

Foreword

Chapter 1
Protein quantitation: advancing
research with MS-based
methods

Chapter 2
Global and unbiased protein
quantitation with MS

Chapter 3
Using quantitative MS to
examine protein networks

Chapter 4
High-throughput proteomics
to identify cancer biomarkers

Chapter 5
How biologists can access
quantitative MS

Epilogue
Continued advancements in
MS technology to support
researchers

A biologist's guide to mass spectrometry-based protein quantitation

Table of Contents

Foreword

Chapter 1
Protein quantitation: advancing research with MS-based methods

Chapter 2
Global and unbiased protein quantitation with MS

Chapter 3
Using quantitative MS to examine protein networks

Chapter 4
High-throughput proteomics to identify cancer biomarkers

Chapter 5
How biologists can access quantitative MS

Epilogue
Continued advancements in MS technology to support researchers

Foreword

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Chapter 1

Protein quantitation: advancing research with MS-based methods

Dr. Nick Seyfried
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Chapter 2

Global and unbiased protein quantitation with MS

Dr. Erik Johnson
Emory University School of Medicine

Chapter 3

Using quantitative MS to examine protein networks

Dr. Nevan Krogan
University of California, San Francisco

3

4

8

10

Chapter 4

High-throughput proteomics to identify cancer biomarkers

Dr. Wilhelm Haas
Massachusetts General Hospital and Harvard Medical School 13

Chapter 5

How biologists can access quantitative MS

Dr. Allis Chien
Stanford University 16

Epilogue

Continued advancements in MS technology to support researchers

Daniel Lopez-Ferrer
Thermo Fisher Scientific 19



Foreword

Chapter 1
Protein quantitation: advancing research with MS-based methods

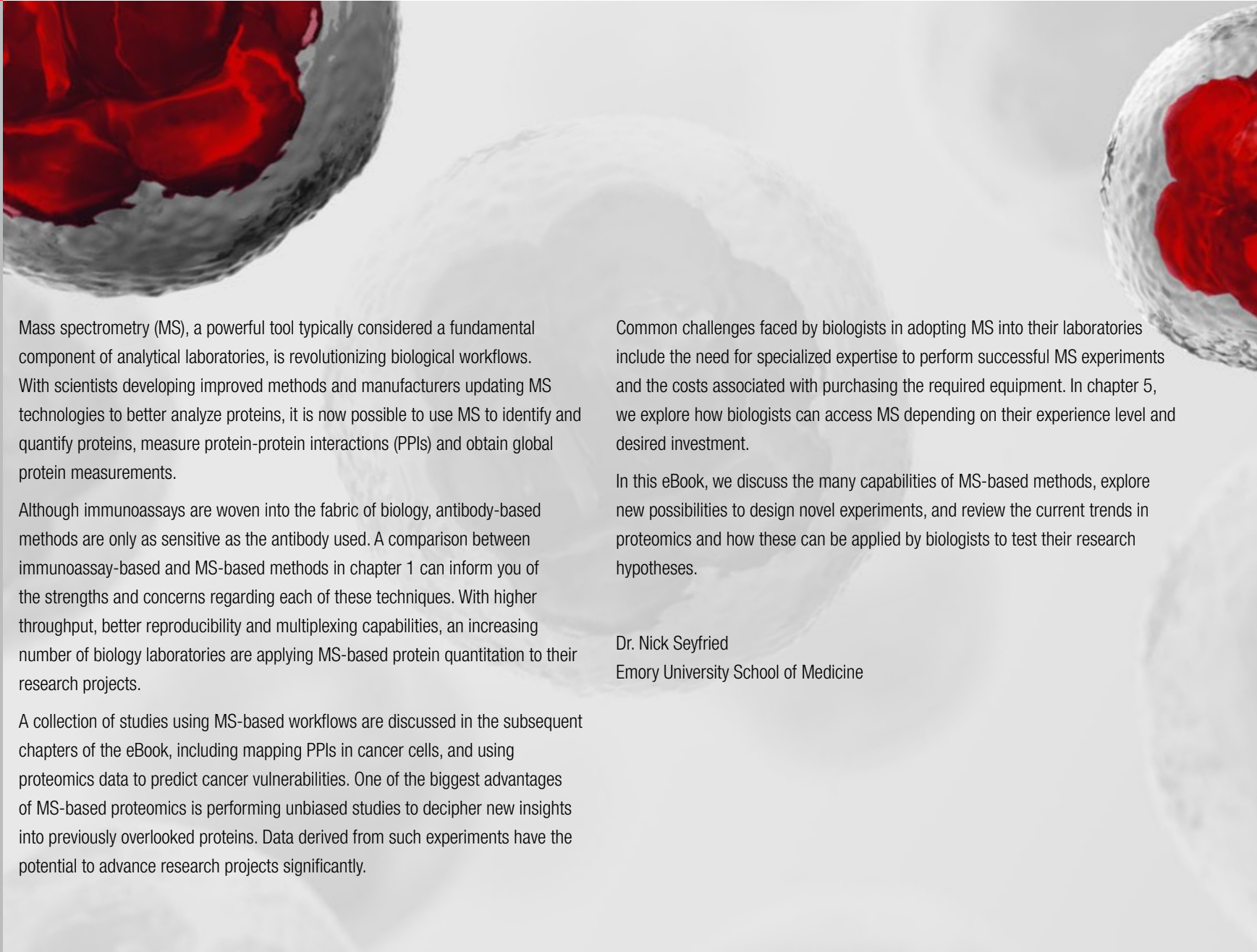
Chapter 2
Global and unbiased protein quantitation with MS

Chapter 3
Using quantitative MS to examine protein networks

Chapter 4
High-throughput proteomics to identify cancer biomarkers

Chapter 5
How biologists can access quantitative MS

Epilogue
Continued advancements in MS technology to support researchers



Mass spectrometry (MS), a powerful tool typically considered a fundamental component of analytical laboratories, is revolutionizing biological workflows. With scientists developing improved methods and manufacturers updating MS technologies to better analyze proteins, it is now possible to use MS to identify and quantify proteins, measure protein-protein interactions (PPIs) and obtain global protein measurements.

Although immunoassays are woven into the fabric of biology, antibody-based methods are only as sensitive as the antibody used. A comparison between immunoassay-based and MS-based methods in chapter 1 can inform you of the strengths and concerns regarding each of these techniques. With higher throughput, better reproducibility and multiplexing capabilities, an increasing number of biology laboratories are applying MS-based protein quantitation to their research projects.

A collection of studies using MS-based workflows are discussed in the subsequent chapters of the eBook, including mapping PPIs in cancer cells, and using proteomics data to predict cancer vulnerabilities. One of the biggest advantages of MS-based proteomics is performing unbiased studies to decipher new insights into previously overlooked proteins. Data derived from such experiments have the potential to advance research projects significantly.

Common challenges faced by biologists in adopting MS into their laboratories include the need for specialized expertise to perform successful MS experiments and the costs associated with purchasing the required equipment. In chapter 5, we explore how biologists can access MS depending on their experience level and desired investment.

In this eBook, we discuss the many capabilities of MS-based methods, explore new possibilities to design novel experiments, and review the current trends in proteomics and how these can be applied by biologists to test their research hypotheses.

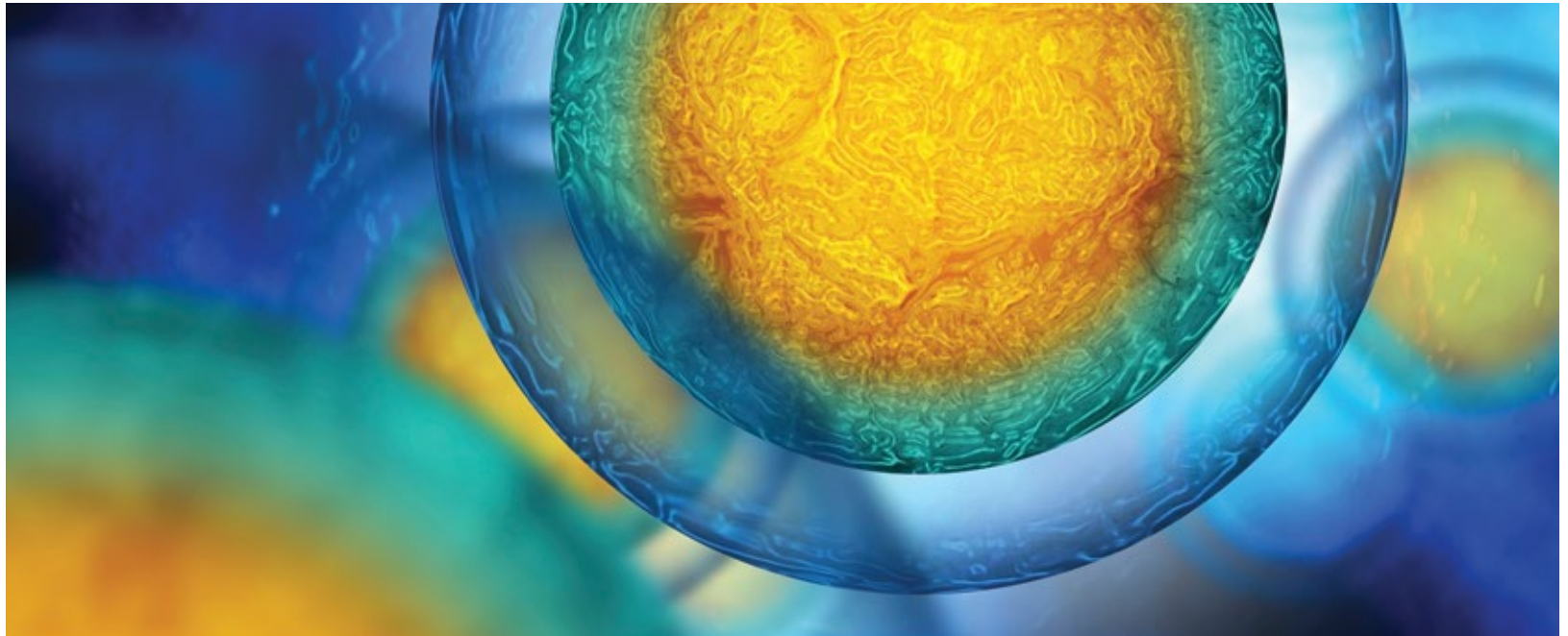
Dr. Nick Seyfried
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Table of Contents

Foreword

**Chapter 1
Protein quantitation: advancing research with MS-based methods**Chapter 2
Global and unbiased protein quantitation with MSChapter 3
Using quantitative MS to examine protein networksChapter 4
High-throughput proteomics to identify cancer biomarkersChapter 5
How biologists can access quantitative MSEpilogue
Continued advancements in MS technology to support researchers

Protein quantitation: advancing research with MS-based methods

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Introduction

In recent years, MS has become an indispensable tool for the identification of proteins, post-translational modifications, PPIs and global protein measurements. With the expanding scale of research projects, biologists often desire more in-depth proteome coverage and better insights into protein biology. Compared to other protein quantitation methods, MS offers immense promise to advance proteomics, and accelerate both basic and translational research.

Choosing between MS-based and antibody-based protein quantitation

Western blots and enzyme-linked immunosorbent assays (ELISAs), are commonly used for protein quantitation (1), yet rely on the availability of highly specific antibodies for the targets of interest. This reliance can result in poor selectivity and reproducibility, so why are these techniques so widely used?

Immunoassays and MS-based methods, each bring evident strengths to a biologist's workflow. At the same time, we also need to consider some of the limitations posed by each. Below is a summary of the strengths and concerns of MS-based and antibody-based protein quantitation methods (1):

Table of Contents

Foreword

**Chapter 1
Protein quantitation: advancing research with MS-based methods**Chapter 2
Global and unbiased protein quantitation with MSChapter 3
Using quantitative MS to examine protein networksChapter 4
High-throughput proteomics to identify cancer biomarkersChapter 5
How biologists can access quantitative MSEpilogue
Continued advancements in MS technology to support researchers

Protein quantitation: advancing research with MS-based methods

Strengths of immunoassay-based protein quantitation

- **Easy to use and widely accepted:** Being relatively easy to perform, immunoassays need minimal training. They are approved for use across a range of basic and clinical research applications. Additionally, their widespread use means that immunoassay workflows are often fully optimized, making troubleshooting more routine.
- **Low equipment cost:** Western blotting equipment and supplies have a fairly low price point. Requiring a basic microplate reader, immunoassays can also be performed without the need for a significant financial investment.
- **Good sensitivity, especially with highly abundant proteins:** Immunoassay readouts, often subjected to signal amplification, provide a high level of sensitivity, especially when antibodies are specific, and the protein of interest is relatively abundant.

Common concerns with immunoassay-based protein quantitation

- **Poor selectivity due to cross-reactivity:** Selectivity of an immunoassay is only as good as that offered by the antibody. Generating highly selective antibodies, with ligand binding efficiencies maintained across several production batches is often difficult and time-consuming.
- **Limited multiplexing capabilities:** Although multiplexing options are available in immunoassays, antibody cross-reactivity and the time-intensive nature of protocols pose challenges. The need for specialized plate readers to process data can also cause limitations.
- **Higher sample volume requirement:** A typical immunoassay requires approximately 100-200 μL of sample volume, making it difficult to analyze and replicate scarce biological samples.
- **Poor reproducibility:** Due to the highly manual nature of the immunoassay workflow, there is a relatively high level of intra- and inter-assay variability, especially with crucial steps, such as antibody incubation times.
- **Lengthy assay times:** Each immunoassay along with antibody incubation and washing steps can take approximately 2-3 hours to complete.

Strengths of MS-based protein quantitation

- **Higher selectivity:** The combination of high-resolution separation offered by liquid chromatography (LC) and the high-resolution accurate mass (HRAM) measurements by modern mass spectrometers provide improved levels of selectivity for even low abundance proteins.
- **Higher sample throughput:** Sample separation by LC and subsequent MS analysis occur concurrently, processing samples much faster. Automation compatibility boosts the throughput rates even further.
- **Better reproducibility:** Unlike immunoassays, the steps involved in MS-based workflows are more precise and better optimized for each experimental output, eliminating variability between runs or even between different users, thereby yielding better data reproducibility.
- **Multiplexing capabilities:** MS protocols inherently offer multiplexing as multiple analytes can be examined at the same time, especially with tandem MS. For instance, thousands of analytes can be measured in a large-scale proteomics study using data-independent acquisition. Plus, developments in reagents and MS methods further enhance multiplexing in quantitative proteomics.
- **Extensive range of analytes:** MS-based methods can identify and quantify a variety of compounds, both organic and inorganic, so long as the molecule can be ionized.

Common concerns with MS-based protein quantitation

- **Significant investment in equipment and setup:** One of the major concerns with MS-based systems is the high cost of LC and MS. With maintenance costs and training requirements, setting up a fully functional LC-MS workflow requires a substantial investment upfront.
- **Need for expertise and staff training:** Unlike immunoassays, LC-MS workflows aren't necessarily intuitive or easy to perform without staff training. Sophisticated instrument setup, elaborate troubleshooting steps and difficult-to-interpret data make MS-based workflows challenging for biologists to adopt.
- **Complex sample preparation steps:** The complexities of certain samples, such as plasma with a huge dynamic range of proteins, make it necessary to carefully optimize sample preparation steps. Moreover, not all buffers used in protein sample preps are compatible with MS.



Table of Contents

Foreword

**Chapter 1
Protein quantitation: advancing research with MS-based methods**Chapter 2
Global and unbiased protein quantitation with MSChapter 3
Using quantitative MS to examine protein networksChapter 4
High-throughput proteomics to identify cancer biomarkersChapter 5
How biologists can access quantitative MSEpilogue
Continued advancements in MS technology to support researchers

Protein quantitation: advancing research with MS-based methods

Although MS-based experiments haven't displaced immunoassays, the recent advances in MS technology have provided scientists with simplified workflows. The undeniable advantages of MS over antibody-based methods have also encouraged biologists to embrace, adopt and develop MS-based workflows for protein identification and quantitation.

Types of MS-based protein quantitation

Researchers can perform protein quantitation by using either peptide labeling or label-free methods. An important question that researchers need to ask before choosing between the different MS-based approaches is 'what level of proteome coverage do I need to answer my research question?'

An important question that researchers need to ask before choosing between the different MS-based approaches is 'what level of proteome coverage do I need to answer my research question?'

Types of MS-based protein quantitation	Details	Considerations
<i>Peptide labeling</i>		
Metabolic labeling		
Stable isotope labeling with amino acids in cell culture (SILAC)	Cells are treated with either light (natural) or heavy (labeled) amino acids. The mass differences in the resulting peptides yield relative protein quantitation data.	<ul style="list-style-type: none"> • Easy to perform • Limited to cell cultures
Chemical labeling		
Isobaric labeling with tandem mass tags (TMTs)	The TMTs label primary amino groups (the N-terminus and lysine side chains) of peptides. In the presence of higher-energy collisional dissociation (HCD) or synchronous precursor selection (SPS) in tandem MS, the tags are cleaved to release reporter ions of different masses that are ultimately detected by the mass spectrometer.	<ul style="list-style-type: none"> • Offers multiplexing capabilities of up to 16-plex • Ratio distortion caused by reporter ions can affect accuracy
<i>Label-free quantitation</i>		
	Proteins are digested into peptides and separated by LC. Precursor protein peptides from a particular m/z window are further dissociated in a tandem MS/MS. Experiments can be targeted using data-dependent acquisition or with data-independent acquisition.	<ul style="list-style-type: none"> • Can be used for protein identification and protein quantitation simultaneously • Results in a better depth of proteome coverage • Has inherent multiplexing with the possibility of identifying thousands of different proteins in a single run



Table of Contents

Foreword

**Chapter 1
Protein quantitation: advancing
research with MS-based
methods**Chapter 2
Global and unbiased protein
quantitation with MSChapter 3
Using quantitative MS to
examine protein networksChapter 4
High-throughput proteomics
to identify cancer biomarkersChapter 5
How biologists can access
quantitative MSEpilogue
Continued advancements in
MS technology to support
researchers

Protein quantitation: advancing research with MS-based methods

Setting up for success: The basic requirements for MS protein quantitation

A generic MS-based proteomics experiment involves the following stages:

1. isolation of proteins either by fractionation or affinity selection,
2. enzymatic degradation of proteins into peptides,
3. high-pressure LC (HPLC) systems to separate the peptides and elute into an electrospray ion source,
4. modern, high-resolution mass spectrometers capable of accurate mass measurements, such as the Thermo Scientific™ Orbitrap™ mass analyzer, with tandem MS capabilities,
5. state-of-the-art software to perform identification and quantitation.

Successful MS-based protein quantitation relies on high-performing instruments and optimized protocols. It is crucial to have access to a high-resolution mass spectrometer capable of tandem MS to detect isobaric reporter tags or perform label-free analysis, as well as a robust LC system to separate peptides in a reproducible manner. Using reliable external standards to run at the beginning, middle and end of experiments ensures reproducibility. Additionally, methods to isolate proteins from complex biological mixtures and sample preparation prior to MS are key steps that can influence the success of the experiment.

Adopting MS: How to get started

With a substantial number of basic requirements, dozens of variables to control and a challenging workflow, getting started with MS-based protein quantitation can seem overwhelming. Once mastered, however, MS experiments can provide invaluable insights into research projects involving proteomics, biomarker discovery and beyond.

To take the first step in adopting MS, it's important to get familiar with the acronyms and terminology used in the field to better appreciate published findings. Consider attending a training course to get better acquainted with a specialized proteomics software, such as MaxQuant, to learn data analysis and visualization. Tap into the pool of established MS experts at your organization or at conferences to begin discussions on experimental design and initiate future collaborations.

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Foreword

Chapter 1
Protein quantitation: advancing
research with MS-based
methods

Chapter 2
**Global and unbiased protein
quantitation with MS**

Chapter 3
Using quantitative MS to
examine protein networks

Chapter 4
High-throughput proteomics
to identify cancer biomarkers

Chapter 5
How biologists can access
quantitative MS

Epilogue
Continued advancements in
MS technology to support
researchers

Global and unbiased protein quantitation with MS

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Introduction

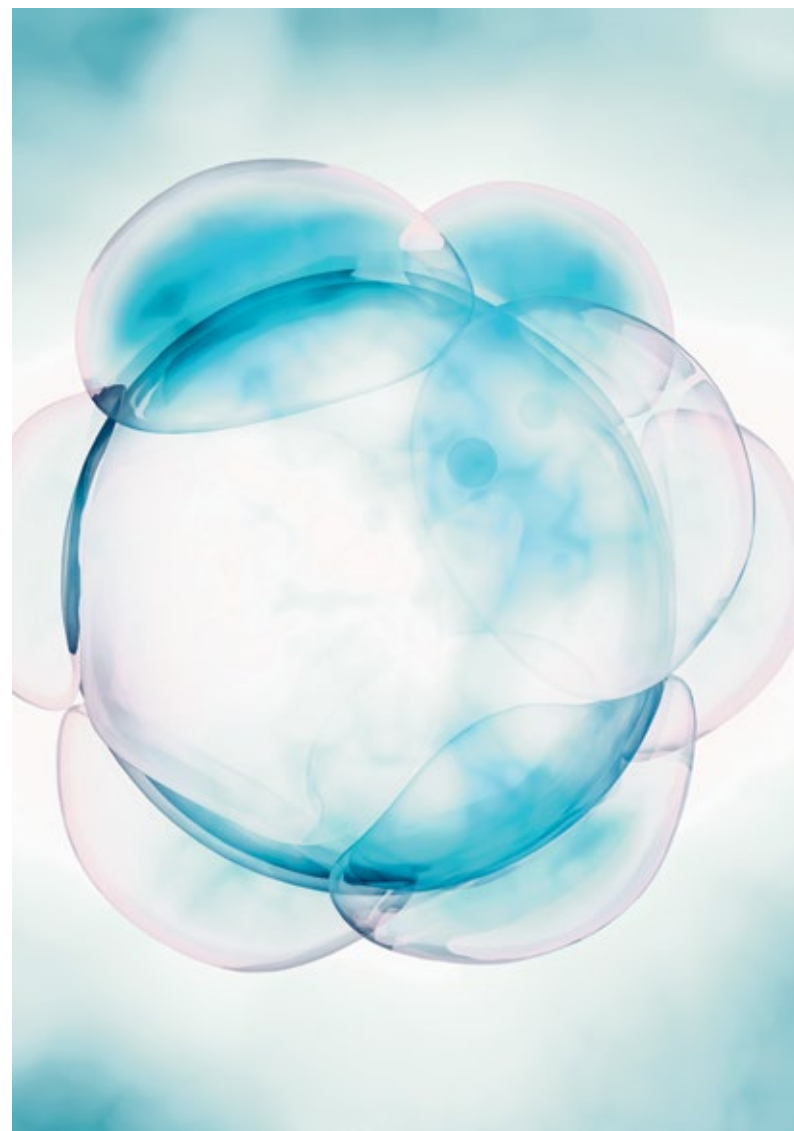
Researchers typically perform protein identification or quantitation experiments to answer different research questions. Protein quantitation experiments are performed to examine the effect of genetic, environmental or pharmacological manipulation in either cells or animal models, whereas protein identification helps determine which protein is responsible for a genotypic or phenotypic effect.

MS can perform both protein identification and quantitation, making it easier to ask both broad and specific research questions. Moreover, proteomics offers a global perspective on protein biology compared to more targeted approaches, such as antibody-based immunoassays, allowing for additional insights into the biological system under study.

The value of unbiased protein quantitation

A biased approach to protein quantitation starts with having a preconceived notion about which proteins will change in a diseased state or in response to a stimulus. Biased approaches, typically using antibody-based methods, target only a limited number of proteins. In complex diseases such as cancer or Alzheimer's Disease (AD), multiple protein networks contribute to the underlying pathophysiology. Having a biased or targeted approach to study these diseases in the discovery phase can limit the scope of one's subsequent hypotheses.

An unbiased analysis with MS, especially using label-free quantitative (LFQ) proteomics, offers an opportunity to identify causal effects at a systems-level rather than only learning the effects of selected proteins. MS-based proteomics provides a global, unbiased, high-throughput protein analysis capable of covering the whole proteome, ultimately transforming the approach towards basic and clinical discovery.



Global and unbiased protein quantitation with MS

Table of Contents

Foreword

Chapter 1
Protein quantitation: advancing research with MS-based methods

**Chapter 2
Global and unbiased protein quantitation with MS**

Chapter 3
Using quantitative MS to examine protein networks

Chapter 4
High-throughput proteomics to identify cancer biomarkers

Chapter 5
How biologists can access quantitative MS

Epilogue
Continued advancements in MS technology to support researchers

History of MS-based protein quantitation

The early days of protein quantitation involved counting protein spectral matches between two samples, but this method was biased towards abundant proteins. As chromatography methods improved, offering better separation of peptides, so did the quantitation capabilities. With LFQ proteomics, made possible by advances in MS technology, researchers can now focus on even lower abundant proteins in a complex protein mixture. To date, developments in protein quantitation methods have allowed researchers to identify around 10,000 proteins in individual samples using LFQ when previously only ~100 proteins could be identified.

SILAC and other labeling techniques offer a direct comparison of internal heavy labeled peptides to examine fold change differences. Isobaric labeling using TMTs, with its ability to tag multiple proteins in a single run, enables substantial multiplexing capabilities. With current efforts to further develop TMTs and expand multiplexing, this can be considered the next generation of MS-based proteomics.

Current trend: deeper proteome coverage with isobaric tags

Isobaric tags are becoming more routine in MS-based proteomics, displacing SILAC labeling methods. A recent experiment characterized an optimized workflow for global proteome analysis using isobaric tags across three independent laboratories, which enabled the assessment of two distinct breast cancer subtypes derived from patient xenograft models (1).

With 10,000 proteins quantified per sample, the method also allowed scientists to distinguish human-derived and mouse-derived proteins from the xenograft tissue. Showing <7% deviation across replicates and laboratories, the method successfully maintained quantitative reproducibility and depth in proteome analysis.

In the quest to gain better coverage of the proteome, researchers are updating methods to overcome previously experienced limitations. In a proteomic analysis of human brain tissues corresponding to asymptomatic and symptomatic stages of AD, despite identifying >5,000 proteins, only 2,736 proteins using a label-free approach were previously quantified (2).

The reduction in quantifiable proteins, a common problem with data-dependent acquisition (DDA) in MS, can be attributed to stochastically choosing ions without maintaining consistency across runs or not effectively matching the peptide precursors across the runs – leaving out parts of the proteome. To resolve the “missing value” problem that limits quantifiable proteins, the team developed a new analysis pipeline consisting of isobaric TMTs, offline prefractionation and mass spectrometers with MS³ capabilities (3). This ultimately doubled the depth of brain proteome coverage, with 6,533 proteins from within the same sample cohort quantified.

Data-independent acquisition (DIA) methods in proteomics also allows a comparable depth of proteome coverage without the added costs of chemical tags. In DIA, all analytes in a predefined *m/z* window are fragmented and subsequently analyzed. Not confined by selected precursors, DIA fragments every peptide in the sample within the window, providing an unbiased approach to protein profiling.

Unbiased biomarker discovery using MS-based methods

A single MS experiment can yield unexpected biomarkers, setting the foundation for several resulting projects. Serendipitous findings from a one-time MS analysis have the potential to change the focus of research laboratories. For example, the discovery of proteolytic sites on tau protein shifted the entire research focus of the Ye laboratory at Emory University to asparagine endopeptidase (4). Using multimodal approaches to look at the genome, transcriptome and proteome can help dissect disease-related pathways in multicellular tissues and in different cell types, further advancing biomarker research.

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Using quantitative MS to examine protein networks

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Table of Contents

Foreword

Chapter 1
Protein quantitation: advancing research with MS-based methods

Chapter 2
Global and unbiased protein quantitation with MS

Chapter 3
Using quantitative MS to examine protein networks

Chapter 4
High-throughput proteomics to identify cancer biomarkers

Chapter 5
How biologists can access quantitative MS

Epilogue
Continued advancements in MS technology to support researchers

Introduction

Proteins rarely act as isolated species in carrying out physiological functions. To better understand the role of proteins in biological systems as well as in disease pathogenesis, it is important to study proteins in the context of PPIs. In studying PPIs, the two commonly used methods include yeast two-hybrid screening (Y2H) and affinity purification coupled with MS (AP-MS). The Y2H method involves designing individual bait and prey plasmids for all interactions to be tested, making

the process binary and inherently biased towards bait and preys included in the screen. Additionally, important PPIs might be missed if they depend on post translational modifications not present in the exogenous yeast expression system.

MS-based methods, on the other hand, offer an unbiased way to screen for endogenous PPIs in mammalian cells. By coupling protein enrichment techniques with the quantitative features of MS, it is possible to target proteins of interest and perform large-scale interactome studies.

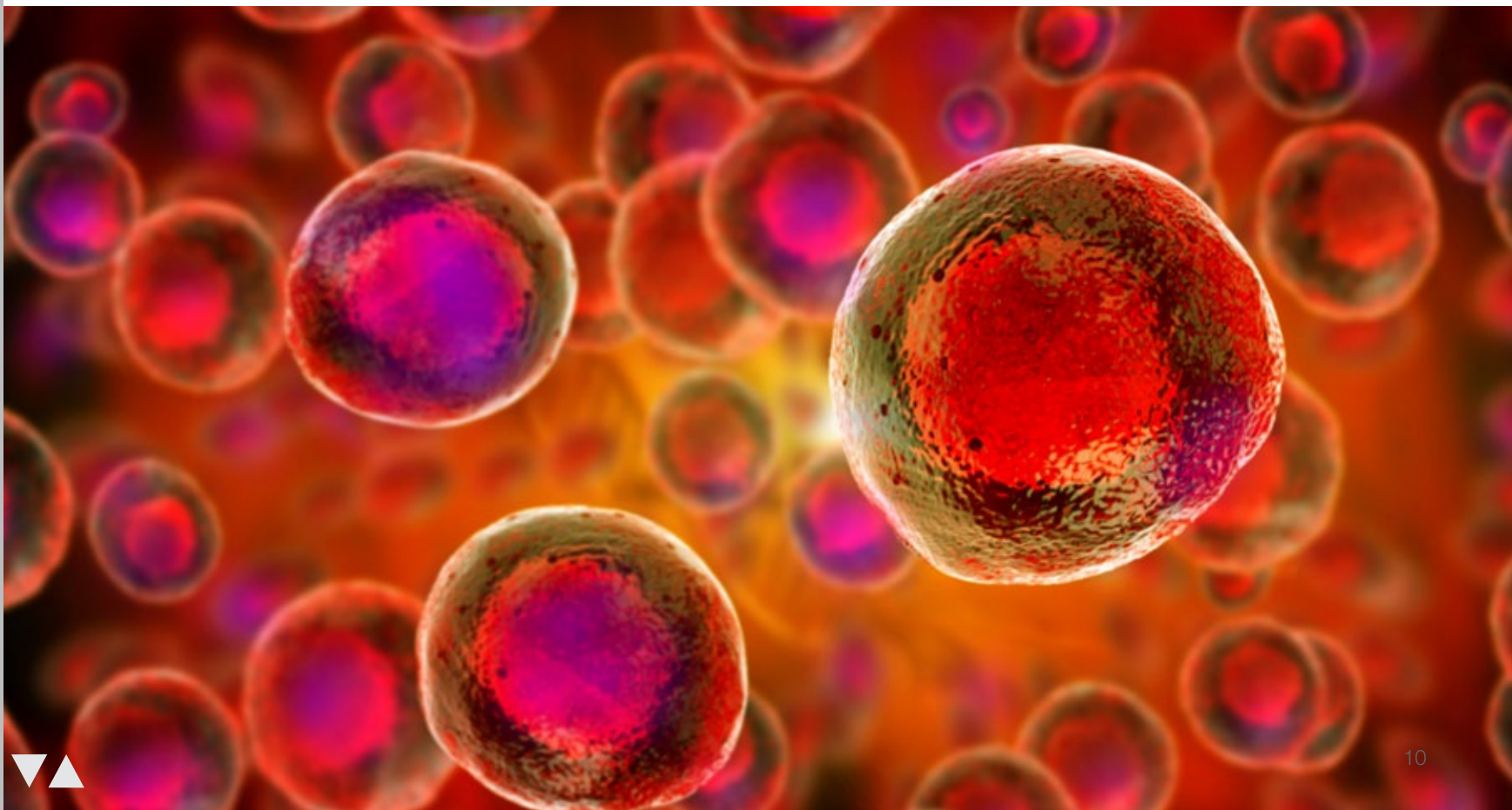


Table of Contents

Foreword

Chapter 1
Protein quantitation: advancing research with MS-based methods

Chapter 2
Global and unbiased protein quantitation with MS

Chapter 3
Using quantitative MS to examine protein networks

Chapter 4
High-throughput proteomics to identify cancer biomarkers

Chapter 5
How biologists can access quantitative MS

Epilogue
Continued advancements in MS technology to support researchers

Using quantitative MS to examine protein networks

Studying protein-protein interactions using MS-based methods

With MS serving as the foundation for protein identification and quantitation, several technologies, coupled with MS, facilitate the study of stable and transient PPIs. Importantly, these techniques allow for the purification not only of direct interactors like with the Y2H method, but the identification of larger protein complexes co-purifying with the protein of interest.

- **Affinity purification mass spectrometry (AP-MS)**

Affinity purification helps capture proteins of interest by using affinity tags, such as FLAG-tag, Strep-tag, HA-tag or green fluorescent protein (GFP). The protein of interest, fused to the tag of choice, can then be captured as bait using a small molecule or antibody bound to a matrix, such as sepharose beads. By using gentle lysis and wash buffers, interacting partners are retained as prey bound to the protein of interest. Adding a second purification step with proteins that are doubly tagged can introduce more stringency to the set of purified proteins. MS analysis is then used to identify the interacting partners and followed by computational analysis to distinguish true interactors from co-purifying background.

In 2006, AP-MS was first used to identify 7,123 PPIs involving 2,708 proteins in yeast, covering 72% of the yeast proteome (1). Improvements in high-throughput cloning and MS analysis methods have enabled researchers to significantly scale up these efforts. For example, the BioPlex 3.0 interactome currently contains almost 120,000 PPIs between 15,000 human proteins in 293T cells (2).

Additionally, researchers have used AP-MS extensively to study interactions between pathogens and their human host cells. For example, recently completed PPI maps for Ebola virus led to the discovery of a peptide that severely decreases virus replication in lab settings, as well as almost 200 additional potential drug targets to fight Ebola infection (3).

- **Proximity-labeling mass spectrometry (APEX-MS)**

Transient PPIs are hard to capture by traditional AP-MS experiments, a drawback that can be remedied by including proximity-labeling techniques in the workflow. Here, the protein of interest is fused to an enzyme that facilitates the promiscuous

covalent labeling of proteins with an exogenously supplied small molecule substrate.

For example, ascorbic acid peroxidase (APEX) in combination with phenol-biotin catalyzes the covalent biotinylation of interacting proteins in a radius of 20 nm upon treatment with H₂O₂. Enrichment of biotinylated proteins can then be performed under more stringent conditions than regular AP would allow. With a significantly faster rate of labeling than other available enzymes, APEX offers a higher temporal resolution to identify PPIs in response to stimuli.

- **Cross-linking mass spectrometry (XL-MS)**

Weak or transient PPIs that may be lost during native AP-MS experiments can also be resolved using chemical crosslinkers, which join components of interacting protein partners to “lock them” in place. When combined with AP, XL-MS offers the study of less stable PPIs.

The biggest strength of XL-MS is that it provides information on which members of a co-purifying protein complex are in direct physical contact, and thus, serves as a source of complementary information to the wider networks obtained by traditional AP-MS studies. Through the location of individual crosslinks, it also reveals details of protein-protein interfaces, thereby providing medium-resolution structural information.

Importance of network mapping: A combinatorial strategy for studying diseases

Quantitative MS offers invaluable translational avenues when combined with genetic, structural and computational methods. MS-based proteomics and CRISPR-based genetics can examine the underlying biology of different diseases, such as infectious diseases, cancer and neurological disorders.

Combinatorial approach case study: The Cancer Cell Map Initiative

Despite increased knowledge about cancer genomes, what mutations drive an individual patient's cancer and how they modulate pathogenesis remains largely unknown. With a few exceptions, most mutations are rare and vary across patients



Using quantitative MS to examine protein networks

Table of Contents

Foreword

Chapter 1
Protein quantitation: advancing research with MS-based methods

Chapter 2
Global and unbiased protein quantitation with MS

Chapter 3
Using quantitative MS to examine protein networks

Chapter 4
High-throughput proteomics to identify cancer biomarkers

Chapter 5
How biologists can access quantitative MS

Epilogue
Continued advancements in MS technology to support researchers

with the same phenotypical cancer. The fact that different mutations across tumors target the same hallmark molecular pathways, however, emphasizes the need to study the biological underpinnings of cancer pathogenesis.

The goal of The Cancer Cell Map Initiative (CCMI) (4) is to serve as a resource that can be used for cancer genome interpretation. The project aims to systematically identify cancer-driving protein networks and pathways to be studied in greater detail. To this end, cell maps are created by comprehensively identifying protein-protein and genetic interactions in healthy as well as cancer cells.

The CCMI project combines a cancer's phenotype with its genotype by using data from protein interaction studies and CRISPR-based genetic interaction screens to offer a better understanding of molecular cancer networks. By performing functional validation and MS-based analysis of key protein interactions, the resulting cell maps promise to uncover the details of tumor biology.

Mindset shifts in protein quantitation approaches

MS-based proteomics, complemented with genomics and computational analysis, empowers scientists to study the underlying biology of a variety of diseases.

The powerful capability of MS in quantifying proteins and providing insights into changes in PPIs based on different disease states offers clear advantages over yeast two-hybrid screens, which only detect binary interactions among a pre-defined set of proteins.

Although requiring the right technical expertise, unbiased quantitative MS methods allow scientists to study disease-associated mutations in different contexts, providing a more holistic picture of a disease's underlying biology and mechanism. Including quantitative MS approaches to study changes in protein abundance and post translational modifications, such as phosphorylation and ubiquitination, further completes this picture. Future efforts to develop intuitive software and stronger algorithms can alleviate the complexity associated with MS data analysis, opening it up to an even more diverse range of applications.

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Table of Contents

Foreword

Chapter 1
Protein quantitation: advancing research with MS-based methods

Chapter 2
Global and unbiased protein quantitation with MS

Chapter 3
Using quantitative MS to examine protein networks

**Chapter 4
High-throughput proteomics to identify cancer biomarkers**

Chapter 5
How biologists can access quantitative MS

Epilogue
Continued advancements in MS technology to support researchers

High-throughput proteomics to identify cancer biomarkers

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Introduction

MS-based proteomics enables the study of many aspects of the proteome, including protein concentration, post-translational modification and PPIs. As the proteome is a cell's main functional entity, MS has the capacity to serve as a major tool in understanding the molecular underpinnings of complex diseases, such as cancer. However, most systematic efforts in studying cancer were built solely on genomics methods: DNA and RNA sequencing.

The historical performance gap between genomics and proteomics can be attributed to the dramatically smaller sample throughput in proteomics. Introducing multiplexing in proteomics, most often based on isobaric labeling, helped overcome this caveat. Currently, up to 16 samples can be quantified simultaneously, allowing a proteome-wide protein quantification in less than three hours per sample.

Here, we discuss the multiplexing capabilities of proteomics and how high-throughput proteomics is currently being used to define the cancer proteome.

The evolution of MS-based proteomics

In the early stages of MS, proteomics involved the identification of proteins isolated and fractionated over gels, providing advantages over then used technologies, such as Edman sequencing. Although MS-based proteomics was fast developed into a technology allowing whole proteome quantification and mapping of all types of post-translational modifications, its main role as the driving force in biological studies remained limited to gel band analyses as genomics methods continued to dominate the scientific landscape.

The situation changed through the rise of isobaric labeling methods and developments in MS instrumentation capabilities, which further expanded MS-based proteomics analysis. One isobaric labeling strategy is based on TMTs used to label peptides on the N-terminus and lysine side chains. The elegance of this strategy is that even upon pooling multiple proteome samples labeled with multiple

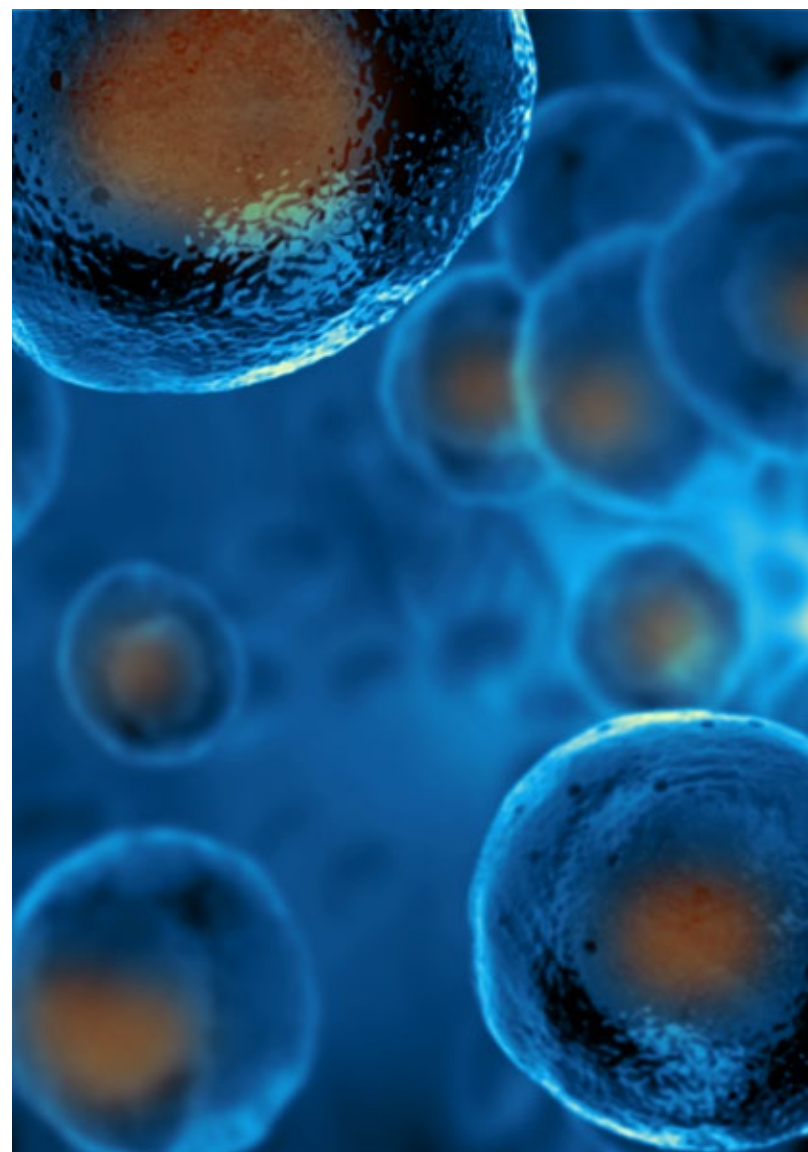


Table of Contents

Foreword

Chapter 1
Protein quantitation: advancing research with MS-based methods

Chapter 2
Global and unbiased protein quantitation with MS

Chapter 3
Using quantitative MS to examine protein networks

Chapter 4
High-throughput proteomics to identify cancer biomarkers

Chapter 5
How biologists can access quantitative MS

Epilogue
Continued advancements in MS technology to support researchers

High-throughput proteomics to identify cancer biomarkers

different reagents, only one signal per peptide is shown in the full MS spectrum. Subjecting the ions to fragmentation produces unique reporter ions representing each individually labeled sample.

Isobaric labeling makes the full MS data more manageable, allowing the isolation and fragmentation of multiple peptide ions during analysis. This can be used to quantify relative protein abundance across multiple experimental conditions, providing improved scalability. Being compatible with proteins obtained from cells, tissues or biological fluids, TMTs are applicable to a variety of projects.

Multiplexing quantitative proteomics for a higher throughput

Multiplexing enables researchers to process many samples treated in different experimental conditions all at once, improving sample throughput and boosting productivity. Using 16-plex isobaric mass tags, researchers can now compare up to 16 different samples in a single run.

Ratio distortion, a common side-effect of using isobaric tags, results from the interference caused by isobaric species. When isobaric species co-elute and co-fragment with the ions of interest during MS analysis, they generate reporter ions that interfere with the readout, causing significant loss of quantitative accuracy and precision.

The multistage MS³ approach involving sequential MS/MS analyses eliminates this isobaric interference (1). Here, users can identify the *m/z* window corresponding to the contaminating isobaric species in the MS² spectrum (and exclude it from the MS³ phase). The fragment ions consisting of the target ions of interest can then be selected and advanced into MS³. While successful in eliminating interference, this method also decreases the sensitivity of the experiment, significantly reducing the overall number of quantifiable peptides.

SPS-based MS³ was developed to restore the sensitivity lost with MS³ while maintaining quantitative accuracy with isobaric labeling (2). SPS, equipped in modern mass spectrometers, significantly improves protein quantitation by allowing the isolation of several MS² precursors at the same time, eliminating isobaric

interference and increasing reporter ion signals. The simultaneous quantitative analysis of five cancer cell proteomes in one single MS experiment (3) with isobaric labeling demonstrates how multiplexing can be used for proteome comparisons.

High-throughput quantitative proteomics can predict cancer vulnerabilities

Quantitative proteomics can reveal cancer vulnerabilities, helping researchers identify novel treatment targets and potentially support personalized cancer patient treatment. Isobaric labeling was used with 10-plex TMTs to map dysregulations in the global landscape of PPIs in 41 breast cancer cells (4). The co-regulation of protein concentrations across cancer cell lines is a powerful and accurate predictor of PPIs, ten-fold superior to co-expression analysis using RNA concentrations.

With quantitative MS-based proteomics, 6,911 proteins were quantified and a network with 14,909 protein-protein associations identified (4). This global interactome mapping using MS takes a fraction of time and effort when compared to using established technologies, such as AP-MS or yeast two-hybrid screening. Using an MS-based approach, a method to map interactome dynamics at a throughput previously unattainable with established methods was developed.

These experimental methods uncovered significant findings. Aberrations in protein networks due to dysregulated PPIs were (i) enriched in essential proteins specific to the affected cancer model and (ii) predicted responses to treatments with drugs targeting the affected pathways (4).

Making the most out of quantitative MS

There is enormous potential in using MS-based proteomics to better understand complex diseases. Multiplexed proteomics has the potential to serve as a key driver in translational research, enabling scientists to process large volumes of biological and clinical samples, offering high-throughput capabilities. There is scope to further automatize the time-consuming hands-on steps in proteomics workflows to make large-scale projects even more productive.



Table of Contents

Foreword

Chapter 1
Protein quantitation: advancing research with MS-based methods

Chapter 2
Global and unbiased protein quantitation with MS

Chapter 3
Using quantitative MS to examine protein networks

Chapter 4
High-throughput proteomics to identify cancer biomarkers

Chapter 5
How biologists can access quantitative MS

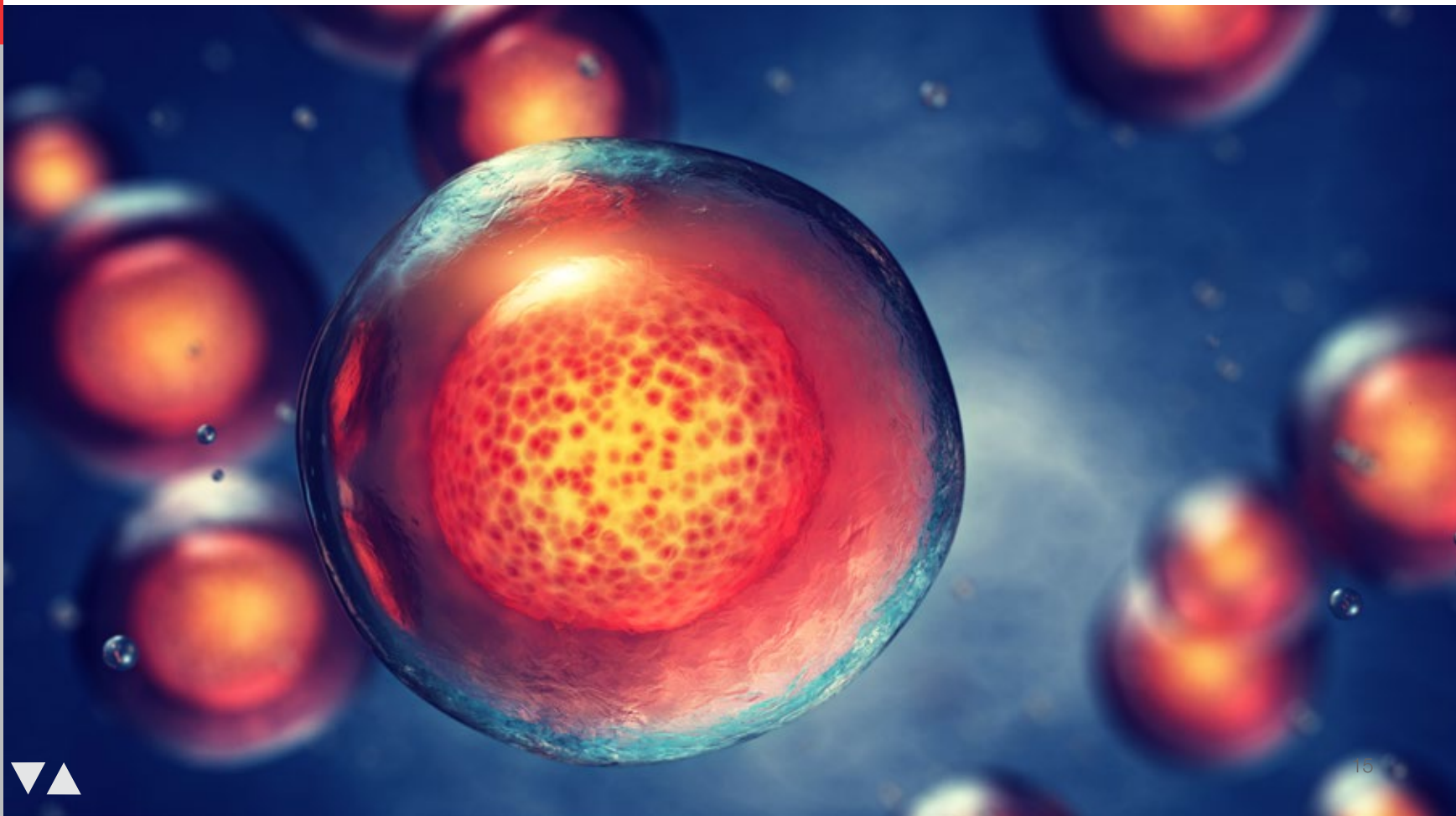
Epilogue
Continued advancements in MS technology to support researchers

High-throughput proteomics to identify cancer biomarkers

For biology labs, seamlessly incorporating MS into existing projects can be challenging due to the need for in-lab MS expertise and committing to a significant investment on equipment. To circumvent these challenges, researchers can consider collaborating with existing MS experts or make use of MS core laboratory that offer proteomics services at a reasonable price.

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How biologists can access quantitative MS

Dr. Allis Chien
Stanford University

Table of Contents

Foreword

Chapter 1
Protein quantitation: advancing research with MS-based methodsChapter 2
Global and unbiased protein quantitation with MSChapter 3
Using quantitative MS to examine protein networksChapter 4
High-throughput proteomics to identify cancer biomarkers**Chapter 5**
How biologists can access quantitative MSEpilogue
Continued advancements in MS technology to support researchers**Introduction**

When incorporating quantitative MS into research projects, biologists confront two key challenges: high equipment costs and a lack of specific expertise. Fortunately, there are untapped opportunities to make MS accessible to even first-time users. Here, we offer options for biologists to consider for gaining access to MS capabilities depending on their long-term goals, team expertise and the desired level of investment.

Using core laboratory at universities

The most straightforward way to incorporate MS into research projects, without having to become an MS expert, is to work with a core laboratory. Equipped with in-house MS experts and diverse instrumentation, core laboratories enable researchers to seek advice and perform a range of experiments with minimal investment.

For instance, the Stanford University Mass Spectrometry (SUMS) core laboratory offers diverse experimental capabilities to researchers on campus. With over 15 MS instruments of diverse flavors, including gas chromatography-mass spectrometry (GC-MS), LC-MS, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), triple quadrupole, and both Thermo Scientific™ Hybrid and Tribrid™ Orbitrap™ MS, among others, researchers can access a wide array of experiments utilizing the instruments that best fit their research needs.

Another advantage of collaborating with a core laboratory is the ability to tap into the expertise of highly skilled staff. Experts in core laboratories typically include professional mass spectrometrists, analytical chemists, reagent and sample preparation experts, and bioinformaticians trained and experienced in their respective specialties, offering a wealth of knowledge on various aspects of MS. Biologists therefore benefit from this expertise at every step of the MS workflow, from experimental design, sample preparation, method development, quality control

and troubleshooting, all the way through to data analysis and visualization, enabling a seamless experience.

Core laboratories offer consistent performance, enabling greater reproducibility. With service contracts for instrument maintenance and access to technical specialists who can monitor instrument performance and troubleshoot skillfully, researchers can focus on the scientific question instead of delving into the technical know-how.

As experimental needs expand, the available MS options, too, can evolve. When projects require certifications such as clinical laboratory improvement amendments (CLIA), good laboratory practice (GLP) or good manufacturing practice (GMP) that are not typically supported by core laboratories, third-party MS services may become an option. Researchers who wish to focus on the methodology of MS techniques or who have massive projects that can occupy an instrument full time may benefit from purchasing their own MS system customized to their specific research needs.

Equipped with in-house MS experts and diverse instrumentation, core laboratories enable researchers to seek advice and perform a range of experiments with minimal investment.

Outsourcing to third party MS services

Accessing quantitative MS via third-party commercial services can be a viable option for experienced researchers who need fast, high-throughput servicing. While core laboratories emphasize collaborative interaction on individual experiments,

Table of Contents

Foreword

Chapter 1
Protein quantitation: advancing research with MS-based methods

Chapter 2
Global and unbiased protein quantitation with MS

Chapter 3
Using quantitative MS to examine protein networks

Chapter 4
High-throughput proteomics to identify cancer biomarkers

Chapter 5
How biologists can access quantitative MS

Epilogue
Continued advancements in MS technology to support researchers

How biologists can access quantitative MS

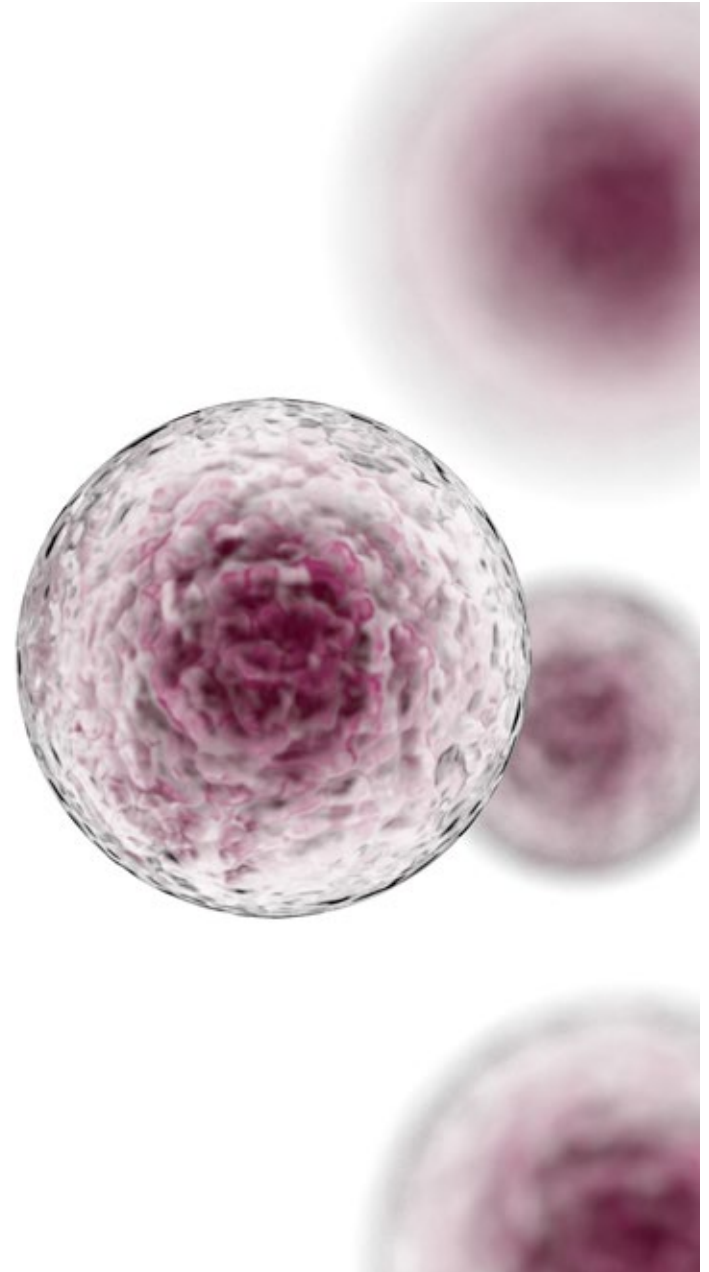
commercial contract labs generally have less flexibility, but much higher capacity. Outsourcing to contract labs with industry certifications, pre-validated methods and turn-key facilities can help move projects from research-scale to production-scale.

Purchasing MS systems for your laboratory

While laboratories typically start with MS experiments used as stepping-stones for other downstream experiments, sometimes MS can become the primary research focus. When MS experiments start becoming all-consuming, researchers may consider having an MS system in their own laboratory customized to perform specific experiments to further develop MS-based methodology. Upon gaining proficiency with the technology, having a dedicated MS system offers flexibility to design, develop and troubleshoot experiments in-house.

As each MS system requires significant financial and technical investment, researchers will need ask some important questions before purchasing:

- Will the current and future focus of the laboratory necessitate the consistent use of MS?
- Which available MS technology will best serve the laboratory's current and future research questions?
- How will the required physical infrastructure – space, electrical power, gas supplies, exhaust, temperature and humidity control – be provided?
- Are financial resources available to support the key 3 S's: (1) service contracts for ongoing maintenance and upkeep; (2) salaries for personnel with the expertise to use the instrument effectively; and (3) supplies for the instrument and associated workflows (e.g. sample preparation, reagents, standards, chromatography columns and consumables)?
- Are team members sufficiently trained to setup, operate and troubleshoot the instrument and associated systems (e.g. ultra-performance liquid chromatography (UHPLC))? When students and postdoctoral researchers advance in their careers, how will institutional memory and expertise be preserved to operate and maintain the MS system?



How biologists can access quantitative MS

Table of Contents

Foreword

Chapter 1
Protein quantitation: advancing research with MS-based methods

Chapter 2
Global and unbiased protein quantitation with MS

Chapter 3
Using quantitative MS to examine protein networks

Chapter 4
High-throughput proteomics to identify cancer biomarkers

Chapter 5
How biologists can access quantitative MS

Epilogue
Continued advancements in MS technology to support researchers

Sometimes a hybrid model can be negotiated, where the principal investigator purchases an instrument to place in the core laboratory, thus gaining the capabilities and benefits of the instrument while retaining support from core lab personnel.

Factors influencing MS-based experiments for biologists

With the diverse available options for accessing MS-based protein quantitation, deciding which path to take can depend on a few key factors. Staying informed about available resources, learning what boosts or impedes MS success, and choosing the right protein quantitation technique can empower biologists to make better decisions about MS-based experiments.

Whether MS is the very next step in the project or simply a consideration for the future, here are some factors that can give biologists an edge in benefitting from MS capabilities:

Knowledge of available resources: Enquire whether your university has a core laboratory; if not, a nearby university may have the resources you need. Mitigate risk by conducting small-budget pilot experiments in collaboration with the core laboratory experts. Take advantage of educational opportunities – many core laboratories run workshops, seminars, and/or symposia.

Cost: Compared to antibody-based immunoassay kits, quantitative MS can be relatively more expensive. To make an educated choice among the available options for quantifying proteins, determine the final goal of protein quantitation experiments. Consider scale, both in the number of proteins and number of samples to analyze, as well as the specificity and precision required to answer your research question. Generally, classic immunoassays are better suited for measuring one to a few proteins. However, antibody quality is crucial and may cause cross-reactivity. MS methods can measure thousands of proteins with unfailing specificity with methods available for both relative and absolute quantitation.

Communicate and plan ahead: Before committing to using valuable samples, consult the university MS core or an MS expert, perhaps along with a biostatistician. Share your project goals and plan the optimal experiment to reach those goals. Begin with experimental design and consider specific details of the workflow. Don't assume anything – communicate and discuss all stages of the experiment. Projects have been doomed by factors as seemingly trivial as buffer selection and storage temperature. Envision the final outcome – find out what data and results will be provided at the end of the experiment. Some core laboratories provide relatively basic reports while others have bioinformaticians on staff to help transform the results into actionable information. Collaborative research is key to maximizing future potential for quantitative MS and other downstream applications.



Table of Contents

Foreword

Chapter 1
Protein quantitation: advancing research with MS-based methodsChapter 2
Global and unbiased protein quantitation with MSChapter 3
Using quantitative MS to examine protein networksChapter 4
High-throughput proteomics to identify cancer biomarkersChapter 5
How biologists can access quantitative MS**Epilogue**
Continued advancements in MS technology to support researchers

Continued advancements in MS technology to support researchers

Daniel Lopez-Ferrer
Thermo Fisher Scientific

As scientists continue to develop, troubleshoot and report impending limitations in MS techniques, manufacturers pay attention, and design newer solutions to drive innovation. With the arrival of highly sensitive techniques in biological MS, researchers can now obtain relative quantitation of proteins at a proteome-wide scale. However, certain fundamental challenges persist, limiting scientists from getting reliable answers to specific questions that would further advance their research projects.

The dogma, 'one gene, one protein,' has been proved inaccurate. Proteins participate in most biological processes and exist as proteoforms, forming complexes with other proteins and ligands. Bottom-up proteomics, the most common proteomics workflow, based on digesting proteins into peptides, unfortunately does not capture proteoform information. Proteins undergo physiological changes, such as post-translational modifications, endogenous proteolysis, alternative splicing, etc., adding variation and complexity to proteoforms. Furthermore, different matrix types used in MS experiments exhibit a varied dynamic range of protein expression and other biomolecules, introducing laborious sample preparation steps to maximize the yield for MS analysis.

Another challenge in current MS techniques is the lack of statistical power and loss of information. Although the latest proteomics experiments can identify and quantify over 10,000 proteins reproducibly, the number of replicate measurements for a given protein is limited. Factoring in the loss of information due to "missed values" occurring in even well-designed MS experiments reduces the statistical power further, producing data bias.

The best way to address this challenge is to have a more targeted protein quantitation approach that minimizes biases during the MS analysis. Such an approach would target a set of predefined signaling pathways implicated in diseases. Traditional methods of targeted protein quantitation that involve antibody-based techniques don't offer the sensitivity, reproducibility and throughput provided by MS.

To overcome this limitation, a new paradigm for targeted protein quantitation using MS has now been developed. These modern turn-key MS workflows offer completely optimized steps, starting with sample preparation all the way through to data analysis to enable biologists to quantify targeted proteins with highly sensitive MS. For example, kit-based workflows, such as Thermo Scientific™ SureQuant™ Targeted MS Assay Kits, enable simultaneous enrichment and quantitation of a wide range of total and phosphorylated proteins. Moreover, by leveraging labeled internal-standard peptides, the system further boosts quantitation reliability in real-time.

Manufacturers are continually advancing MS technologies to eliminate common pain-points and save valuable time for researchers. Features such as built-in templates and ready-to-use presets in these turn-key systems have made MS readily accessible for biologists with limited time or minimal expertise to adopt MS and develop MS-based assays without any complications.



Table of Contents

Foreword

Chapter 1
Protein quantitation: advancing
research with MS-based
methods

Chapter 2
Global and unbiased protein
quantitation with MS

Chapter 3
Using quantitative MS to
examine protein networks

Chapter 4
High-throughput proteomics
to identify cancer biomarkers

Chapter 5
How biologists can access
quantitative MS

Epilogue
Continued advancements in
MS technology to support
researchers



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