2009 (39)

[a] Other peptides may have been monitored but listed peptides were the ones used for LOD/LOQ determination and quantification.
[b] Q1/Q3, selected transition *m/z* values; SCX, strong cation exchange; PC, prostate cancer; BPH, benign prostate hyperplasia.
[c] Both LOD and LOQ or 1 of the 2 has been provided based on available data.
[d] Although monitored, this peptide was not detectable in the samples.
[e] Five other nonhuman proteins were also multiplexed in this study.

limit of quantification (LOQ) and specificity of MRM assays are hindered by the large dynamic range and complex nature of direct digests *(41, 44)*. Coupling and various combinations of prior purification steps such as enrichment of target analytes, depletion of high-abundance proteins, and prefractionation have been shown to reduce sample complexity and decrease the limits of detection (LODs) of MS-based assays. A schematic representation of the 3 strategies is shown in Fig. 2. Next we review these strategies and their applicability to candidate verification.

Coupling of Target Analyte Enrichment to MS-Based Quantification

TARGET PROTEIN ENRICHMENT

Enrichment of target proteins before MRM has proven useful for reliable quantification in the low microgram per liter range, with several studies showing LOQs below the microgram per liter range *(46, 49, 50)*. For instance, immunoextraction through antibodies immobilized on a hydrazide resin, followed by digestion of the immunoprecipitated protein to peptides and MRM with incorporation of stable isotope-labeled standards, has resulted in low microgram per liter quantification for proteins such as carcinoembryonic antigen in lung cancer sera, other proteins such as tissue factor pathway inhibitor 2, and secretory leukocyte peptidase inhibitor spiked into normal sera *(49)*. In addition, sub–microgram per liter quantification has been shown for PSA (LOQ of 1 μg/L, LOD of 0.1 μg/L) through antibody capture on a 96-well ELISA plate *(46)* by use of a product ion–monitoring assay performed on a linear ion-trap mass spectrometer. It should be noted that product ion monitoring is a full-scan tandem-MS acquisition mode that can take upwards of 100 ms/scan. Although this method enables more thorough detection of interferences and increases the reliability of the transitions measured, the time required is considerably longer than the scan time allocated for typical MRM-MS scans (approximately 5–10 ms/transition) *(46)*. Sample preparation for immunoextraction can require up to 2–3 days, and in a more recent study this was reduced to a single day through the use of microwave-assisted enzymatic digestion *(50)*.

Prior coupling of protein immunoextraction to MS (immuno-MS) through antibody capture can greatly enhance sensitivity, and although investment in the development and optimization of such methods may be warranted for highly prioritized candidates for which 1 antibody (but not 2 for the development of an ELISA) is available, these methods are not applicable for the majority of novel candidates that lack antibodies altogether. Immuno-MS methods often emulate ELISAs in that antibodies immobilized on various platforms are used for capturing target analytes, with mass spectrometers acting as "secondary antibodies" aiding in subsequent detection and quantification. In this re-

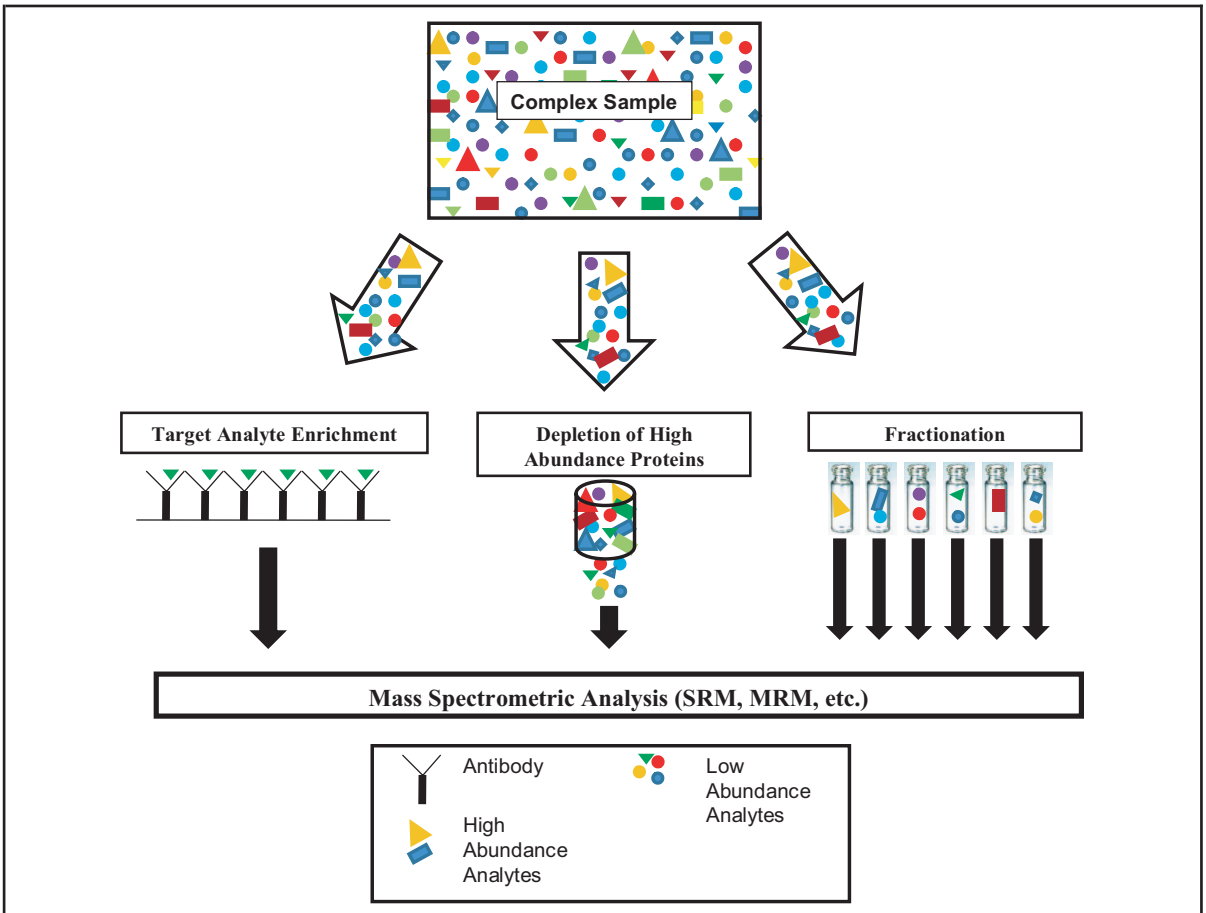

Fig. 2. Strategies to minimize sample complexity and improve detection.

Three primary approaches prior to MRM or MS-based analysis—target analyte enrichment, depletion of high abundance proteins, and fractionation—decrease sample complexity and purify analytes. SRM, selected reaction monitoring.

spect, the limited multiplexing capabilities of ELISAs are translated to immuno-MS approaches at the stage of analyte capture. Enriching for subsets instead of singular proteins in plasma, based on characteristic moieties (such as the enrichment of secreted or shed proteins based on N-glycosite moieties) *(51, 52)*, may facilitate multiplexing while still reducing sample complexity and enabling microgram per liter quantification; however, such methods are also not universally applicable for all proteins.

TARGET PEPTIDE ENRICHMENT

An alternate approach to analyte enrichment is capture at the peptide level through antipeptide antibodies. The production of novel antibodies for a protein or peptide first requires the production of the target analyte *(18)*. Because synthetic peptides can be more readily manufactured in comparison to proteins, which require lengthy purification procedures, researchers have turned to the use of analyte capture via antipeptide antibodies. An application of this approach is stable isotope standards with capture by antipeptide antibodies (SISCAPA) *(53)*. In SISCAPA, antipeptide antibodies are immobilized on supports, which are then packed into affinity columns. Subsequently, digested plasma samples spiked with stable isotope standards are passed through, resulting in the binding of both endogenous proteotypic peptides and their spiked standard counterparts. MS analysis through MRM, followed by peak integration, enables quantitative analysis of targeted proteins *(53, 54)*.

A pilot study using polyclonal antibodies for capture of 4 peptides demonstrated the ability to enrich for standard counterparts of the peptides by approximately 120-fold *(53)*. However, these results were not very translatable to endogenous forms of the peptides, for which only 2 of 4 peptides targeted, those from proteins of high abundance in plasma (g/L range),were

detected, whereas peptides from proteins of low abundance (interleukin 6 and tumor necrosis factor α in the ng/L range) were undetected *(53)*. In a subsequent reported study, the authors developed a more robust antibody support configuration that used magnetic beads *(54)*. In this newer arrangement, free antibodies were first incubated with the peptides (i.e., plasma digest) offline and captured by magnetic beads. Subsequent washing of beads and elution of peptides was coupled online to the liquid chromatography–tandem MS system. The offline binding of antibodies allowed for more efficient capturing of peptides and the online wash/elution setup minimized loss of low abundance peptides. Using this magnetic bead system, the authors were able to produce 1800- and 18 000-fold enrichments of α_1-antichymotrypsin and lipopolysaccharide-binding protein, respectively, relative to albumin, a high-abundance serum protein *(54)*. Although this newer setup addressed many previous limitations, binding of nonspecific peptides to the magnetic bead material was an issue, and the use of less adsorbent materials was suggested for future experiments. The use of higher affinity monoclonal antibodies may also aid in improving specificity, although potentially confounding effects caused by presence of nonspecific peptides have been shown to be avoidable at the MRM step *(53, 54)*.

In a proof of principle study, SISCAPA was used to quantify plasma cardiac troponin I and interleukin 33, by multiplexing *(55)*. Although interleukin 33 concentrations were below the LOD (1 μg/L) of the assay, cardiac troponin I concentrations were quantified in increasing concentrations from 0.6 μg/L to 8 μg/L over a time course of 24 h in patients undergoing planned heart attacks for hypertrophic obstructive cardiomyopathy. Both assays showed good linearity, and cardiac troponin I results with SISCAPA correlated well with those from ELISA ($R = 0.89$) *(55)*. An additional study used a similar approach for the quantification of thyroglobulin, a low-abundance protein and established tumor marker for thyroid cancer *(56)*. The authors noted a good correlation ($R^2 = 0.81$) with immunoassay results and were able to reliably detect concentrations within the clinically relevant range for certain thyroid disorders *(57)*. In addition, reduction in interference from nonspecific heterophilic antibodies and endogenous immunoglobulins was also noted (a major challenge for clinical immunoassays) *(56)*.

SISCAPA and similar approaches demonstrate LOQs in the low microgram per liter range, with apparent quantification of certain proteins at sub–microgram per liter concentrations. With improvements in multiplexing and the implementation of initiatives such as the Human Proteome Detection and Quantification project *(58)*, which aims for quantification of all known proteins in the human proteome at a 1 μm/L LOQ through the production of stable isotope-labeled peptide standards and corresponding antipeptide antibodies, SISCAPA and similar methods might emerge as more feasible means for verification studies across laboratories.

Coupling of Immunodepletion and Prefractionation to MS-Based Quantification

Immunodepletion and prefractionation reduce sample complexity and thereby improve detection by removing interfering elements such as proteins of high abundance or by dividing analytes into more manageable components for analysis (Fig. 2). In a recent study, Keshishian et al. *(41)* evaluated the effect of immunodepletion and prefractionation of plasma on reproducibility, LOD, and LOQ of MRM assays using stable isotope dilution MS for 6 proteins. Although depletion and prefractionation alone showed quantification capability in the microgram per liter range, combination of the 2 enabled penetration into the nanogram per liter range, an approximate 1000-fold increase compared with the milligram per liter detection in untreated plasma *(41)*. In addition, depletion of 12 proteins of high abundance showed overall superiority in terms of LOQs, LODs, and CVs (reproducibility) compared to the depletion of 7 proteins, although both methods allowed for quantification in the microgram per liter range.

In a subsequent study, the same group used a combination of 12 protein depletions with minimal fractionation by strong cation exchange for the multiplexed quantification in the low microgram per liter range of 6 proteins relevant to cardiac injury *(42)*. Although good correlation was noted compared to ELISA, the MRM-generated values were consistently lower than ELISA results, a difference attributable for the most part to sample loss during processing, which the authors suggested could be circumvented through the addition of standards earlier in the procedure. Through coupling of an optimized solid-phase extraction with mixed cation exchange to fractionate samples, another group quantified PSA concentrations in 9 clinical serum samples from patients with benign prostate disease and prostate cancer *(39)*. Clinically relevant PSA concentrations ranging from 4 μg/L to 30 μg/L were quantified with excellent correlation to ELISA results ($R^2 = 0.99$ for cancer and benign disease independently and $R^2 = 0.96$ when considered together). These results, as tested by the authors, were comparable to the variability observed between different ELISA kits for PSA *(39)*.

Depletion and/or fractionation can improve detection, and, as exemplified by the study mentioned above, allow for quantification in clinically relevant

concentrations for some proteins. However, recovery of proteins is of great concern, especially with immunodepletion, because proteins of interest are also sometimes subtracted along with proteins of high abundance, resulting in the underestimation of concentration values (as per above *(42)*. Although some depletion methods showed excellent recovery of proteins, others appear to have failed. For example, in a study in which only albumin was immunodepleted from plasma samples *(39)*, recovery of proteins from different columns/methods ranged from >90% to 70% and even 5%. There were also instances in which protein recovery was good overall (80%–100%), but select proteins showed poor recovery. PSA showed a recovery of 30%, likely due to complex formation with certain proteases *(39)*. Taken together, these inconsistencies underscore the need to optimize the depletion method when coupling this strategy to MRM. In terms of the utility of this method for candidate verification, whereas multiplexing capabilities are largely unhindered *(43)*, increased processing can limit throughput of samples. However, for verification phases in which candidates are assessed in moderate sample sizes, coupling of depletion/fractionation can allow for sensitive and reproducible relative quantification between groups. If through such means candidate biomarkers show potential, more extensive assay development and optimization can be warranted for further verification and possibly validation. Automation of sample processing can also increase the utility of this process for candidate verification.

Multiplexing Capabilities

Multiplexing for purposes of protein quantification is the ability to quantify multiple proteins during a single MS analysis *(41–44)*. This feature can be invaluable for verification phases because it enables the assessment of many proteins in parallel, facilitating the quantification of large numbers of candidates. Multiplexing can also help in minimizing the amount of sample used, an important consideration when working with precious samples. In addition, the shift from individual markers toward biomarker panels to achieve higher diagnostic sensitivity and specificity has underscored the need for the development of multiplexed assays. Although ELISAs at present surpass MS-based quantification in terms of sample throughput, MS-based MRM assays show superior multiplexing capabilities *(41–44)*.

A study by Hunter et al. *(43)* demonstrated the ability to quantify 47 proteins of medium and high abundance in plasma (dynamic range of 4.5 orders of magnitude) through multiplexing by using a mixture of stable isotope standards and a 1-h method protocol. In this investigation the quantification showed good reproducibility (CVs of 2%–22% in 10 runs, with more that three-quarters of the proteins showing CVs <10%). In plasma samples without prior enrichment or depletion, LOQs were in the low milligram per liter range. Improved performance of MRMs was noted subsequent to depletion of 6 high-abundance proteins; however, only a few proteins undetectable in the nondepleted samples were detectable upon depletion. A combinatory approach of abundant protein depletion with limited fractionation may aid in achieving increased detection capabilities, although this approach would markedly increase processing time *(41)*.

In a more recent study by the same group *(44)*, 45 of the same proteins previously reported were multiplexed and quantitatively analyzed without prior depletion. This more recent study also used concentration-balanced mixtures of isotopically labeled standards with MRM, whereby the amount of standard added was close to the mean endogenous concentration of the peptide in the sample as opposed to the addition of equimolar amounts of peptides for all proteins. Kuzyk et al. *(44)* initially used this approach to achieve better linearity in their results; however, as added benefits they also noticed increased signal intensity and decreased analytical variation, with the majority of the peptides assessed having CVs <4%.

The importance of direct plasma or serum digest analysis without prior sample processing, such as enrichment or depletion, was highlighted in these studies. As stated by the authors, with the imminent enhancements in mass spectrometers themselves, lower LOQs should be possible, and MRM-MS should be expandable for rapid, multiplexed quantification of 100 or more proteins in plasma. Such a feat would aid the rapid verification of large numbers of candidates and alleviate the bottleneck in the biomarker pipeline.

Points of Concern for MRM-Based Protein Quantification

The majority of studies discussed above used MRM, and although this technique has many advantages, there are also several points of concern, some of which are outlined below.

SELECTION OF PROTEOTYPIC PEPTIDES

MRM-based quantification relies heavily on the monitoring of selected "proteotypic" peptides, or peptides that are precise and highly reproducible representations of the intact protein of interest, and their product ions (Fig. 1). In several studies outlined above, discrepancies in quantification of the same protein by different peptides was noted, and as an extension, different LOQs *(39, 41, 42)*. For instance, in a study by Keshishian et al. *(41)*, the LOQ for myelin basic protein was

115 μg/L with 1 peptide and 67 μg/L with another. In addition, for PSA, which was also studied, of the 2 peptides monitored, only 1 was detectable. Although some of these variations are inarguably due to sample loss and possible purity issues of the proteins during processing, it brings into question the selection of proteotypic peptides and perhaps the need for increased stringency in choosing appropriate surrogates.

Several avenues to aid in the selection of representative peptides have been described in detail previously *(29, 59–63)*. These can be sources built upon empirical data such as available databases like the Global Proteome Machine *(59)* and peptide libraries *(60)*, which allow researchers to select peptides repeatedly produced by enzymatic digestion and identified in MS runs. There are also various software options *(24, 29, 61–63)* that allow for automated selection of proteotypic peptides as well as prediction of optimized parameters for the MS procedure, such as collision energy and cone voltage in the absence of relevant empirical data. The capabilities of such software greatly reduce the time required for the development and optimization of novel assays, and can facilitate the rapid development of numerous protocols for further optimization. At present, researchers often utilize a combination of both empirical guidance and software predictions and for the time being, this seems the best option. For example, a recent study showed that although some of the peptides chosen through empirical guidance were the same as those chosen by an available software type, overall the empirically guided selection of peptides and optimal parameters resulted in an 11.4-fold increase in signal intensity compared to their software-predicted counterparts *(44)*. In a recent multisite assessment of the reproducibility and transferability of MRM assays *(45)*, it was also noted that although proteotypic peptides are generally transferable between sites, (also seen in Table 2 for PSA, for which several groups used the same peptides), some optimization is required based on the different MS instrumentation used. Certain peptides, however, resulted in poor quantifications, and selection of proteotypic peptides as suggested by the authors, at least at more advanced stages, will most likely require multisite cooperation *(45)*.

SELECTIVITY ISSUES

Selectivity of MRM-based methods has also come into question *(64)*. In various studies it has been noted that nonspecific peptides coelute with the proteotypic peptides being monitored *(33, 39, 41, 43, 44)*. Although sequence specificity for the target protein is a criterion for the selection of proteotypic peptides, specificity of *m/z* ratios is more difficult to discern, and in complex mixtures other peptides with isobaric or very similar *m/z* values to target peptides and their product ions can slip through the mass window of the analyzers, resulting in an overestimation of peptide concentration *(41)*. With MRM, this is typically compensated for by the use of multiple peptide transitions for the same protein, as well as the use of stable isotope standards that coelute with the target peptide, allowing for the identification of the target signals by comparison of retention time *(41, 44)*. Furthermore, online liquid chromatography adds an additional barrier acting to separate potentially redundant *m/z* peptides. However, until increased mass accuracy and improved resolution of instruments occur, *m/z* redundancy is a serious concern, and the possible incorporation of false-positive rates for MRM assays has been recently suggested *(64)*.

REPRODUCIBILITY AND TRANSFERABILITY ACROSS LABORATORIES

Although reproducibility of MRM assays within laboratories has been adequate, as shown by the generated CVs (Table 1), many questions have arisen as to the reproducibility between laboratories. In addition, for MRM-based assays to be effective in alleviating the bottleneck to discovery, proteotypic peptides and assay parameters must be easily transferable between laboratories. In this respect, a landmark study by the Clinical Proteomic Technology Assessment for Cancer network assessed reproducibility and precision between different laboratories and the interlaboratory transferability of MRM assays *(45)*. Eight laboratories took part in the study and analyzed an equimolar mixture of proteins in unfractionated plasma prepared at a central site. The potential for variability was increasingly introduced by 3 study designs, and procedures for sample preparation, data acquisition, and analysis were standardized and provided to each location, as were MRM assay parameters and *m/z* values.

For the most part, all laboratories were able to monitor the centrally chosen transitions, with slight modifications, to achieve maximum detection and specificity based on different instrumentation capabilities. As expected, with increasing complexity of study design and increasing sample handling, the authors noted increasing variability in results, as well as increased sample loss *(45)*. Intralaboratory reproducibility, as measured through CVs of the first 2 study designs, in which samples were processed centrally, were very good, with a median CV of ≤15%. When sample preparation was handed over to the individual sites, reproducibility was slightly reduced but still good, with a median CV of ≤25% for low concentrations, and a median CV of ≤15% at higher concentrations. Interlaboratory reproducibility also showed median CVs of ≤20%, although analysis of lower concentrations showed poorer reproducibility. LOQs and LODs were

in the low mg/L–100 μg/L region, which was consistent with other studies of MRM-based assays in plasma without prior enrichment, depletion, or fractionation. Although clinically relevant assays are required to have CVs of <15%, the authors deemed their results sufficient for verification phases for which candidate biomarkers are in the low– to mid–milligram per liter range with 3 orders of magnitude dynamic range *(45)*. To successfully verify candidates across cancer sites, and at a pace rapid enough to keep up with discovery studies, assays developed by one group should ideally be applicable for use by others. This study shows the potential for MS-based assays to meet such requirements.

Will MS-based quantification replace ELISAs? According to a recent report in *Clinical Chemistry*, the consensus among experts is that ELISAs will likely not be replaced by MS-based methods in the clinic, but will serve in concert with immunoassays for quantification of certain proteins, in particular those for which ELISAs of good quality do not exist, or for those for which quantifying isoforms or posttranslational modifications is required *(65)*. At present, the LOQs of MS-based methods lack reliable quantification in the ranges required for biomarker studies without being coupled with prior enrichment, depletion, and fractionation, as outlined in this review. However, these methods too have their limitations, and a more meaningful solution to alleviate the bottleneck in the biomarker pipeline will likely come about from advances in automated sample preparation, clean-up, and on-line fractionation, as well as improvements in mass accuracy and resolving power of the mass analyzers themselves.

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

Authors' Disclosures of Potential Conflicts of Interest: *No authors declared any potential conflicts of interest.*

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: We thank V. Kulasingam and M. Pavlou for their discussions on the topic. The authors also acknowledge that although many relevant papers were described in this review, due to space constraints not all could be included.

References

1. Boyle P, Levin B. World cancer report 2008. International Agency for Research on Cancer. http://www.iarc.fr/en/publications/pdfs-online/wcr/ (Accessed July 2009).
2. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin 2009;59:225–49.
3. Korner M, Miller LJ. Alternative splicing of pre-mRNA in cancer focus on G protein-coupled peptide hormone receptors. Am J Pathol 2009;175:461–72.
4. Zhu J, Yao X. Use of DNA methylation for cancer detection: promises and challenges. Int J Biochem Cell Biol 2009;41:147–54.
5. Anglim PP, Alonzo TA, Laird-Offringa IA. DNA methylation-based biomarkers for early detection of non-small cell lung cancer: an update. Mol Cancer 2008;23:81–94.
6. Bartels CL, Tsongalis GJ. MicroRNAs: novel biomarkers for human cancer. Clin Chem 2009;55:623–31.
7. 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.
8. Kulasingam V, Diamandis EP. Tissue culture-based breast cancer biomarker discovery platform. Int J Cancer 2008;123:2007–12.
9. Kulasingam V, Diamandis EP. Strategies for discovering novel cancer biomarkers through utilization of emerging technologies. Nat Clin Pract Oncol 2008;5:588–99.
10. Hanash SM, Pitteri SJ, Faca VM. Mining the plasma proteome for cancer biomarkers. Nature 2008;452:571–9.
11. Wang P, Whiteaker JR, Paulovich AG. The evolving role of mass spectrometry in cancer biomarker discovery. Cancer Biol Ther 2009;8:1083–94.
12. Gramolini AO, Peterman SM, Kislinger T. Mass spectrometry-based proteomics: a useful tool for biomarker discovery? Clin Pharmacol Ther 2008;83:758–60.
13. Ludwig JA, Weinstein JN. biomarkers in cancer staging, prognosis and treatment selection. Nat Rev Cancer 2005;5:845–56.
14. Sturgeon CM, Duffy MJ, Stenman UH, Lilja H, Brunner N, Chan DW, et al. National academy of clinical biochemistry laboratory medicine practice guidelines for use of tumor markers in testicular, prostate, colorectal, breast, and ovarian cancers. Clin Chem 2008;54:e11–79.
15. Zhao Y, Lee WN, Xiao GG. Quantitative proteomics and biomarker discovery in human cancer. Expert Rev Proteomics 2009;6:115–8.
16. Paulovich AG, Whiteaker JR, Hoofnagle AN, Wang P. The interface between biomarker discovery and clinical validation: the tar pit of the protein biomarker pipeline. Proteomics Clin Appl 2008;2:1386–402.
17. Carr SA, Anderson L. Protein quantitation through mass spectrometry: the way out of biomarker purgatory? [Editorial]. Clin Chem 2008;54:1749–52.
18. Campbell AM. Production and purification of antibodies. In: Diamandis EP, Christopoulos TK, eds. Immunoassays. San Diego: Academic Press; 1996. p 96–115.
19. Sardana G, Jung K, Stephan C, Diamandis EP. Proteomic analysis of conditioned media from the PC3, LNCaP, and 22Rv1 prostate cancer cell lines: discovery and validation of candidate prostate cancer biomarkers. J Proteome Res 2008;7:3329–38.
20. Brunner E, Ahrens CH, Mohanty S, Baetschmann H, Loevenich S, Potthast F, et al. A high-quality catalog of the Drosophila melanogaster proteome. Nat Biotechnol 2007;25:576–83.
21. Faca VM, Ventura AP, Fitzgibbon MP, Pereira-Faca SR, Pitteri SJ, Green AE, et al. Proteomic analysis of ovarian cancer cells reveals dynamic processes of protein secretion and shedding of extra-cellular domains. PLoS One 2008;3:e2425.
22. Bousette N, Kislinger T, Fong V, Isserlin R, Hewel JA, Emil A, et al. Large-scale characterization and analysis of the murine cardiac proteome. J Proteome Res 2009;8:1887–901.
23. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods 2009;6:359–62.
24. Kitteringham NR, Jenkins RE, Lane CS, Elliott VL, Park BK. Multiple reaction monitoring for quantitative biomarker analysis in proteomics and metabolomics. J Chromatogr B Analyt Technol Biomed Life Sci 2009;877:1229–39.
25. Yocum AK, Chinnaiyan AM. Current affairs in quantitative targeted proteomics: multiple reaction monitoring-mass spectrometry. Brief Funct Genomic Proteomic 2009;8:145–57.
26. Lange V, Picotti P, Domon B, Aebersold R. Selected reaction monitoring for quantitative proteomics: a tutorial. Mol Syst Biol 2008;4:222.

27. Han B, Higgs RE. Proteomics: from hypothesis to quantitative assay on a single platform. Guidelines for developing MRM assays using ion trap mass spectrometers. Brief Funct Genomic Proteomic 2008;7:340–54.
28. Lin S, Shaler TA, Becker CH. Quantification of intermediate-abundance proteins in serum by multiple reaction monitoring mass spectrometry in single-quandrupole ion trap. Anal Chem 2006; 78:5762–7.
29. Fusaro VA, Mani DR, Mesirov JP, Carr SA. Prediction of high-responding peptides for targeted protein assays by mass spectrometry. Nat Biotechnol 2009;27:190–8.
30. Baty JD, Robinson PR. Single and multiple ion recording techniques for the analysis of diphenylhydantoin and its major metabolite in plasma. Biomed Mass Spectrom 1977;4:36–41.
31. Zakett D, Flynn RGA, Cooks RG. Chlorine isotope effects in mass spectrometry by multiple reaction monitoring. J Phys Chem 1978;82:2359.
32. Barr JR, Maggio VL, Patterson DG Jr, Cooper GR, Henderson LO, Turner WE, et al. Isotope dilution mass spectrometric quantification of specific proteins: model application with apolipoprotein A-I. Clin Chem 1996;42:1676–82.
33. Barnidge DR, Goodmanson MK, Klee GG, Muddiman DC. Absolute quantification of the model biomarker prostate-specific antigen in serum by LC-MS/MS using protein cleavage and isotope dilution mass spectrometry. J Proteome Res 2004; 3:644–52.
34. Kuhn E, Wu J, Karl J, Liao H, Zolg W, Guild B. Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and ^{13}C-labeled peptide standards. Proteomics 2004; 4:1175–86.
35. Wu SL, Amato H, Biringer R, Choudhary G, Shieh P, Hancock WS. Targeted proteomics of low-level proteins in human plasma by LC/MSn: using human growth hormone as a model system. J Proteome Res 2002;1:459–65.
36. Kiernan UA, Addobbati R, Nedelkov D, Nelson RW. Quantitative multiplexed C-reactive protein mass spectrometric immunoassay. J Proteome Res 2006;5:1682–7.
37. Janecki DJ, Bemis KG, Tegeler TJ, Sanghani PC, Zhai L, Hurley TD, et al. A multiple reaction monitoring method for absolute quantification of the human liver alcohol dehydrogenase ADH1C1 isoenzyme. Anal Biochem 2007;369:18–26.
38. Yang Z, Hayes M, Fang X, Daley MP, Ettenberg S, Tse FLS. LC-MS/MS approach for quantification of therapeutic proteins in plasma using a protein internal standard and 2S-solid-phase extraction cleanup. Anal Chem 2007;79:9294–301.
39. Fortin T, Salvador A, Charrier JP, Zenz C, Lacoux X, Morla A, et al. Clinical quantitation of prostate-specific antigen biomarker in the low nanogram/milliliter range by conventional bore liquid chromatography-tandem mass spectrometry (multiple reaction monitoring) coupling and correlation with ELISA tests. Mol Cell Proteomics 2009;8:1006–15.
40. Williams DK, Muddiman DC. Absolute quantification of C-reactive protein in human plasma derived from patients with epithelial ovarian cancer utilizing protein cleavage isotope dilution mass spectrometry. J Proteome Res 2009;8:1085–90.
41. 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.
42. Keshishian H, Addona T, Burgess M, Mani Dr, ShiX, Kuhn E, et al. Quantification of cardiovascular biomarkers in patient plasma by targeted mass spectrometry and stable isotope dilution. Mol Cell Proteomics 2009;8:2339–49.
43. Anderson L, Hunter CL. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. Mol Cell Proteomics 2006;5:573–88.
44. 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.
45. Addona TA, Abbatiello SE, Schilling B, Skates SJ, Mani DR, Bunk DM, et al. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. Nat Biotechnol 2009;27:633–41.
46. Kulasingam V, Smith CR, Batruch I, Buckler A, Jeffery DA, Diamandis EP. "Product ion monitoring" assay for prostate-specific antigen in serum using a linear ion-trap. J Proteome Res 2008;7: 640–7.
47. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics 2002;1:845–67.
48. Schiess R, Wollscheid B, Aebersold R. Targeted proteomic strategy for clinical biomarker discovery. Mol Oncol 2009;3:33–44.
49. Nicol GR, Han M, Kim J, Birse CE, Brand E, Nguyen A, et al. Use of immunoaffinity-mass spectrometry-based approach for the quantification of protein biomarkers from serum samples of lung cancer patients. Mol Cell Proteomics 2008; 7:1974–82.
50. Berna M, Ackermann B. Increased throughput for low-abundance protein biomarker verification by liquid chromatography/tandem mass spectrometry. Anal Chem 2009;81:3950–6.
51. Zhang H, Li X, Martin DB, Aebersold R. Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. Nat Biotechnol 2003;21:660–6.
52. Stahl-Zeng J, Lange V, Ossola R, Eckhardt K, Krek W, Aebersold R, et al. High sensitivity detection of plasma proteins by multiple reaction monitoring of N-glycosites. Mol Cell Proteomics 2007;6: 1809–17.
53. 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.
54. Anderson NL, Jackson A, Smith D, Hardie D, Borchers C, Pearson TW. SISCAPA peptide enrichment on magnetic beads using an in-line bead trap device. Mol Cell Proteomics 2009;8:995–1005.
55. Kuhn E, Addona T, Keshishian H, Burgess M, Mani DR, Lee RT, et al. Developing multiplexed assays for troponin I and interleukin-33 in plasma by peptide immunoaffinity enrichment and targeted mass spectrometry. Clin Chem 2009;55: 1108–17.
56. Hoofnagle AN, Becker JO, Wener MH, Heinecke JW. Quantification of throglobulin, a low-abundance serum protein, by immunoaffinity peptide enrichment and tandem mass spectrometry. Clin Chem 2008; 54:1796–804.
57. Wunderlich G, Zophel K, Crook L, Smith S, Smith BR, Franke WG. A high-sensitivity enzyme linked immunosorbent assay for serum thyroglobulin. Thyroid 2001;11:819–24.
58. Anderson NL, Anderson NG, Pearson TW. Borchers CH, Paulovich AG, Patterson SD, et al. A human proteome detection and quantitation project. Mol Cell Proteomics 2009;8:883–6.
59. Craig R, Cortens JP, Beavis RC. Open source system for analyzing, validating, and storing protein identification data. J Proteome Res 2004;3: 1234–42.
60. Lazar IM, Yang X. MRM screening/biomarker discovery with linear ion trap MS: a library of human cancer-specific peptides. BMC Cancer 2009;9:96.
61. Mallick P, Schirle M, Chen SS, Flory MR, Lee H, Martin D, et al. Computational prediction of proteotypic peptides for quantitative proteomics. Nat Biotechnol 2007;25:125–31.
62. Sherwood C, Eastham A, Peterson A, Eng JK, Shteynberg D, Mendoza L, et al. MaRiMba: a software application for spectral library-based MRM transition list assembly. J Proteome Res 2009;8:4396–405.
63. Mead JA, Bianco L, Ottone V, Barton C, Kay RG, Lilley KS, et al. MRMaid, the web-based tool for designing multiple reaction monitoring (MRM) transitions. Mol Cell Proteomics 2009;8:696–705.
64. 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.
65. Diamandis EP, Hanash S, Lopez M, Carr S, Petricoin EF 3rd. Protein quantification by mass spectrometry: is it ready for prime time? Clin Chem 2009;55:1427–30.