Method of the Year 2012

New method and tool developments are helping to bring targeted proteome analysis technologies to a broader array of biologists.

At the close of 2012 at *Nature Methods*, we continue our annual celebration of biological research methods with our choice of targeted proteomics as Method of the Year.

Our choice is a little different from those of previous years, when we highlighted very new, cutting-edge techniques such as next-generation sequencing (2007), super-resolution microscopy (2008) and optogenetics (2010). Broadly speaking, methods for targeted protein analysis date back to the introduction of the radioimmunoassay in the 1960s. Antibody reagents have long been indispensable research tools, allowing biologists to pull out their protein of interest from a complex biological sample or detect their protein in a tissue slice or in a western blot.

In modern research applications such as immunofluorescence microscopy, flow cytometry and protein microarray technology, antibodies continue to be vitally important. But reliance on antibodies for protein detection has drawbacks. The biggest limitations are that antibodies vary greatly in availability and quality. Large-scale efforts such as the Human Protein Atlas, Antibodypedia and the US National Institutes of Health Protein Capture Reagents Program, among others, seek to address these limitations through the systematic generation and characterization of antibodies with respect to human protein targets.

But there is another way. Mass spectrometry, perhaps most familiar for its use in discovery-based proteomics, can also be applied to specifically analyze target proteins of interest. In the most mature technology for targeted analysis, known as selected (or multiple) reaction monitoring, a mass spectrometer called a triple quadrupole is programmed to detect specific peptides that uniquely represent proteins of interest, allowing researchers to quantitatively monitor these proteins with high sensitivity and reproducibility. A Primer on page 23 provides a brief overview of this technology and compares and contrasts the targeted mass spectrometry workflow with discovery-based proteomics analysis.

Mass spectrometry has an advantage over antibodies in that developing a new targeted assay is much faster than generating a new antibody, and issues of detection specificity (that is, antibody cross-reactivity) are greatly minimized. Antibodies still have the upper hand in terms of sensitivity of detection for low-abundance proteins. But a highly positive trait of mass spectrometry is its inherent ability to unambiguously detect multiple proteins in one experiment, allowing, for example, a systems biology researcher to look at what happens to protein levels upon perturbation of a protein network or a clinical researcher to measure how a panel of proposed biomarkers changes in a disease state.

Getting to the current state of affairs in the mass spectrometry–based targeted proteomics field has taken considerable time. The triple quadrupole mass spectrometer was developed in the 1970s, and the first demonstrations that it could be used to detect peptides were published in the 1980s. Interest in applying targeted mass spectrometry technology on a broader scale has been rapidly escalating in the past several years, and new methods, tools, resources and next-generation approaches are helping to bring the technology to a wider community of researchers. In a News Feature on page 19, Vivien Marx describes how mass spectrometry–based targeted proteomics technologies are on the cusp of translation to biological laboratories.

Mass spectrometry–based proteomics has long been viewed as far too complex for anyone but specialists to apply. A discovery-based proteomics experiment requires highly sophisticated bioinformatics aptitude to extract reliable results from the data. On the other hand, targeted mass spectrometry experiments are in principle simple to perform, once reliable protein assays are available, and data analysis is relatively straightforward. Targeted approaches also allow biologists to carry out fundamentally different kinds of experiments—driven by hypotheses—in contrast to discovery-based investigations. In a Commentary on page 24, Ruedi Aebersold, Paola Picotti and Bernd Bodenmiller make a strong case for the important role that mass spectrometry can play in hypothesis-driven research, perhaps even eventually replacing the tedious western blot in basic research labs.

Though *Nature Methods*' focus is on methods for basic biological research, we cannot ignore the important impact that targeted mass spectrometry methods are having on the field of clinical proteomics. In a Perspective on page 28, Steven Carr and Michael Gillette discuss how selected reaction monitoring is being used to sensitively detect proteins in plasma and tissue and how it is highly suited for verification of human disease biomarker candidates.

And finally, as every year, we look to the future with our selection of Methods to Watch (starting on page 35), presenting a mixture of diverse technologies that we are keeping our eyes on: some poised to make an impact in the very near future, others in the longer term.

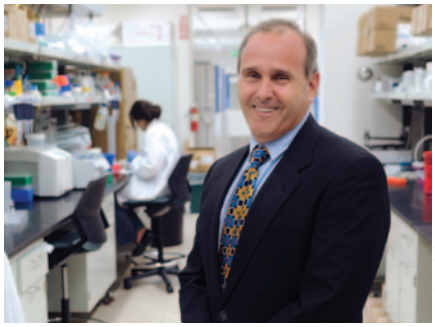
We hope you enjoy our special feature. To all readers, a happy new year!

Targeted proteomics

Analysis of a preselected group of proteins delivers more precise, quantitative, sensitive data to more biologists. Vivien Marx reports.

Although the number and identity of protein-coding genes in humans and many other organisms are known to a certain level of approximation, the numbers of proteins produced by each of these genes remains a mystery. Further complicating matters, given the many possible splice forms and post-translational modifications, the potential number of proteins is "staggering," says Arizona State University researcher Josh LaBaer, who is also president-elect of the US Human Proteome Organization. A protein is also dynamic. "It's phosphorylated this minute; it's not phosphorylated the next minute," he says. This is fascinating science, but it makes proteins in a complex, dynamic sample hard to precisely measure.

Understanding disease-related changes, for example, calls for reliable, quantitative ways of assessing protein levels, and mass spectrometers are instruments able to nail that task. But the data from so-called discovery proteomics experiments in which mass spectrometry is used to identify a large number of proteins in a sample are not always useful to biologists. Enter targeted proteomics, in which the analysis focuses on a subset of proteins of interest in a sample—an approach that has been steadily gaining traction over the last few years.



J. LaBaer/ASU

Targeted proteomics points the field toward high-throughput biology, says Josh LaBaer.

"I personally can't wait until we stop hearing about someone describing how big of a list of proteins, peptides or phosphopeptides they detected," says one researcher critical of discovery proteomics who did not wish to be identified. Proteomics has been doing "my list is bigger than your list" for far too long. "It is way more important to measure the one right protein than 10,000 wrong ones."

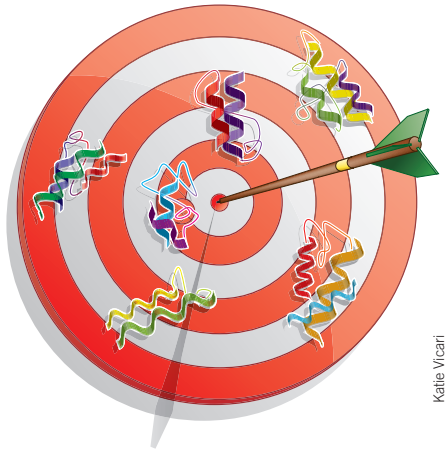
Scientists wanting to follow well-founded hunches about dozens or hundreds of proteins seek a focused, reproducible, quantitative view of a small subset of the whole proteome in their lab vials. High-throughput biology experiments, which include DNA sequencing, genome analysis and gene expression analysis, are generating massive data sets pertaining to particular genes and pathways active in disease or in signaling processes of interest. The shifting of proteomics closer to data mountains in biology is taking it "exactly where it needs to go," says LaBaer. Targeted proteomics helps researchers build on this knowledge and focus their experiments on the subset of proteins important to their line of inquiry.

ETH Zurich

Ruedi Aebersold hopes many laboratories will adopt targeted proteomics.

Targeted proteomics using mass spectrometry promises to deliver data to help address specific biological questions in a way that makes it fundamentally unlike discovery proteomics.

Analysis of any given sample with a discovery-based approach runs into challenges. Two people might do the same experiment, or one person might do the same experiment twice, but "the results are not inherently the same," says Ruedi Aebersold, from the Institute of Molecular Systems Biology at the Swiss Federal Institute of Technology in Zurich. Neither person is necessarily wrong: the contradiction stems from their measurement of different subsets of the whole proteome, he says. "Because the space to sample is so huge, then the mass spectrometer pulls out, every time, a slightly different subset."



Katie Vicari

Targeted proteomics detects proteins of interest with high sensitivity, quantitative accuracy and reproducibility.

"What I like about targeted proteomics is that you answer the question that you are interested in," says Michael MacCoss of the University of Washington. Added significance comes from a "mental shift that our field is beginning to take," he says. Rather than trying to detect all the proteins in a mixture, as in a discovery-based approach, a targeted approach "lets us build quantitative assays to specifically answer hypothesis-driven questions," he says.

The 2012 Method of the Year "tips the hats to the biologists" in their quest to probe, detect, identify and quantify specific aspects of the complex and vast proteome under many conditions, says Brad Gibson from

the Buck Institute for Research on Aging in Novato, California. For instance: knowledge of proteins and their concentrations in a sample can reveal that a signaling pathway is active, giving biologists clues about how the changing components fulfill a physiological function. In clinical applications, to take another example, specific proteins in the blood of a lab animal can indicate how a tumor responds to a drug candidate.

Riding the workhorse

Although the application of mass spectrometry in targeted proteomics studies is relatively recent, it has largely depended on a mature workhorse: the triple quadrupole mass spectrometer. The instrument was originally developed in the late 1970s[1].

A native to analytical chemistry, the triple quadrupole has been widely used in pharmaceutical research, giving researchers ways to standardize how they identify and quantify drug metabolites in blood, says Rob Moritz, an Aebersold collaborator from the Institute for Systems Biology in Seattle. "It's a very highly reproducible standard technique that's used across the industry." The instrument's precision has been a necessity for drug companies, which have worked with mass spectrometer manufacturers to obtain the reproducible, precise measurement results needed to pass muster with the US Food and Drug Administration.

S. Carr /Broad Institute

"The giants who preceded us were the folks in the small-molecule field," says Steven Carr.

"The giants that preceded us were the folks in the small-molecule field," agrees Steven Carr, who directs the proteomics platform at the Broad Institute of MIT and Harvard. To get to less complex mixtures for analysis, the chemists removed proteins from their samples. Researchers in targeted proteomics need the proteins, but they, too, must also reduce sample complexity. They use mass spectrometry to do so.

As Carr explains, adoption of targeted mass spectrometry approaches for peptides dates back to the 1980s, such as in work by University of Tennessee researcher Dominic Desiderio, who sought a specific and selective way to assay peptides found in the brain[2].

Besides the availability of the triple quadrupole, the development of techniques such as electrospray ionization, used to produce gas-phase ions, and other general mass spectrometry advances also paved the way for the adoption of mass spectrometry for targeted peptide and protein analysis.

The triple quad allows researchers to use a processing technique called selected reaction monitoring (SRM), also known as multiple reaction monitoring (MRM), which focuses the mass spectrometer for the detection of a preselected group of analytes.

This SRM approach is similar in concept to immunoassays, in which antibodies latch onto and therefore identify proteins in a specific fashion. Some scientists point out that immunoassays allow greater sensitivity of detection than does mass spectrometry, and there are supporters for each approach (**Box 1**). But antibody-based methods are not easy to scale up. And immunoassays have their limitations because antibodies have varying availability and quality. "I think people would love to have it so that we don't have to rely on antibodies anymore," says MacCoss.

For finding a single protein in 10,000 patients, however, antibody-based tech-

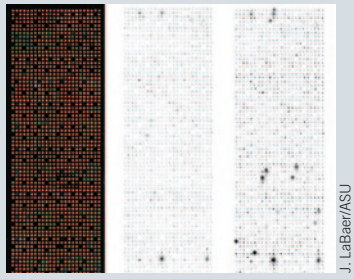
BOX 1 ANTIBODIES TARGET PROTEINS

Historically, researchers seeking quantitative information on proteins and peptides have relied on antibodies or other affinity reagents to provide the results they need. One challenge is that antibodies do not exist for all proteins, and those that do exist vary widely in their quality. A number of current initiatives are addressing this shortcoming, such as the Human Protein Atlas (http://www.proteinatlas.org/), an effort to generate at least two validated antibodies for each protein-coding gene in the human genome; Antibodypedia (http://www.antibodypedia.com/), a database of available antibodies developed in collaboration with Nature Publishing Group; and the US National Institutes of Health Protein Capture Reagents program (http://commonfund.nih.gov/proteincapture/), which focuses on developing renewable affinity reagents.

As a way to study many proteins in a targeted way, LaBaer uses protein arrays, which do not measure the actual protein in a sample but rather use the immune response to find the proteins. They can, for example, help to study antibodies against so-called "self-proteins," he says, which have been found to play a role in autoimmune disease, diabetes and cancer.

LaBaer sees value in hybrid approaches that use combinations of antibodies and mass spectrometry to target specific proteins. Peptides ionize with different efficiencies, he says, which could allow some of them to go missing in the mass spectrometry analysis. And even highly sensitive mass spectrometry–based methods such as SRM still have difficulty detecting low-abundance proteins as compared to antibodies. "If it's a rare thing you're looking for, you could miss it," he says.

One such hybrid approach, explains LaBaer, is stable isotope standard capture with anti-peptide antibodies (SISCAPA)[9], developed by Leigh Anderson, who directs the Plasma Proteome Institute in Washington, DC. This method, in which an antibody is used to pull targeted peptides out of a sample, is one that researchers Amanda Paulovich at the Fred Hutchinson Cancer Research Center and Steven Carr at the Broad Institute harness in their proteomics research.



J. LaBaer/ASU

Protein arrays can use the immune response to detect and find disease-specific signals. After confirmation of consistent protein display (left), identical arrays are probed with plasma from a healthy woman (center) and a breast cancer patient (right).

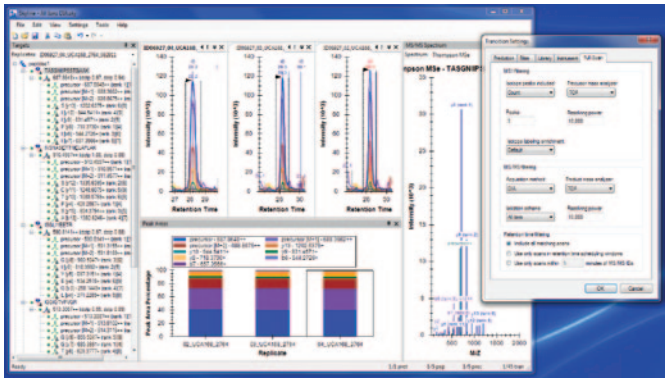
SISCAPA antibodies allow researchers to multiplex experiments and gain analysis depth, too. "In a one-step process, you can pull out things that are at the bottom of the nanogram-per-milliliter range in blood, which is a very challenging biofluid to deal with," says Carr.

Antibodies work with sensitivity and specificity, he says. With mass spectrometry, "we have to do analytical handstands in order to get down to really low detection limits."

niques "will not easily be beat," says Albert Heck, a mass spectrometrist at Utrecht University in The Netherlands. To find 10,000 proteins in a single experiment, discovery-based mass spectrometry is most appropriate, he says. The SRM technology fills a niche between antibody-based detection and discovery-based mass spectrometry and "may cover everything in between, typically about 100 proteins in 1,000 patients or conditions," he says.

Out of the quagmire

In the discovery-based proteomic approach, researchers must plow through databases, matching up the experimental spectra to *in silico*–derived spectra from protein sequence databases to infer which proteins are in a sample, explains Moritz.



M. MacCoss/Univ. Washington

Skyline is open-source software that works across mass spectrometry platforms to help users build targeted methods and analyze the resulting data.

"Quagmire proteomics" is how he refers to the discovery-based approach. It identifies highly abundant proteins in a straightforward manner, "but we could never get below those" to the low-abundance proteins, he says, which are often biologically important.

The complex data analysis steps in discovery-based proteomics have kept the ranks of those proficient in the art and the science quite thin. "It's a small number of labs that generate the bulk of the data," Aebersold says. "It should be the opposite." Recent advances and applications highlight that targeted proteomics is poised to affect the way a growing number of biologists detect and quantify proteins of interest. In contrast to discovery-based proteomics, targeted proteomics simplifies the downstream analysis, doing away with the complex bioinformatics required by discovery proteomics to analyze the mass spectrometry data. "I believe a much larger number of people can do it," he says.

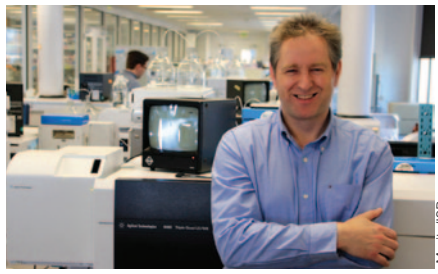
Aebersold has high hopes for targeted proteomics. "My dream would be that it is headed into many laboratories." Those labs might be currently using western blots to confirm hypotheses. "I would hope they would use targeted mass spectrometry," he says. Another application area is in systems biology, where scientists study networks of proteins under many conditions and need consistent data and high sensitivity for complex samples[3].

Whether a targeted or discovery-based workflow is used, the experimental analysis steps are similar: proteins are first preprocessed into smaller components—short amino acid sequences called peptides—that are ionized, separated and sorted according to mass and charge and then shot through a detector that quantifies the ions and delivers peptide spectra to the researcher.

With the SRM approach, scientists first determine which proteins they are interested in for a given sample. Then they program the triple quadrupole with so-called SRM assays, which enable the selection of certain signature peptides known as proteotypic peptides. These peptides not only uniquely represent the target proteins but also have favorable physicochemical properties, allowing the mass spectrometer to more readily detect them[4]. The mass spectrometer thus fragments and analyzes only those peptides that it has been programmed to process.

Being sensitive

Sensitivity is a major advantage of the SRM approach over discovery-based methods. "If we tell the mass spectrometer to only look at the fragments we know are there, then we can dive into the dynamic range and get down to these very low–copy number proteins," of which there may only be ten or fewer per cell, Moritz says. Proteotypic peptide selection and assay development are crucial to ready the technique to "be the most sensitive for the proteins you want to



R. Moritz/ISB

Targeted approaches are a way out of "quagmire proteomics," says Rob Moritz.

study, using the best identifier of the protein that you think of," says Heck.

Reproducibility is another advantage researchers cite for targeted proteomics over discovery-based approaches. SRM assays can perform "exactly the same from lab to lab, machine to machine, so there's no need to go back and do this database searching anymore," says Moritz. Every lab can use the same assays, which helps to make results comparable across labs, he says.

Carr and his colleagues demonstrated the reproducibility of SRM assays in eight laboratories across the United States with different instrument platforms in place, reporting "multisite robustness" and "very good" precision for blood-sample analysis[5]. Gibson, a coauthor on the paper, says he and others had expected it to work, but no study had shown this degree of reproducibility before. Carr says that the study "demonstrated to the community that this is not something that only a few specialized laboratories can do" and that SRM technology delivers "value because of the ability to target and repeatedly measure."

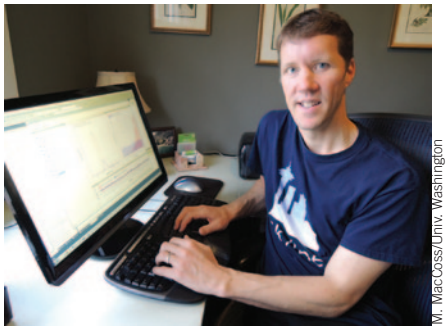
Another advantage of SRM is that, once SRM assays have been generated, it is "much, much faster" than a typical discovery-based experiment, and measurements can "be highly multiplexed so you can measure several hundred and now even several thousand peptides in a single analysis," says Aebersold. New developments over the past few years have enabled rapid proteotypic peptide selection and SRM assay development for new proteins or new splice forms[6].

The quantitative and targeted nature of this approach links proteomics more explicitly to hypothesis-driven research in biology, such as when researchers have hunches about a signaling pathway, says Aebersold. Certain proteins may be involved, and they might increase, decrease or be phosphorylated, all of which become testable hypotheses. Scientists can use SRM to obtain credible and reproducible results, "but you don't

discover a new protein," Aebersold says. If a scientist knows little or nothing about a pathway, then targeted proteomics "is not the method for you."

Meeting and greeting the technology

Over the years, "vendors have done a great job" at improving the sensitivity, dynamic range and specificity of these high-resolution and accurate mass spectrometry instruments for proteomics, says MacCoss. These technology strides, however, have yet to be met by the right analysis tools for targeted proteomics. As a result, he, software developer Brendan MacLean and their colleagues created Skyline[7], open-source software that works across mass spectrometry platforms and helps users build SRM methods. When they use the tool to visually assess their data, they are able to focus on biology rather than the method, MacCoss says.

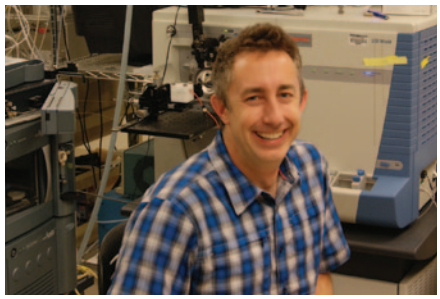


M. MacCoss/Univ. Washington

Brendan MacLean, software developer in the MacCoss lab, managed development of the widely used software tool Skyline.

Skyline, which has been installed 13,000 times, is software that "nearly everyone in the field is using as an indispensable tool for setting up and performing the assays," says Heck. It's becoming a kind of gold standard, says Gibson. As targeted proteomics techniques progress, new analysis bottlenecks emerge that call for new tools and methods.

Other resources moving targeted proteomics ahead include online portals and compendia, such as the SRM Atlas (http://www.srmatlas.org/). As Moritz explains, for the last few years he, Aebersold and other proteomics researchers have built and validated over 170,000 SRM assays for human, mouse and yeast proteins that will soon be available to the broader community via the website.



M. MacCoss/Univ. Washington

"What I like about targeted proteomics is that you answer the question that you are interested in," says Michael MacCoss.

A swath of options

Another new development over the last year has been the emergence of 'SWATH' mass spectrometry, developed by Aebersold in collaboration with mass spectrometer manufacturer AB SCIEX. "We have always known we were frustrated by dynamic range," Gibson says. Traditional discovery-based experiments use a data-dependent approach, in which the instrument's control software selects the top peaks in a mass spectrometry run for further fragmentation and identification.

The SWATH method relies on data-independent acquisition (DIA), a concept originally developed in John Yates's lab at The Scripps Research Institute[8]. Technology developments are helping propel it forward because newer instruments can perform many more scans per second and are more sensitive, Gibson says. With DIA, all peptides within a predetermined mass window are fragmented, which allows a "march up the mass range," giving a "more comprehensive sampling" of many more peptides.

"I think that data-independent acquisition is a great way to interrogate your data," says MacCoss, who believes DIA will become a significant method for proteomics in the near future. The SWATH approach moves targeted proteomics into a higher-throughput space, allowing scientists to analyze more proteins and peptides than the SRM approach. MacCoss, along with other scientists, is pursuing software tool development for DIA, evolving Skyline for these more comprehensive data acquisition strategies.

Separately, Gibson and his colleagues are exploring how to use SRM scheduling to include more peptides in a single experimental run. A mass spectrometer will cycle through the crowd of peptides in the sample every second for an hour, he says. Building on the knowledge that certain peptides ionize at certain times, he and his colleagues target a time window to capture the subset of peptides. Gibson says this approach offers the chance to "quadruple the number of peptide analytes that could be effectively targeted."

Carr is exploring an alternative approach using fractionation methods coupled with mass spectrometry. "The idea is to present a less complex sample to the mass spectrometer," he says. Normally, the most abundant proteins dominate mass spectrometry analysis, hiding the less abundant proteins. Fractionation allows researchers to sample deeper into the proteome to find proteins that might be overlooked or even undetectable initially.

As technology and methods develop and mature, Carr believes sensitivity, specificity, an improved ability for quantification and high levels of confidence about the achieved analytical results will push targeted proteomics ahead. "It's been a long, fun ride, and it's only going to continue to get better."

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Mass spectrometry–based targeted proteomics

A brief overview of mass spectrometry technology for targeted proteomics applications is presented.

A number of technologies can be used to study proteomes, but arguably none is more powerful than mass spectrometry. There are two fundamentally different mass spectrometry–based strategies for analyzing proteomes: discovery-based identification and targeted quantification.

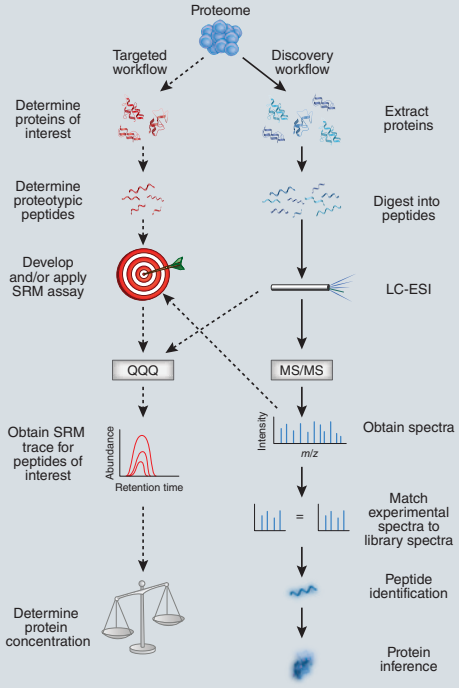
To discover or to target

With a discovery-based strategy, the goal is usually to identify as many proteins as possible. The goal of a targeted proteomics experiment is to monitor a select few proteins of interest with high sensitivity, reproducibility and quantitative accuracy.

In a standard workflow for either type of experiment, proteins are extracted from a sample using biochemical methods, and a protease is used to snip the protein into peptides at defined amino acid residues. The peptides are separated by liquid chromatography, and the fractions are analyzed by electrospray ionization coupled to mass spectrometry. The mass spectrometer measures and reports the mass-to-charge (m/z) ratio of the peptide ions.

In a typical discovery-based experiment, peptide ions are automatically selected in the mass spectrometer for fragmentation on the basis of their signal intensities. Fragmentation generates rich but complex tandem mass spectra for each peptide sequence. The experimental spectra are matched to library spectra to infer the peptide sequences and, by extension, the proteins—a process involving sophisticated bioinformatics tools and careful scrutiny of the results.

In a targeted workflow, the mass spectrometer is programmed to detect specific peptide ions derived from proteins of interest. This requires more up-front investment than a discovery-based experiment, but once a reliable assay is generated for a specific protein, analysis of the mass spectrometry data is relatively straightforward.



Discovery-based versus targeted proteomics workflows using mass spectrometry. LC-ESI, liquid chromatography–electrospray ionization; MS/MS, tandem mass spectrometry.

An old technology made new

The triple quadrupole mass spectrometer (QQQ) was developed more than 30 years ago for small-molecule analysis. It operates as a dual mass filter that allows molecular ions of predetermined masses to be selected for fragmentation in the instrument. In recent years the use of the QQQ for targeted proteomics applications has escalated as methodological advances have made the technology more widespread.

In a targeted proteome analysis, peptide ions travel into the first QQQ mass filter, which can be programmed to select specific 'precursor' ions (on the basis of their m/z ratio) for fragmentation. In the second mass filter, target 'product' ions are selected and then guided to the detector for quantification, resulting in a trace of signal intensity versus retention time for each precursor ion–product ion pair. This process is called selected reaction monitoring (SRM) or multiple reaction monitoring (MRM).

SRM 'assays' are generated by defining a signature set of peptide fragment coordinates. A detectable precursor ion–product ion pair is referred to as a 'transition', and several suitable transitions constitute an SRM assay for detection and quantification of a target peptide and, by extension, the target protein. By spiking the sample with heavy isotope–labeled reference peptides, it is possible to achieve absolute quantification of the targeted peptides. The SRM technique is best suited for analyzing about 50–100 proteins concurrently.

A major bottleneck has been the development of robust SRM assays for reliable protein detection and quantification. Not all peptides are equally analyzed by mass spectrometry: some are better separated, ionized and detected than others owing to their physicochemical properties. Peptide sequences must also be carefully chosen to ensure that they uniquely represent one of the targeted proteins. Several prediction tools and methods have been recently developed to identify these 'proteotypic' peptides, often using information from discovery-based experiments. Once proteotypic peptides have been chosen, the optimal SRM transitions must be determined and rigorously validated. However, once generated, SRM assays can be reproducibly deployed across samples and across laboratories.

Next-generation targeted proteomics

SRM is the most mature mass spectrometry–based technology for targeted proteome analysis, but new methodologies that obviate the need for laborious SRM assay optimization are on the horizon. With an approach called SWATH, complex mass spectra generated by data-independent acquisition (in which peptides are selected for fragmentation without regard to signal intensity) are queried for the presence of specific peptides using libraries of qualified peptide fragment spectra. With another new approach called parallel reaction monitoring, all transitions are monitored in parallel in a single analysis. Continuing methods and software development will be key for bringing targeted proteomics technology to biologists.

Allison Doerr

ACKNOWLEDGMENTS

We thank R. Moritz for his help in preparing this Primer.

Proteomics meets the scientific method

Paola Picotti, Bernd Bodenmiller & Ruedi Aebersold

By delivering precise, reproducible quantification of proteins of interest in biological samples, targeted proteomics approaches are allowing researchers to apply the scientific method using mass spectrometry.

Much of today's scientific knowledge has been generated by what has colloquially been termed the 'scientific method'. The Oxford English Dictionary defines the scientific method as "a method or procedure that has characterized natural science since the 17th century, consisting in systematic observation, measurement, and experiment, and the formulation, testing, and modification of hypotheses." The essence of the scientific method is the formulation of a hypothesis based on prior information or intuition, the rigorous challenge of the hypothesis by accurate and reproducible measurements and the refinement of the hypothesis based on the thus generated results.

Applied to the field of molecular biology, the scientific method requires the quantitatively accurate and reproducible measurement of molecules that are relevant to a particular hypothesis. Among the different classes of biomolecules, proteins are particularly interesting because they are indispensable for virtually any biological function. Proteins are essential structural components of cells; they catalyze cellular reactions as enzymes; they are central to cellular information processing by constituting signaling networks of receptors, kinases, phosphatases and their substrates; and they control gene expression as transcription factors. Consequently, a major portion of life science research over the last decades has been centered on understanding proteins, their functions, their interplay and their changes in abundance in response to internal and external cellular cues.

Although each protein is encoded by a gene, genomic and gene expression data accurately predict neither the abundance nor the activity of proteins. Post-transcriptional mechanisms such as allosteric regulation, post-translational modifications, alternative splicing and dynamic protein-protein interactions modulate the quantity, precise chemical composition and activity of the proteins that are translated from a particular primary mRNA transcript. Thus, direct measurement—that is, identification and quantification of proteins that constitute and control a biological process—is vastly superior to the inference of these parameters from nucleic acid data. It is therefore not surprising that considerable efforts have been dedicated to the detection and quantification of proteins, ever since the functional significance of proteins was discovered almost 90 years ago[1]. This typically involved the generation of a specific assay for a protein in question and the subsequent use of this assay to detect and quantify the protein, and to possibly determine its activity in a large range of samples. Traditionally, protein identification and quantification assays have been based on affinity reagents that are specific for a target protein, whereas assays to measure enzyme activities have frequently been based on chromogenic or fluorescence detection methods that quantify the enzyme's product. To quantify proteins, affinity reagents have been used in a multitude of measurement methods, including western blotting, ELISA, antibody array methods and, most recently, flow cytometry.

In the 1990s the human genome project significantly changed the landscape of biomedical research in three fundamental ways[2]. First, it became possible to define, in principle, all the biomolecules encoded by the genome and their products, thus confining the chemical space within which all biological processes must occur. Second, it catalyzed the development of an array of powerful technologies for the large-scale measurement of these molecules at high throughput. Third, it led to the insight that most biological processes operate as integrated systems of interacting molecules, the founding hypothesis of systems biology.

Mass spectrometry, more specifically liquid chromatography–tandem mass spectrometry, operated in a discovery or 'shotgun' mode emerged as the method of choice for the large-scale analysis of proteins[3]. In a typical shotgun workflow, proteins are first digested using a specific protease, and the resulting peptides are separated using liquid chromatography. The peptides eluting from the chromatography column are ionized and directly injected into a mass spectrometer, where the mass-to-charge (m/z) ratios of the molecular ions are determined. From such a survey scan, peptide ions are chosen on the basis of an abundance-guided heuristic and subjected to fragmentation. The mass of each peptide (precursor) and those of its fragment ions are then assigned by a database search tool to the best-matching peptide sequence in a database[3]. Changes in protein abundance between samples can be quantified by directly comparing the ion abundance between subsequent mass spectrometric analyses (label-free quantification)[4]. Alternatively, quantification can be achieved with isotope dilution strategies, whereby proteins or peptides from different samples are labeled with unique stable isotopes and mixed before mass spectrometric analysis; comparison of the isotope

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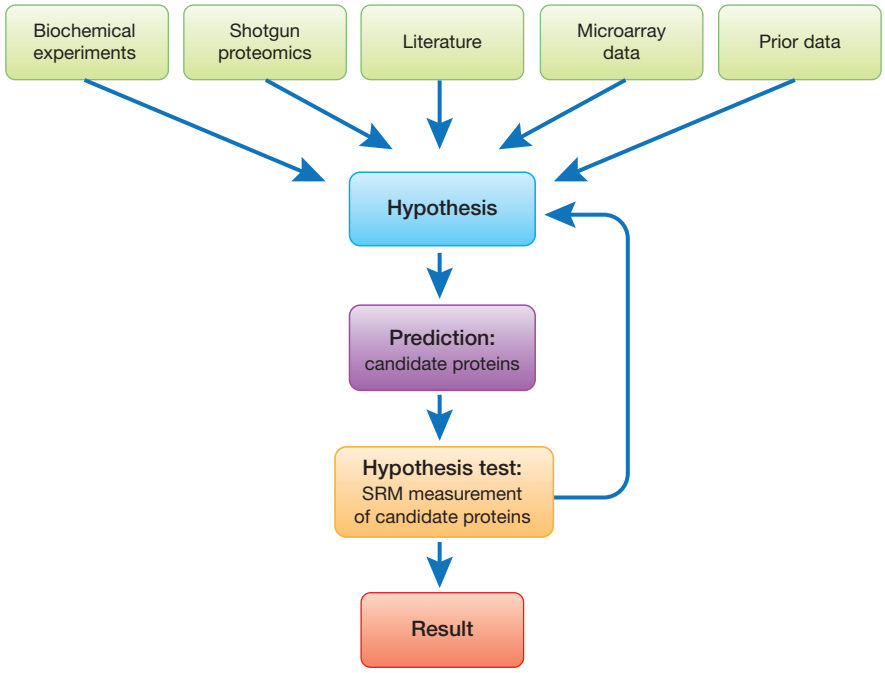

Figure 1 | The scientific method and proteomic analyses. A hypothesis is generated based on biochemical data, shotgun proteomic screens, microarray data, literature knowledge or other prior data that predicts the response of a set of candidate proteins to a selected set of perturbations. These predictions are tested in suitably selected sample sets by SRM to challenge the hypothesis. Depending on the results of the measurement, the hypothesis is rejected or accepted.

pairs in the mass spectrometric scans allows researchers to determine changes in peptide abundance[5–7].

The net result of this approach is the enumeration and relative quantification of the proteins contained in an unknown sample. When mass spectrometry is coupled with specific enrichment steps, post-translational modifications can also be identified on a large scale[8]. As the cumulative result of numerous technical advances, close to complete proteome coverage can now be achieved if a complex proteome is fractionated and the fractions are exhaustively sequenced[9].

In spite of its impressive strengths as a proteome discovery engine, shotgun proteomics has not been broadly interfaced with the scientific method, for several reasons. First, the critical step of precursor-ion selection is stochastic, making the results obtained with the method inherently poorly reproducible as long as the number of peptides in the sample tested significantly exceeds the available sequencing cycles of the mass spectrometer. Second, the method remains technically challenging, beyond the reach of nonspecialist laboratories. Third, no a priori biological knowledge is used for this discovery method. Hypothesis testing is applied after data acquisition and is hampered by the incomplete proteome coverage of most data sets generated to date. Therefore, for proteomics the somewhat paradoxical situation has arisen that highly sophisticated and powerful mass spectrometric platforms are applied in an increasing number of specialist laboratories for the perpetual rediscovery of the proteome, whereas the vast majority of research groups applying the scientific method continue to quantify proteins by western blotting using the limited repertoire of available antibodies. A stunning consequence of this situation was recently reported by Edwards and colleagues[10]. They compiled a distribution of research papers reporting findings about specific human proteins for a period before and after the completion of the human genome sequence. Two observations stood out. First, the vast majority of research papers are focused on a small number of proteins, whereas the vast majority of proteins remain essentially unexplored. The second observation was that the genome sequence and the powerful (proteomic) techniques it catalyzed have not noticeably changed this distribution. To explain these results, the authors plausibly postulated that most protein-related research has been focused on those proteins for which specific assays are readily available: that is, those that can be reproducibly, precisely and easily quantified[10].

We firmly believe that targeted proteomics approaches have the potential to fundamentally change this situation. The main purpose of a targeted strategy is the accurate and reproducible quantification of any protein or sets of proteins in any biological sample. Following the tradition of other methods that support the scientific method, targeted proteomics requires the generation of a definitive assay for the quantification of a targeted protein and the subsequent use of this assay to quantify the protein in a biological sample (**Fig. 1**). To date, targeted proteomics has been most commonly based on a mass spectrometric technique called selected reaction monitoring (SRM), also referred to as multiple reaction monitoring[11,12]. SRM data are acquired by setting the two mass analyzers of a triple quadrupole mass spectrometer to stabilize only the trajectories of ions with predefined *m/z* values. As applied to proteins, multiply protonated ions of peptides unique to the target protein(s) are measured. The two-level mass filtering of the SRM method increases selectivity, and the nonscanning nature of the technique allows for a high signal-to-noise ratio for the target analytes. SRM can be thought of as the mass spectrometrist's ELISA. It targets proteins using a predetermined assay with high sensitivity and selectivity. It does not, however, require immunoassays. Additional advantages of SRM over antibody-based workflows are a higher degree of multiplexing (up to 100 proteins can be concomitantly quantified[13]) and faster and cheaper assay development (assays for ~250 proteins can be developed in about a day)[14]. Another attractive feature of SRM is that it enables the development of specific assays for the quantification of basically any subform of a given protein in a sample, as long as the different forms are characterized by a mass shift, and even for species that cannot be easily distinguished using antibodies (for example: protein isoforms, post-translationally modified proteins, splice variants and mutated forms[15–17]). Further, the SRM workflow eliminates the stochastic component of shotgun proteomic approaches, thus enabling the consistent quantification of the proteins of interest across a multitude of samples. The high precision and reproducibility of SRM measurements of proteins were recently demonstrated by a

comparative interlaboratory study[18]. The high sensitivity of SRM allows the detection of proteins at concentrations below 100 copies per cell in unfractionated yeast proteomes[19], ~7,500 copies per cell in unfractionated human proteomes[20] and down to a high several-hundred nanogram-per-milliliter value in undepleted human plasma[18]. To further improve the limit of detection, researchers can take advantage of the method's compatibility with all commonly used sample fractionation methods[12].

Overall, given a proteome-wide set of definitive SRM assays (see below), the method has the potential to quantify any set of proteins required to test a hypothesis in any biological sample with high throughput. We expect, therefore, that it will broaden protein-based research on two important levels. First, it will dramatically expand the subset of the proteome that is accessible to the scientific method; and second, it will vastly increase the number and type of research groups that will be able to generate high-quality proteomic results.

Currently, the most suitable application areas of SRM are analyses in which reasonably small (~100) sets of proteins, their isoforms or modifications can be defined to test specific hypothesis. Frequently, in translational research these are lists of candidate biomarkers that have to be quantified across large sets of clinical samples to test their validity as clinically relevant markers. Particularly exciting is the capability of SRM to distinguish highly similar protein sequences. This can be applied to quantify disease-associated protein species, including somatically mutated[21] proteins that drive tumorigenesis, fusion proteins or aberrantly modified proteins. Similarly, in systems biology, SRM supports the concurrent quantification of all the proteins constituting a particular biological process across a set of conditions (for example, dosage series or time courses, gene deletions, or sets of chemical modulators), to test the reaction of the system to external perturbations. Another powerful application of SRM, which has emerged only recently[22], is quantitative trait locus (QTL) analysis. QTL studies aim at correlating genetic variation to protein abundances so as to ultimately identify genetic loci that control the expression levels of specific proteins. Understanding these relationships is crucial for unraveling the molecular mechanisms underlying complex traits, such as those associated with common diseases like cardiovascular or neurodegenerative disorders, diabetes or cancer. However, systematic detection of genetic differences affecting protein levels is particularly challenging because it relies on the capability to precisely measure protein concentrations throughout large numbers of samples to achieve the required statistical power. SRM is well suited to fulfill these requirements when the list of target proteins is of moderate size.

Despite its potential and increasing number of successful applications, targeted protein analysis by SRM still suffers from several caveats. As mentioned above, the detection of low-abundance proteins in mammalian or similarly complex proteomes requires sample separation or enrichment steps, which reduce the throughput of the technology. Another limitation is the inability of SRM to support ultra–high throughput multiplexed analyses, a task that is trivial for ELISA assays. Because of the relative novelty of the SRM technology, it has not yet been widely accepted as reliable. Although multiple studies have shown that quantitative results obtained by SRM match those obtained with antibody-based assays[23–28], there is still skepticism from the broader scientific community about the ability of SRM to reliably quantify proteins. Last, an important limitation that needs to be addressed in the near future is the accessibility of the technology to nonspecialized laboratories, a continuing issue for all mass spectrometry–based analysis techniques. Despite tremendous improvement in the implementation and automation of SRM experiments (such as tools to automate assay development and data analysis), an SRM-based platform for non–mass spectrometrists has not been established. Concerted efforts from both academia and mass spectrometry vendors are required to address this issue to transition SRM measurements from technology-oriented research or service units to biological and clinical labs. Certainly, the generation of publicly accessible libraries of SRM assays for complete proteomes, currently undertaken for different species, will help eliminate this limitation[22].

Where is targeted mass spectrometry headed in the future? We believe that in many instances it will support the scientific method by replacing antibody-based methods such as western blots, as a larger number of proteins and their modifications can be concurrently quantified in sample sets. A promising step toward increasing the number of proteins quantified in a sample is the implementation of the targeted concept on high-resolution and accurate-mass instruments. One strategy involves the generation of data-independent fragment-ion maps for all the analytes contained in a sample and their targeted analysis using SRM-like coordinates, an approach also referred to as SWATH-MS[29]. Similarly, a direction currently being explored to reduce the number of upfront assay development steps is the parallel detection of all product ions of a target peptide on quadrupole-Orbitrap mass spectrometers and the reconstruction of their chromatographic profiles (parallel reaction monitoring)[30,31].

In terms of sample throughput, improvements in the chromatographic setup might enable the use of short gradient times, which should expand the throughput of the SRM approach and make it applicable to screening applications in which thousands of samples are analyzed. Further, the targeting capabilities of SRM could be exploited for the reliable quantification of marker peptides or chemical probes for the activity of specific classes of proteins[32], such as proteases or kinases. Another area in which SRM could greatly increase the amount of information gained and overcome current limitations is the field of mass spectrometry–based tissue imaging[33]. Here, typically matrix-assisted laser desorption/ionization is used to ionize cellular molecules or peptides (after on-tissue trypsin digestion), and subsequently the measured m/z ratios are used to identify the analytes. Currently, the sensitivity and dynamic range of the approach is low because chromatography cannot be used to separate the analytes, and thus stronger matrix effects are observed. SRM could improve the sensitivity of the approach while also enabling the targeted and reproducible detection of molecules of interest across multiple tissue samples. Recently, a new targeted technique called mass cytometry was described, coupling flow cytometry to mass spectrometry and allowing quantification of up to 100 proteins and phosphorylation sites on the single-cell level with high sensitivity and throughput[34,35]. In mass cytometry, pure metal isotopes are coupled to antibodies, which in turn detect and bind to defined biomolecules of interest in a targeted manner on single cells. Then the labeled single cells are introduced into an inductively coupled plasma mass spectrometer that measures the metal isotope amount

and composition of each single cell, thereby revealing the single-cell abundances of the antibody targets[34,35].

Over the past ten years, discovery-based proteomics has rapidly advanced to identify ever-increasing numbers of proteins in biological samples. In contrast, targeted proteomics has been developed to challenge hypotheses in the scientific method. This approach is extending the application of mass spectrometry toward the reproducible quantification of significant segments of a proteome and toward protein measurements in single cells.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available at http://www.nature.com/doifinder/10.1038/nmeth.2291.

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Quantitative analysis of peptides and proteins in biomedicine by targeted mass spectrometry

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Targeted mass spectrometry (MS) is becoming widely used in academia and in pharmaceutical and biotechnology industries for sensitive and quantitative detection of proteins, peptides and post-translational modifications. Here we describe the increasing importance of targeted MS technologies in clinical proteomics and the potential key roles these techniques will have in bridging biomedical discovery and clinical implementation.

Proteomics comprises the large-scale, systematic study of protein structure and function, usually with respect to a defined entity: a pathway, organelle, cell, tissue or organism. Whereas any methods or technologies to systematically interrogate large numbers of proteins can justifiably be considered proteomic approaches, the term is increasingly being used to designate work in which MS is the central technology platform. Clinical proteomics is a loose assemblage of proteomics initiatives unified by their translational nature: that is, their impetus to progress along the path from basic research to medical application. Clinical proteomics experiments typically involve the characterization of proteomes of normal or diseased tissues or biological fluids, thus detailing and quantifying the protein differences that associate with, define or cause the diseased state to illuminate pathobiology, improve disease classification or identify new therapeutic targets. Proteomic biomarker discovery is a familiar instance of clinical proteomics research in which MS-based proteomic approaches are used to identify peptides, proteins or post-translational modifications that support early disease detection, facilitate diagnosis, inform prognosis, guide therapy or monitor disease activity. The ultimate objective of any translational enterprise is clinical implementation, in which knowledge previously gleaned is used to directly drive clinical decision making and intervention. When that implementation involves MS-based measurement of one or more protein-derived analytes, it represents the fullest realization of clinical proteomics.

A defining advantage of MS for discovery or hypothesis generation in clinical proteomics is the capability to confidently identify thousands of proteins in complex biological samples without prespecification of the analytes to be measured. With this broad and unbiased coverage comes the cost of reduced sensitivity and stochastic sampling. As one moves along the translational path, findings must be verified and hypotheses must be tested, requiring that sensitive quantitative protein measurements be made precisely and reliably every time. This crucial phase of clinical proteomics is increasingly achieved by focusing the resources of the mass spectrometer on a defined subset of analytes, an approach called targeted MS.

Targeted MS in the spectrum of MS methods

For over four decades, targeted MS approaches have been used to increase the speed, sensitivity and quantitative precision of biomolecule analysis[1–3]. Targeted MS technologies have been developed, in large part, to overcome the sampling limitations of conventional data-dependent scanning MS analysis used in a discovery-based strategy (**Fig. 1**). In both approaches, analytes (small molecules, metabolites or peptides) are infused or eluted from a reversed phase column attached to a liquid chromatography instrument and converted to gas phase ions by electrospray ionization. Analyte ions are fragmented in the mass spectrometer (a technique known as tandem MS or MS/MS), and fragment and parent masses are used to establish the identity of the analyte. In data-dependent acquisition, ions are automatically selected for MS/MS based on their signal intensity in the preceding full-scan MS spectrum. Interpretation of the MS/MS spectra provides the amino acid sequences of the selected peptide ions; sequence and parent ion mass–directed database search allows peptide identification.

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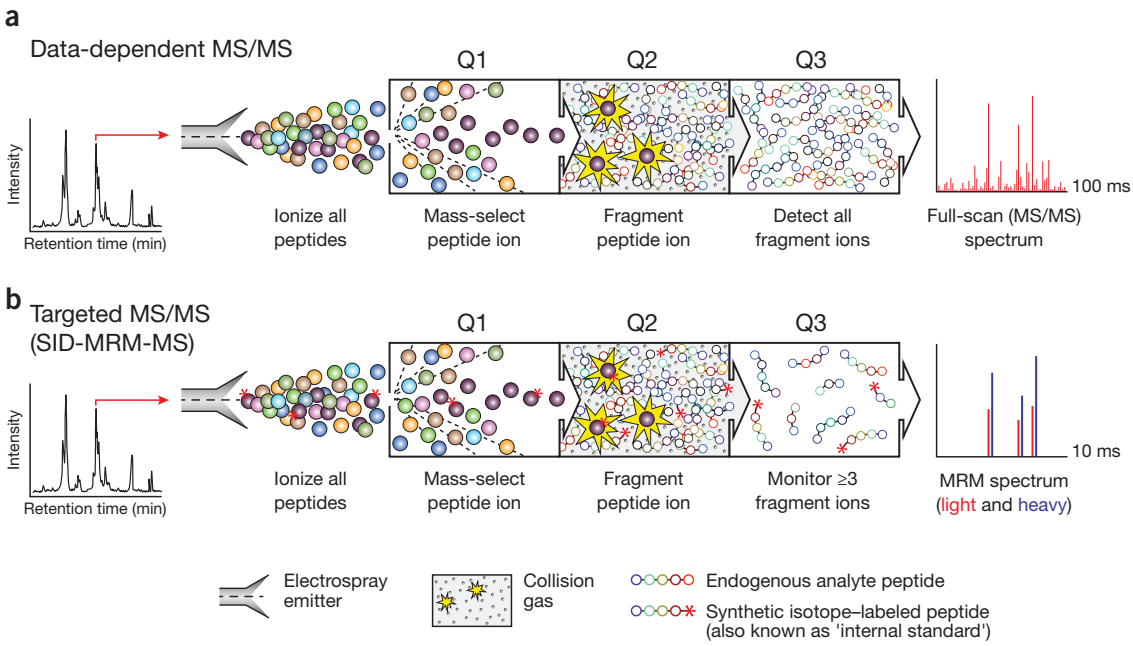

Figure 1 | Comparison of conventional data-dependent analysis to targeted MRM-MS on a triple quadrupole mass spectrometer. (**a**) In a data-dependent MS experiment, digested proteins are loaded on a reversed-phase column attached to a liquid chromatography setup and eluted via electrospray to yield gas-phase ions. At any given point in the chromatographic separation many tens to hundreds of peptides are eluting nearly simultaneously. A full-scan MS spectrum is acquired and informs collection of subsequent MS/MS scans in which 4–10 ions observed in the MS spectrum are automatically selected on the basis of their signal intensity (Q1) for fragmentation by collision with inert gas (Q2). The complete array of fragment ions is detected (Q3), which constitutes the full-scan MS/MS spectrum (far right). (**b**) In an SID-MRM-MS analysis, proteotypic peptides uniquely representing proteins of interest are predefined together with their most informative fragment ions. Peptides are selected for fragmentation (Q1 and Q2), and fragment ions are selected for detection (Q3) based on a user-specified list of targeted precursor-fragment pairs ('transitions'). Synthetic peptides containing stable-isotope labels can be spiked in as standards (asterisks). Comparing labeled to unlabeled peak area (far right) provides precise relative quantification of the endogenous analyte.

This data collection cycle (typically 2–3 s in duration) is repeated over the entire course of the liquid chromatography (LC)-MS/MS analysis. The principle behind the alternative approach of targeted acquisition is simple: guided by a reference spectrum, an analyte can be identified using only a few selected fragment ions rather than the entire complex content of the MS/MS fragmentation spectrum.

In the earliest implementation of targeted MS, multiple ion monitoring, signals for a few selected ions were extracted from previously collected full-scan MS data and used to identify and quantify analytes[1]. With the development of the triple quadrupole mass analyzer[4], it became both possible and practical to accomplish this during data collection, by rapidly mass selecting and fragmenting specific precursor ions representing analytes of interest and monitoring signals for only a few predefined fragment ions for each analyte (**Fig. 1b**). In a contemporary multiple reaction monitoring (MRM) experiment (also commonly referred to as selected reaction monitoring), each fragment ion from an analyte needs to be sampled for only a few milliseconds to obtain interpretable spectra. More than 100 precursor-product ion pairs (referred to as 'transitions') can thus be recorded per second in MRM, enabling targeted MS analysis of many tens of analytes in a time frame much shorter than the peak width of their chromatographic elution. Whereas in early MRM implementations the instrument cycled continuously through the entire set of transitions, recent software and hardware improvements now support scheduled analysis of subsets of transitions based on established retention times of the analytes in the chromatographic system used[5], allowing hundreds of different analytes to be targeted and analyzed in a single MRM analysis. In a properly designed and implemented MRM 'assay', analytes are consistently measured by their specified transitions; absence of detection means that the analyte is below detection limits. In contrast, absence of detection in a data-dependent MS analysis (**Fig. 1a**) can mean either that the analyte is below detection limits or that it was not sampled.

Analysis of proteins and their post-translational modifications by MRM is based on detection of peptides derived by digestion of the protein (**Fig. 2**). The highest detection confidence and measurement precision for peptides in complex samples is obtained by combining stable isotope dilution (SID)—an analytical chemistry technique in which a known concentration of an isotope-labeled compound is added to the sample before analysis—with MRM to yield SID-MRM[5–9]. In this approach, synthetic versions of each analyte peptide containing an amino acid labeled with a stable isotope (for example, ^{15}N or ^{13}C) are used as internal standards. The labeled internal standards separate by chromatography and fragment identically to their native counterparts but are distinguished in the MS and MS/MS spectra by the increased masses of the peptide and of fragment ions containing the labeled amino acid. Confidence that the correct analyte is measured using just a few fragment ions from the peptide is increased by the requirements that the labeled internal standard and endogenous analyte elute together in chromatography and that their monitored fragment ions have the same relative abundance. The sensitivity of MRM-MS is greater than that of discovery proteomics methods because the signal from the selected ions accumulates for longer periods of time in the mass spectrometer.

Peptide and protein concentrations are derived from ratios of the peak areas measured for each internal standard and endogenous

peptide (**Fig. 1b**). These measurements typically have high precision, but they may have low accuracy as the release efficiency of analyte peptides from a protein during proteolysis depends on sequence context and is highly variable and because labeled peptides are not subjected to all the same sample preparation steps as the endogenous sample. In most cases today MRM is used to precisely and consistently measure relative changes in the levels of targeted analytes across samples rather than to accurately establish the amount or concentration in a sample. In cases where the accurate (or 'absolute') amount of a peptide and the corresponding protein are needed, such as in clinical measurements, stable isotope–labeled proteins or protein surrogates in which the tryptic peptide analytes are surrounded by their native sequences can be used as reference standards for accurate quantification when added at the start of sample processing[10].

Targeted MS in biomedicine and its adoption in proteomics

The recent explosion of interest in clinical and translational applications of targeted MS for peptide and protein measurements has its roots in a technology revolution a generation ago. In the 1960s, immunoassays were used routinely to measure small molecules such as metabolites, hormones and drugs in blood, urine and other body fluids[3]. Because the emerging targeted MS methods offered decisive advantages, they progressively replaced immunoassays in clinical research laboratories even though these changes required deployment of costly instrumentation and knowledgeable personnel to run the instruments and analyze the data. In contrast to immunoassay-based methods, developing MS-based assays for new analytes was simple and fast, and, rather than measuring only one analyte at a time using different tests, a single LC-MS/MS analysis could be used to measure many tens of small molecules. Today, millions of LC-MS/MS assays are run annually in clinical laboratories worldwide, the vast majority based on MRM[1,3].

The adoption of targeted MS for analysis of peptides and proteins has been driven by many of the same positive attributes that were recognized for small molecules. Desiderio and colleagues first demonstrated the use of targeted MS for the analysis of bioactive peptides[2] in the 1980s. Application of these methods to the analysis of proteins and post-translational modifications began to accelerate in the late 1990s and has rapidly expanded over the past decade, enabled by improvements in the capabilities of LC-MS systems and in methods to prepare tissue and biofluid samples for MS analysis.

Experimental challenges

The most substantial challenge in using MRM-MS for targeted peptide analysis in clinical proteomics applications is the prevalence of interferences from other peptides and small molecules in the sample matrix. This problem, although well studied for small-molecule analysis, is both less well recognized and far more severe for peptide analysis[11], chiefly because peptide MRM analyses are typically carried out in an ocean of many hundreds of thousands to millions of peptides produced by digestion of the 10,000 or more proteins (not counting modified forms) found in blood and other tissues. Interference manifests itself in two ways: ion suppression and transition interference. Suppression decreases the ion current response of an analyte in an unpredictable and nonreproducible way. Suppression effects increase with the complexity of the biological matrix. Transition interference is caused by peptides or other sample constituents that have both a precursor mass and one or more fragment ion masses that are identical or nearly identical to those being monitored for an analyte of interest. Software has been introduced that facilitates detection of interference and identification of unreliable transitions[11,12]. These programs use additional information inherent to the analysis to build confidence in assignment, including co-elution of analyte and standard based on simultaneous appearance of their respective transitions; the relative ratio of the selected fragment ions to one another compared to those observed for the internal standard; statistical measures of assay precision; and other independent scoring methods.

Although assays can be configured without the use of labeled internal standard peptides[5], in this case measurement precision and confidence in identification both suffer, especially in complex plasma and tissue matrices. Expense is frequently raised as an objection to the use of isotope-labeled peptides that currently cost $250–500 for 1–2 milligrams of purified and quantified material. However this amount of peptide is enough to run 10,000 or more assays, potentially bringing the cost of peptide on a per-analyte, per-assay basis down to a few pennies. Additional substantial savings can be achieved by configuring initial assays with unpurified labeled peptides now offered by many companies. In our view, when the cost of biological and especially clinical follow-up on an incorrect identification is considered, the cost of labeled peptides needed for internal standards becomes unsubstantial and well-justified. Furthermore, transferability of assays across laboratories to measure proteins in plasma is presently only possible through the use of labeled internal reference standards[9]. Certainly it is likely that clinically deployed MRM assays will require the use of labeled internal standard peptides and/or labeled proteins to establish that the test is measuring the analyte of interest and is free of interference. We anticipate that use of isotope-labeled peptides as internal standards will continue to increase, leading to additional reductions in the cost of synthetic peptides.

Probing the depths of the plasma and tissue proteomes

Once an MRM assay been developed it has a high probability of being applicable in any context in which measurement of the target protein is desirable. This has motivated the development of public repositories containing configured MRM assays[13,14]. However, although MRM assays can be configured to measure peptides and modified peptides from nearly any protein, developing assays that achieve the desired sensitivity in clinical proteomics applications is not guaranteed. The problem is not primarily one of insufficient instrument sensitivity. Rather, it is signal-to-biological-noise ratio caused by sample complexity and the wide dynamic range of protein abundance in sample matrices such as plasma and tissue. Multiple strategies have therefore been developed to improve sensitivity and specificity of peptide detection and quantification in complex matrices (**Fig. 2**).

In the case of plasma and solid tissue (which is often admixed with or contaminated by plasma), depletion of the 12–70 most abundant plasma proteins using immunoaffinity depletion columns has become standard, despite inherent risks that the depleted abundant proteins are informative or that informative lower-abundance proteins might be bound to the targeted proteins and incidentally lost. Depth of detection can be additionally increased using two or more dimensions of chromatography-based separation[8,15]. By combining one or more of these sample-processing steps with MRM, it is possible to quantitatively measure proteins in blood that are present in

the high picogram to low nanogram per milliliter concentration. Even greater sensitivities have been achieved for small numbers of analytes using highly targeted and focused chromatographic isolation methods[16,17], but the generality of these approaches has yet to be demonstrated.

Immunoaffinity enrichment of target analytes short-circuits the need for abundant protein depletion and fractionation before SID-MRM-MS. In such an approach (**Fig. 2**) either the intact protein is captured using an anti-protein antibody[18] or a peptide derived from the protein is captured using an anti-peptide antibody that has been raised to the target peptide of interest[19–21]. In either case only a single capture antibody is required as the mass spectrometer substitutes for a secondary detection antibody, providing absolute sequence specificity. The protein capture method is limited by the availability of antibodies capable of selective and sensitive immunoprecipitation of the protein from tissues and plasma. Immunoaffinity enrichment of peptides usually requires generation of custom antibodies for each target. Although this is a lengthy and expensive process, the combination of peptide immunoaffinity enrichment with MRM is very versatile. Both the labeled reference and endogenous forms of the analyte peptide are simultaneously enriched with the anti-peptide antibody in a method called stable isotope standards and capture by anti-peptide antibodies (SISCAPA); after elution their relative amounts can be measured by SID-MRM. The affinity of the anti-peptide capture antibody needs to be high, but its selectivity need not be high because the mass spectrometer can readily distinguish and quantify the analyte peptide of interest despite the binding of other peptides in the digested sample. SISCAPA assays can be highly multiplexed[22] and throughput can be improved by coupling SISCAPA to magnetic bead–handling robotics to automate peptide capture, wash and elution steps[20]. These assays have proven to be robust and reproducible across laboratories, with detection limits of about one nanogram of protein per milliliter of plasma and assay coefficient of variation of 15% or less[23]. Of note, comparable advantages accrue to the immuno-MALDI–time of flight MS method, which couples affinity enrichment to the alternate matrix-assisted laser desorption and ionization (MALDI) MS interface[24].

Targeted MS in verification of candidate biomarkers

The portfolio of targeted MS in translational science is expanding dramatically, for instance, with its increasing use to monitor nodes in signaling cascades, canonical cancer pathways or other biologically important networks[13]. With a trio of reagents for each node (a conventional proteotypic peptide to control for global expression, and phosphorylated and nonphosphorylated forms of a phosphopeptide to assess phosphorylation stoichiometry), pathway activity can be dissected and precisely quantified. Despite such important new roles, biomarker candidate verification remains among the signal applications of targeted MS in clinical proteomics.

Over the past decade proposed biomarkers have been derived largely from genomics and proteomics experiments in which very large numbers of transcript or protein measurements are made on comparatively small numbers of samples, creating conditions that favor detection of spurious differences unrelated to the disease-specific variables of interest. Cancer, the focus of many biomarker discovery efforts, poses a special undersampling problem because the often extreme molecular heterogeneity of disease means that even informative markers may be expressed to a different extent in only a subset of cases. These challenges are compounded in MS-based proteomics discovery experiments because stochastic sampling of complex proteomes means that proteins are inconsistently observed across samples and when observed are likely to have their abundance estimated by different component peptides, with attendant quantitative imprecision. Discovery based on

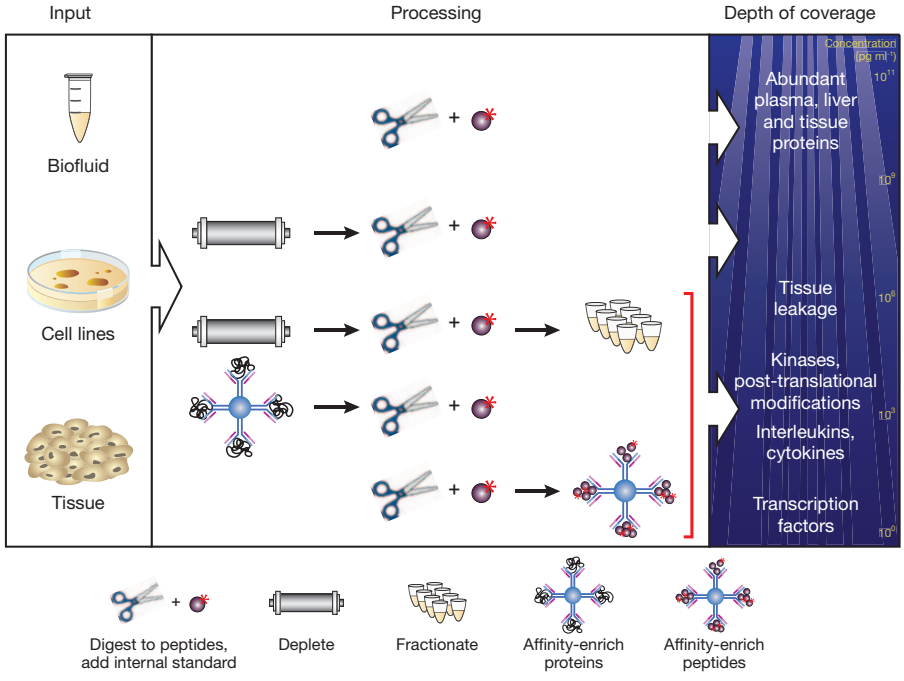

Figure 2 | Protein and peptide enrichment strategies to increase sensitivity and specificity of analyte detection in SID-MRM-MS. After extraction from tissues, cell lines or biofluids (left), proteins are digested into peptides. To achieve the highest detection confidence and measurement precision for peptides in these complex samples, synthetic versions of each analyte peptide containing an amino acid labeled with a stable isotope (for example, ^{15}N or ^{13}C) are added as internal standards (top line, center). Additional strategies may be required to achieve desired detection sensitivity in complex matrices. In plasma, depletion of the 12–70 most abundant plasma proteins using immunoaffinity depletion columns can result in limits of detection of ~100 ng ml^{-1} (second line, center), representing 'middling' depths in the plasma peptide ocean (right). Biomarkers in diseases such as cancer and cardiovascular disease are commonly present in the low nanogram per milliliter range and can be robustly detected by coupling depletion to limited fractionation (third line, center), albeit at the expense of reducing throughput and introducing variability and analytical complexity. A different strategy to enhance sensitivity relies on immunoaffinity reagents to enrich target analytes. In one implementation the intact protein is captured using an anti-protein antibody before digestion (fourth line, center). Alternatively, a proteotypic peptide derived from the protein can be captured using an anti-peptide antibody to the target peptide of interest (bottom line, center).

transcript expression amounts avoids this complication but identifies differences that are upstream of protein expression and so may be (literally) lost in translation. It has therefore become increasingly well recognized that the legions of differentially expressed analytes emerging from 'omics' discovery experiments are candidates, not biomarkers, and require additional credentialing using precise, quantitative methods in larger sample numbers, a process we have called "verification"[25].

The low probability that any particular biomarker candidate will be a functional biomarker suggests that a successful biomarker development enterprise will require a large proportion (ideally, all) of the candidates to be evaluated in verification studies. This highlights a serious mismatch between the verification capacity requirements of biomarker development and the still conventional approach of antibody-based verification. Though antibody-based methods, particularly well-characterized enzyme-linked immunosorbent assays, are suitable tools for verification, qualified antibodies are generally unavailable especially for new biomarker candidates and are prohibitively slow and costly to develop[26], leaving the vast majority of potential markers without a straightforward means for verification. Reversed-phase protein arrays[27] are potentially powerful verification tools but are limited to the few hundred antibodies of sufficient quality that are currently available. Technology solutions ranging from microbead suspensions to planar arrays coupled with microscale or nanoscale fluidics can be used to improve antibody multiplexing and sample economy[28,29] but do not address the fundamental problem of a dearth of highly specific affinity reagents and are probably better positioned for clinical deployment of limited multianalyte assays or real-time point of care tests than as verification solutions. Aptamer-based approaches have theoretical advantages of specific, stable binding without substantial crossreactivity and so hold promise for a range of proteomics applications[30,31], but their utility for biomarker candidate verification has not yet been convincingly demonstrated.

In contrast, targeted MS methods using MRM-MS approaches are in many respects ideally suited to candidate verification[25,32,33]. Analytically validated MRM assays that use internal standards can be configured for up to tens of proteins per month (depending on the extent of enrichment required for detection[23]), much faster than immunoassay development. Multiplexing is straightforward, with the ability to quantify hundreds of analytes in a single LC-MRM-MS run[5] and up to 50 different analytes by peptide immuno-MRM[20,22]. Multiplexing capacity may be increased and overall platform robustness may be enhanced through the use of higher flow rates (such as 0.5 milliliters per minute) with ultra-high-performance liquid chromatography rather than the nanoflow chromatography (≤300 nanoliters per minute) that is typically used in this application. The consequent reduction in overall sensitivity of quantification can be ameliorated by loading more sample onto the larger columns used with ultra-high-performance liquid chromatography. This substitution could facilitate verification as well as the later stages of clinical validation and implementation[34].

Several studies have now demonstrated the central importance and effective deployment of MRM-MS as a verification tool for candidates in the context of a comprehensive discovery-to-verification biomarker pipeline. Working in a mouse model of breast cancer, Whiteaker *et al.*[35] developed conventional MRM assays to 56 protein targets including 49 high-confidence biomarker candidates. The median analytical coefficient of variation for all assays was 5.7% at limits of quantification (LOQ), representative of the capabilities of this method. Using a single multiplexed assay, 46 of the targeted peptide analytes were above LOQ in >50% of plasma samples from mice bearing clinically apparent tumors; 30 proteins were significantly elevated in breast cancer and so were preliminarily verified in the context of the model. Addona *et al.* used MRM for preclinical verification of four new and several established markers of cardiovascular disease derived from short-term longitudinal coronary sinus and peripheral blood sampling of patients undergoing planned therapeutic myocardial injury[15]. Only three of the 52 candidates initially selected for quantitative assay development by MRM-MS had available reagents suitable for construction of enzyme-linked immunosorbent assays. The MRM-MS assays were used to measure the target analytes present at low nanogram per milliliter amounts in plasma.

Other groups have demonstrated the use of MRM for verification of previously described but insufficiently verified biomarker candidates in cardiovascular disease[36] and cancer[37]. Wang[38] used enrichment with variant-agnostic anti-protein antibodies coupled with MRM-MS to measure cancer-specific mutant proteins that were difficult to assess by other means. In the largest-scale demonstration of the potential of targeted MS for candidate verification yet undertaken, Hüttenhain[14] developed MRM assays for 1,157 proteins associated with cancer and measured 73 of the target proteins in a crude plasma digest, 182 in depleted plasma and 408 in urine. Using a subset of MRM assays that were directed to four of the five proteins that comprise the US Food and Drug Administration–approved, antibody-based OVA1 biomarker test for ovarian cancer risk, they showed significant ($P < 0.01$) differential expression in the expected direction in a set of 83 plasma samples from 67 individuals with ovarian cancer and 16 with benign ovarian tumors. In the same analysis they monitored 30 additional proteins for which there was evidence of association with ovarian cancer, showing significant ($P < 0.01$) cancer versus control differences for 19 of them and demonstrating the use of MRM both to confirm and to extend established antibody-based assays.

Clinical implementation of targeted MS

To many in the field of proteomics, the pivotal question has been not whether but when targeted MS will be broadly adopted as a tool for clinical measurement of protein analytes, supplementing not supplanting the current use of immunoassays. This is less hubris or provincialism than a practical assessment of the advantages of the method, similar to what motivated its adoption for small-molecule measurements a generation ago. There are no fundamental technical obstacles to its adoption in clinical laboratories. Data demonstrating key analytical requirements such as low assay coefficients of variation, interlaboratory reproducibility and means to accurately measure amounts of targeted analytes have continued to accrue[9,15,16,20–22]. Clinical translation is therefore largely a problem of engineering rather than radical invention.

The first major inroads have now begun in the predicted fashion—namely, in the context of a clinically important analyte that has been recalcitrant to conventional immunoassays and with assay deployment initially centralized to reference laboratories. The seminal instance involved development of a peptide immunoaffinity MRM-MS assay for thyroglobulin[39]. Thyroglobulin levels are used to monitor disease activity in some subtypes of thyroid cancer. However, 20% of the population has circulating anti-thyroglobulin

antibodies that interfere with the immunoassay and result in erroneous test results. The MS-based method involves digestion of all proteins to peptides before capture of a unique peptide from thyroglobulin using an anti-peptide antibody, thereby removing interferences from anti-thyroglobulin antibodies. Immuno-MRM assays for thyroglobulin are being developed by ARUP Laboratories and Quest Diagnostics, both of which are involved in the large-scale development and application of clinical tests. Lab-based immuno-MRM assays have been developed for similar reasons to measure parathyroid hormone in blood[3] and total pepsin or pepsinogen in saliva[21].

Future developments will likely include the development of diagnostics and clinical predictors based on targeted MS-based detection of multiplexed panels of proteins or modified peptides that are either difficult or costly to translate into conventional immunoassays because suitable antibodies prove too difficult to produce or because the multiplex level required is greater (for example, >5) than immunoassays can readily support. Such tests will face the same requirements for clinical adoption as any other assay (reagents for calibration and standardization, demonstration of accuracy and robustness, and so on), with potential new elements such as standards to assess the digestion efficiency of the clinical sample. More widespread and general deployment, for instance, to hospital laboratories and for a wider range of protein measurements, seems a more distant eventuality. In addition to a greater number of validated targets and high-performance assays based on qualified reagents, the development of robust, turnkey instruments is needed as well as more intelligent software for robust data analysis without expert oversight. This will require cooperation from the MS vendors who will need to clearly see the opportunity before being willing to make the investments. Participation and cooperation from regulatory agencies will also be essential in promoting development of new tests using new devices.

Proteomics continues to rapidly evolve through invention of improved sample-handling methods, use of higher-efficiency chromatography and the introduction of faster, more sensitive and precise MS technologies. These advances have greatly increased sample-analysis throughput while reducing undersampling and providing more consistent and reproducible peptide measurements. As the capabilities of MS-based proteomics technologies continue to improve, the line between what constitutes 'discovery' and 'verification' is blurring, leading to what has been recently described as 'platform convergence'[33]. High-resolution, high-mass-accuracy MS systems once confined to the data-dependent discovery realm are increasingly being used for targeted MS analysis with the benefits of higher specificity and lower potential for false positive identifications[40–42]. These attributes will be of particular importance to clinical adoption of targeted MS approaches. A happy consequence is the anticipated elimination of boundaries between discovery and verification, replaced by a continuum enabled by a single integrated LC-MS/MS platform. This may define the point at which these maturing technologies reach adulthood.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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