A biologist's guide to modern techniques in quantitative proteomics

Introduction

Biology is the study of the dynamic changes that living things undergo over time and in response to external stimuli. Proteins play a crucial role in structure and function of biological systems, from being involved in cellular development, differentiation, responding to therapeutic intervention, transporting molecules within a cell and catalyzing metabolic reactions. To understand the functions of individual proteins and their place in complex biological systems, it is often necessary to measure changes in protein abundance relative to changes in the state of the system. As such, modern proteomics has evolved from an exclusively qualitative technique into one that spans a continuum of qualitative and quantitative. Confident protein identification is a necessary foundation for proteomics, and with protein identifications, high quality quantitative data is also needed (Figure 1). Currently, proteomics researchers are striving to close the gap and ensure all identified proteins are quantified with high precision and accuracy to gain real, significant biological insights.

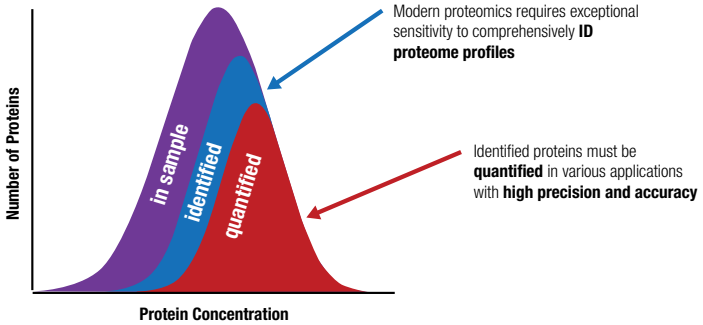


Figure 1. Paradigm shift in proteomics

Traditional vs. new methods for protein quantitation

Traditionally, a large majority of quantitative protein measurements were done by western blotting. However, western blotting almost always requires *a priori* knowledge of the system, the expected changes and the proteins involved to obtain the appropriate antibodies that target the protein, or proteins of interest. Antibodies are often not available, not specific, or cost prohibitive to screen large quantity of relevant targets. Antibodies for site-specific modifications can be even more difficult to obtain with high specificity. In addition, western blotting is sample intensive, semi-quantitative, has limited linear dynamic range, and typically only a single target is quantified in each western blot. Alternatively, liquid chromatography coupled to mass spectrometry (LC-MS) has emerged as a powerful technology for system-wide identification and quantitation of proteins. It can be used for both discovery-based (untargeted) and targeted determination of changes in protein abundance. It can also be used to measure changes in the abundance of protein-specific posttranslational modifications (PTMs) and facilitate identifying the location of the modified residue. Quantitative mass spectrometry (MS) analyses require less sample than western blotting, does not require the use of antibodies and can detect and quantify thousands of proteins in a single experiment across multiple conditions.

Discovery vs. targeted proteomics

MS-based proteomics is divided into two parts: discovery proteomics and targeted proteomics. Discovery proteomics experiments are intended to identify as many proteins as possible across a broad dynamic range, while at the same time measuring the relative protein abundance changes of these proteins across multiple set of samples. Discovery proteomics focuses on optimizing protein identification by spending more time and effort per sample and reducing the number of samples analyzed. This can result in errors in quantitative precision and accuracy, but these errors are well within the acceptable range for researchers. In contrast, targeted proteomics strategies limit the number of proteins that will be monitored and optimizes the instrument method for throughput of hundreds or thousands of samples. This translates to high quantitative precision and accuracy with very little errors in the estimation of the protein abundances. Typically, discovery proteomics requires longer time on analysis (~2 hours), while targeted proteomics make use of shorter MS analysis (~10-20 mins). Discovery proteomics analysis is most often used to inventory proteins in a sample or detect differences in the abundance of proteins between multiple samples; targeted quantitative proteomic experiments often follow discovery proteomics to quantitate specific proteins found during fundamental biology or drug discovery screening (Figure 2).

Coverage, throughput, method development, reproducibility and precision – dilemma

Quantitative proteomics relies on balancing proteome coverage, sample throughput, method development, reproducibility and precision. There are tradeoffs between these analytical criteria and unfortunately, there is no one-size fits all approach. Therefore, depending on the biological question and the specifics of the experimental objectives, an appropriate workflow can be chosen to ensure analytical criteria are met that are of higher importance to the experiment. There are multiple workflows currently available for discovery proteomics. In general they can be split into isotopic labeling or label-free methods. There are tradeoffs for each technique, and proteomics researchers need to choose the one that best fits the question at hand.

Proteomics pipeline

Discovery-based analyses – identify and quantify

With discovery-based quantitative analyses, the goal is to both identify proteins and measure their relative abundance changes across multiple sample sets, usually on a proteome-wide level. The multiple sample sets here could potentially represent different time points in a biological pathway, responses to different stimulus or different cellular location. Several discovery based techniques have been developed, including stable isotope labeling by amino acids in cell culture (SILAC), chemical labeling with isobaric mass tags, and label-free quantitation (LFQ).[1, 2, 3]

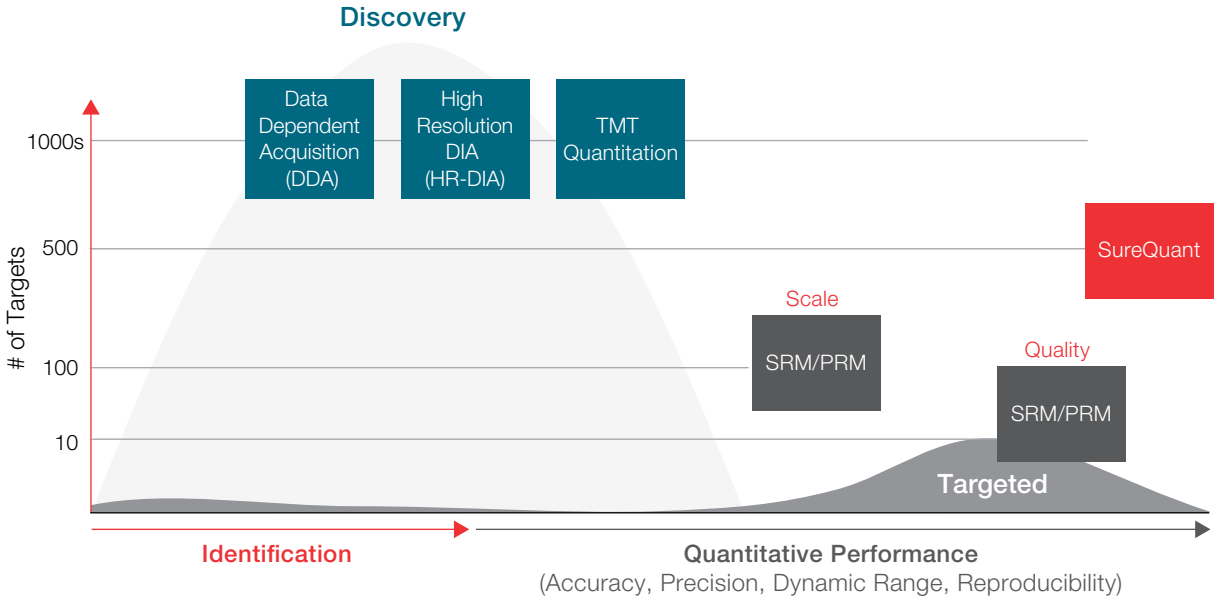


Figure 2. The proteomics continuum

LC-MS techniques for quantitative proteomics

LFQ enables exploration of the proteome in greater depth

LFQ has gained popularity for discovery-based quantitative proteomics. It does not require specific sample preparation and accommodates large numbers of diverse samples. There are two types of LFQ approaches differentiated by how the identification (MS^2) data is acquired. This is done by either data-dependent acquisition (DDA) or data-independent acquisition (DIA).

LFQ – data-dependent acquisition

In this approach, the ions for a given *m/z* range are individually isolated and fragmented. The quantitation involves extracting peptide chromatograms (MS^1 precursor ion) from LC-MS runs and integrating peak areas over the chromatographic time scale or using the intensity at the highest point of the chromatographic peak. The resulting areas or intensities are compared across a sample set (i.e. control vs experimental) for quantitation. LFQ DDA has demonstrated high reproducibility and linearity at both peptide and protein levels.[4] The samples that are part of the comparison study are run individually. Therefore, meticulous sample handling, sample preparation, reproducible chromatography between technical and biological replicates, and sensitive, high-resolution, accurate-mass MS are all essential (Figure 3).

Table 1. Discovery-based quantitation techniques all have specific applications and advantages

Technique	Type	Number of samples per LC-MS	Precision (%CV)	Accuracy	Reproducibility	Coverage	Method Development	Benefits	Drawbacks
LFQ (DDA)	Relative	1	<15-20	Good	Very Good	Medium	Low	• Applicable to any sample type • Cost-efficient sample preparation • Minimal sample handling	• Each sample runs individually (low throughput) • Requires very reproducible LC separations • Requires multiple technical replicates
LFQ (DIA)	Relative	1	<10-15	Good	Very Good	Medium	Low	• Applicable to any sample type • Cost-efficient sample preparation • Minimal sample handling	• Each sample runs individually (low throughput) • Requires very reproducible LC separations • Requires multiple technical replicates • Spectral libraries need to be generated
SILAC	Relative	1-3	<10-15	Good	Very Good	High	Low	• Least susceptible to inter-sample variations in sample handling and preparation • Multiplexing increases MS throughput	• Only readily applicable to cell cultures • Increases MS spectral complexity
TMT	Relative	1-16	<5-10	Very Good	Very Good	High	Low	• Applicable to any sample type • Multiplexing increases MS throughput	• Requires extensive fractionation or long chromatographic gradients

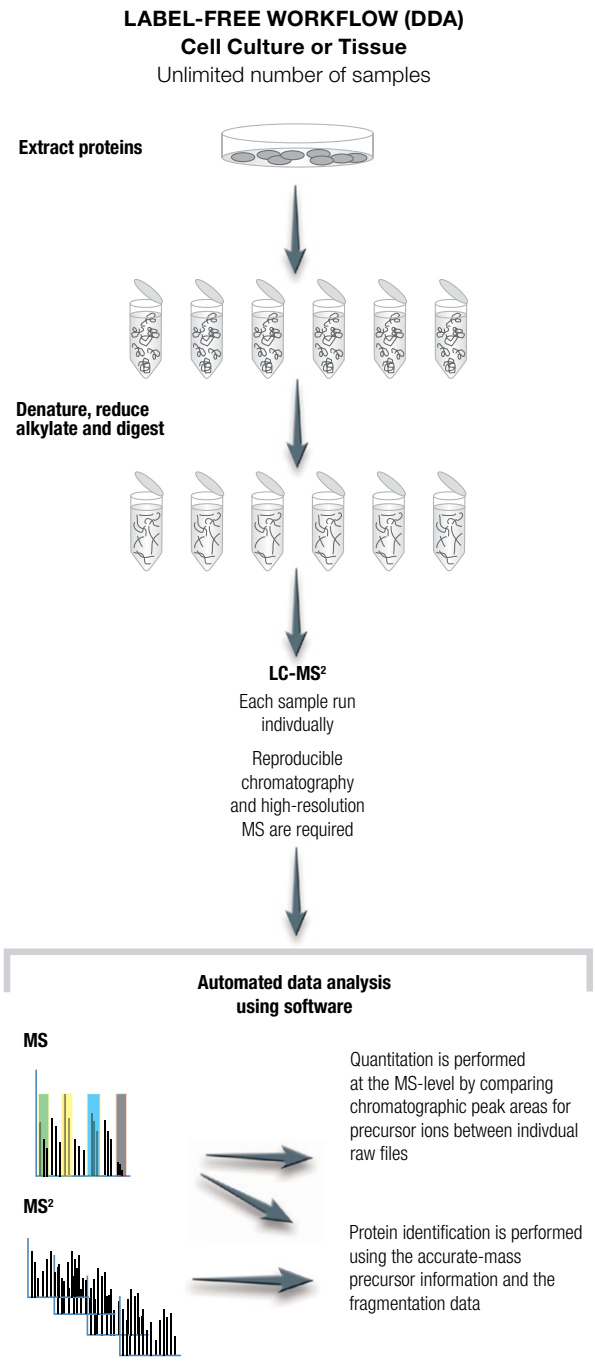
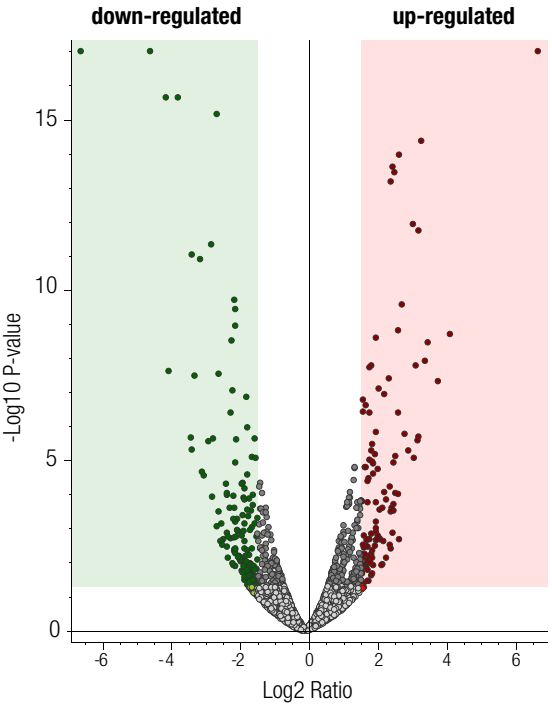


Figure 3. Schematic representation of the LFQ workflow using DDA.

In traditional data-dependent acquisition workflows, consistent pre-cursor and thereby protein quantitation can be challenging to achieve due to the stochastic sampling nature of the mass spectrometer. Innovations in software algorithms have addressed these limitations. These algorithms extract LC-MS peaks in the raw data files and maps them to identified spectra. The algorithm then detects these features across LC-MS data files using retention-time alignment and feature linking. This minimizes 'missing data points' and maximizes quantitative insights.



Application example: LFQ (DDA) analysis of pre-classified prostate cancer tissue compared to matched control (tumor free) tissue. Data shows up-regulation of 334 proteins and down-regulation of 430 proteins compared to healthy tissue. 6,438 proteins were identified across 44 samples in 3 technical replicate analysis (22 healthy vs. 22 prostate cancer tissues) in 120 minutes analysis time.

LFQ – data-independent acquisition

In LFQ DIA experiments, a precursor mass range is selected, usually one that covers the masses of most enzymatic peptides. That range is then divided into a series of relatively wide isolation windows: for example, 25 *m/z* each. Chimeric MS^2 data is acquired from all detected precursor ions in the first isolation window. That is repeated for each consecutive, adjacent isolation window until the entire precursor mass range is covered. MS^2 spectral libraries are used to identify peptides of interest from the acquired data.

DIA significantly increases coverage and reproducibility by acquiring MS^2 data from all detected precursor ions. This also makes possible retrospective data analysis. Because no prior knowledge of expected precursors is required, DIA method development is very simple. Similar to LFQ DDA, LFQ DIA involves extracting peptide chromatograms (MS^1 precursor ion or MS^2 fragment ions) from LC-MS runs and integrating peak areas over the chromatographic time scale. The resulting areas are compared across the sample set for quantitation. The samples that are part of the comparison study are run individually (Figure 4).

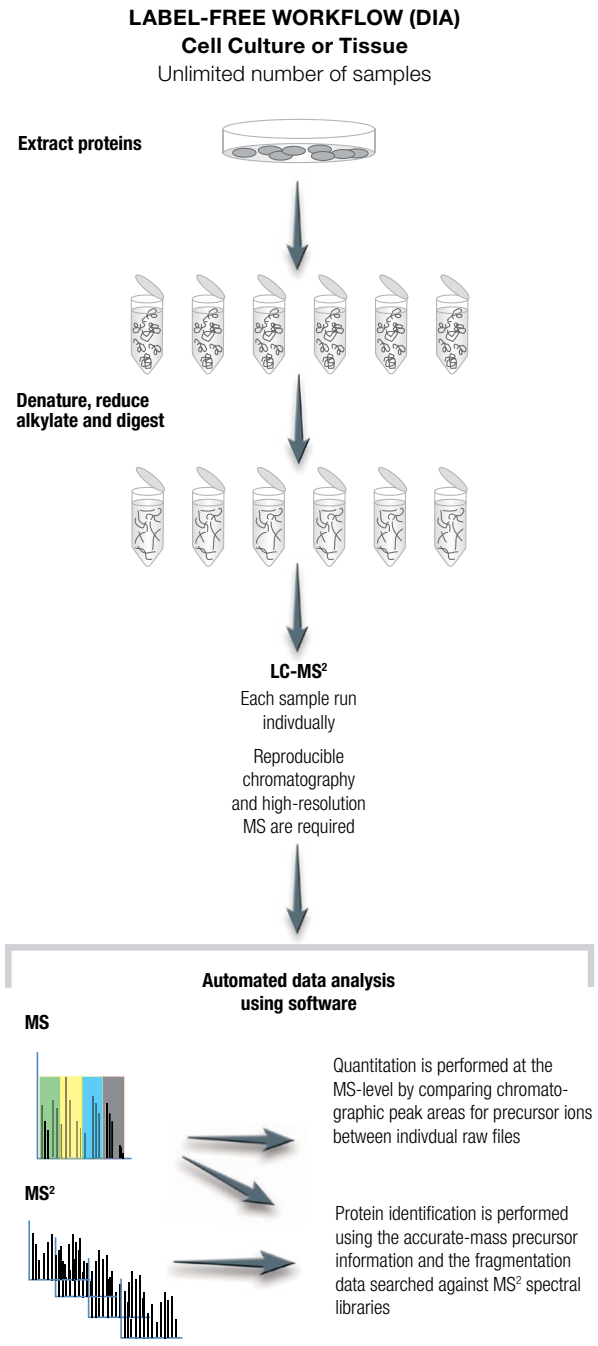
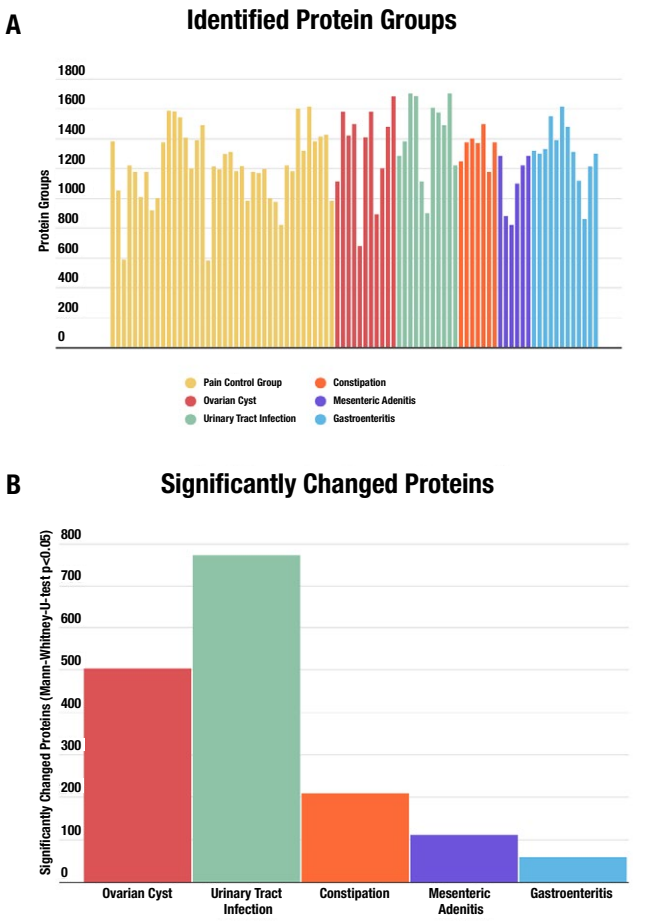


Figure 4. Schematic representation of the LFQ workflow using DIA



Application example: Comprehensive urinary proteome coverage by LFQ DIA workflow

The workflow was applied to a urinary study comprised of 87 samples associated with six different differential diagnoses (abdominal pain controls, ovarian cyst, mesenteric adenitis, constipation, urinary tract infection (UTI), gastritis, gastroenteritis). The samples were obtained from patients with abdominal pain in a pediatric emergency room. On average, 1,301 protein groups (848 -1720, A above) were detected. In total, the DIA approach enabled the detection of 2,456 proteins. Compared to all other samples, 773 proteins were significantly changed in their amount in the UTI samples (non-parametric Mann-Whitney U test, $p<0.05$), 502 in the ovarian cyst samples, 209 in the constipation samples, 111 in the mesenteric adenitis samples and 58 in the gastroenteritis samples (B).

Stable isotope labeled techniques

SILAC improves the throughput of discovery-based quantitative MS analyses and increases accuracy of results

SILAC is a powerful and widely used method of identifying and quantifying relative changes in complex protein samples. It can be applied to complex biomarker discovery and systems biology studies, as well as to isolated proteins and protein complexes. As its name implies, the SILAC method uses *in vivo* metabolic incorporation of "heavy" ^{13}C- or ^{15}N-labeled amino acids in place of the naturally occurring isotopes for one set of samples (experimental), while another sample incorporates the "light" amino acids of natural isotope abundance (control). Equal amounts of protein from both cell populations are combined, digested, separated and identified by MS (Figure 5). Because peptides labeled with heavy and light amino acids are chemically and biologically identical, they behave similarly within the analytical stage, co-eluting at the same time during liquid chromatography (LC). However, they can easily be differentiated by a mass spectrometer owing to the mass differences of the two isotope labels.

The relative peak intensities of multiple isotopically distinct peptides from each protein are used to determine the average change in protein abundance in the treated sample. The main advantage of SILAC is its early incorporation of the isotope labels within the growth medium, which minimizes the number of manipulations, thus, allowing for the least amount of variation between the samples during the preparative procedures. In addition, the mixing of samples permits a variety of enrichment techniques including immunoprecipitation. These can improve the detection of abundance changes for both low-abundance proteins and PTMs such as phosphorylation or glycosylation.

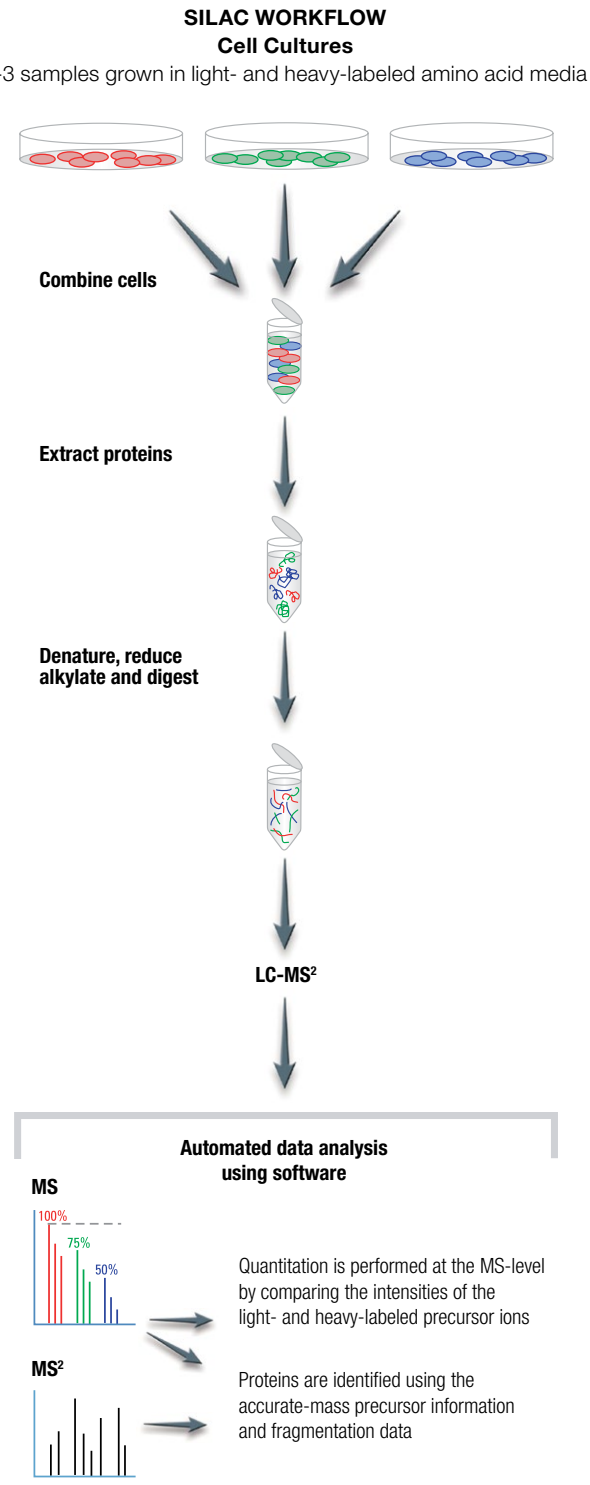
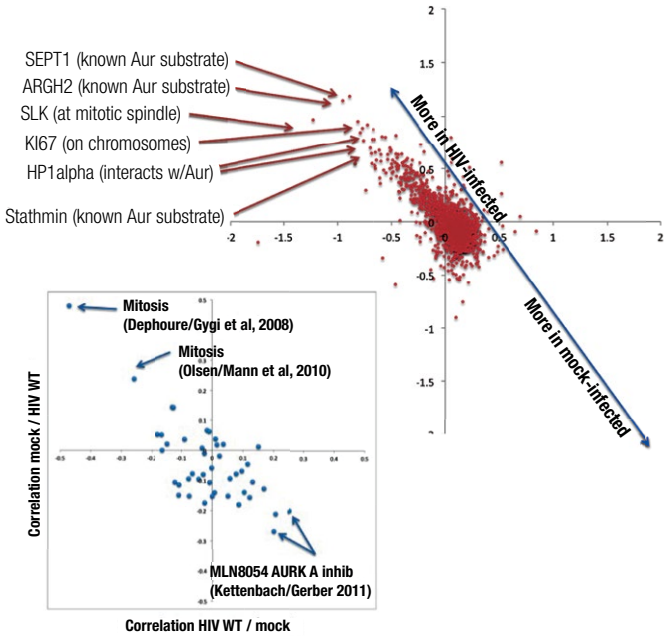


Figure 5. Schematic representation of the SILAC workflow



Application example: SILAC based quantitative proteomic analysis of cells infected by HIV by monitoring the changes in the global phosphorylation status of host protein.[6] Aurora kinases are activated by HIV infection. SILAC phosphorylation profiles comparing HIV-infected to mock-infected Jurkat cells were compared to the PTMfunc database of posttranslational modification. PTMfunc identified a correlation with two independent mitosis phosphoproteomics data sets, and an anti-correlation with an aurora kinase inhibitor data set. Manual inspection of the most heavily phosphorylated peptides confirmed strong activation of aurora kinases in HIV-infected cells.

TMT labeling increases the number of samples analyzed, and peptides identified and quantified, in a single analysis

Isobaric chemical tags are a more universal alternative to SILAC for simultaneous identification and quantitation of proteins in multiple sample sets. They can facilitate relative quantitation of a wide variety of samples including cells, tissues, and biological fluids. Thermo Scientific™ Tandem Mass Tag (TMT) reagents are isobaric mass tags consisting of an MS/MS reporter group, a spacer arm, and an amine-reactive group. Amine-reactive groups covalently bind to peptide N-termini or to lysine residues. After labeling, the peptides are introduced into the mass spectrometer where each tag fragments during MS^2, producing unique reporter ions due to the incorporation of heavy carbon and nitrogen isotopes in the tag structure. Peptide quantitation is accomplished by comparing the intensities of the reporter ions.

However, achieving quantitative accuracy is highly dependent on the purity of the precursor ion population selected for MS^2 analysis. Innovations such as synchronous precursor selection (SPS)-based MS^3 technology and Real-Time Search data acquisition for SPS MS^3 addresses these challenges providing the capability to accurately measure the most subtle changes in low-abundance proteins.[9,10] Currently, up to 16 different samples can be compared and quantified in a single LC-MS analysis (Figure 6).

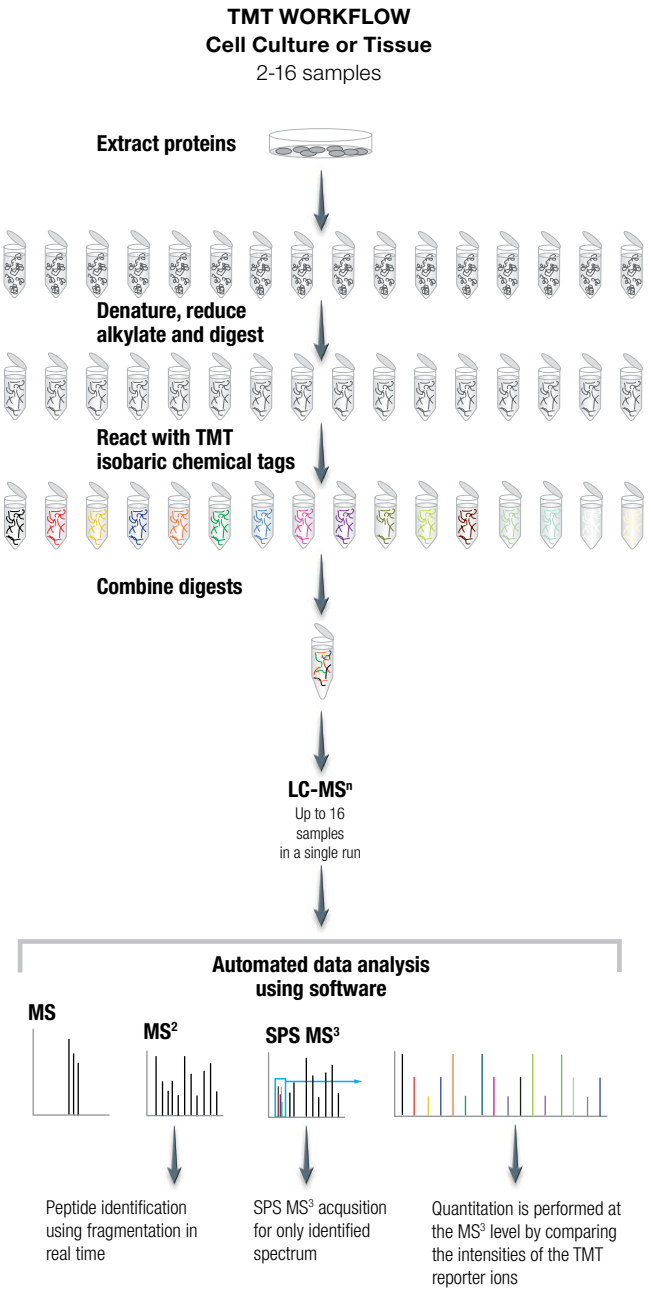
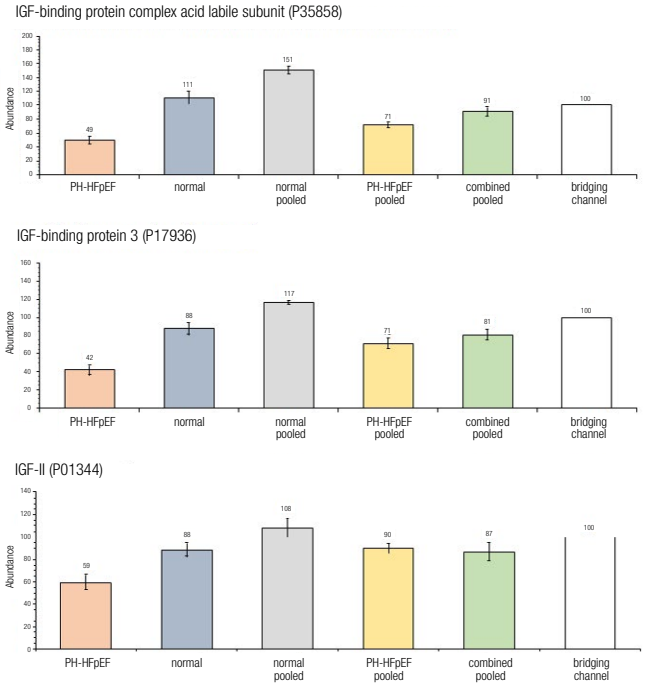


Figure 6. Schematic representation of the TMT workflow

A

11-plex sample sets	reporter ion channels										
	126	127N	127C	128N	128C	129N	129C	130N	130C	131N	131C
Sample Set 1	normal_1	PH-HFpEF_1	normal_2	PH-HFpEF_2	normal_3	PH-HFpEF_3	normal_4	normal_pooled	PH-HFpEF_pooled	mixed_pooled	bridge
Sample Set 2	PH-HFpEF_4	normal_5	PH-HFpEF_5	normal_6	PH-HFpEF_6	normal_7	PH-HFpEF_7	normal_pooled	PH-HFpEF_pooled	mixed pooled	bridge
Sample Set 3	normal_8	PH-HFpEF_8	normal_9	PH-HFpEF_9	normal_10	PH-HFpEF_10	normal_11	normal_pooled	PH-HFpEF_pooled	mixed_pooled	bridge
Sample Set 4	PH-HFpEF_11	normal_12	PH-HFpEF_12	normal_13	PH-HFpEF_13	normal_14	PH-HFpEF_14	normal pooled	PH-HFpEF_pooled	mixed_pooled	bridge
Sample Set 5	normal_15	PH-HFpEF_15	mixed_pooled	normal_16	PH-HFpEF_16	mixed_pooled	normal_17	normal_pooled	PH-HFpEF_pooled	mixed_pooled	bridge

B



Application example: A TMT quantitative study in which thirty-two plasma samples from healthy donors and individuals diagnosed with pulmonary hypertension heart failure with preserved ejection fraction (PH HFpEF) were analyzed in five 11-plexed sets, each set consisting of pooled controls and bridging channels. Protein abundances were compared across all individuals as well as between the two pooled cohorts to identify potential biomarker candidates and gain insight into the mechanism of the development and progression of the PH-HFpEF. Several proteins were shown to have differential abundance in the PHHFpEF patients' samples relative to the normal controls.[11] IGF-binding proteins showed the most significant difference, with the abundance changes of IGF binding protein complex acid labile subunit and the IGF-binding protein 3 having the largest fold change. These were also shown to be lower in the PH-HFpEF cohort. IGF-II was also detected at lower abundance levels, consistent with the relative abundance level of its binding proteins.

Targeted analyses – fast and sensitive quantitation

Mass spectrometry-based targeted quantitation is widely used to determine relative or absolute abundances of peptides of interest. It provides a high degree of accuracy and sensitivity, and allows the profiling of hundreds of targets in a single experiment. It is a powerful, more-flexible alternative to time- and resource intensive, enzyme-linked immunosorbent assays (ELISAs). Targeted quantitation is frequently applied to large sample sets to validate putative biomarkers identified in earlier discovery experiments or to analyze wide-scale changes in biological systems. While target peptides are often selected through analysis of data from previous discovery experiments, hypotheses and *a priori* knowledge of targeted systems can also be used to select proteotypic peptide targets.

Targeted analyses provide improved quantitation sensitivity and, combined with increased speed of analyses, enable analysis of expanded sample sets to assess the validity of the candidate proteins. Spiking biological samples with proteotypic, isotopically labeled peptide standards makes possible the absolute quantitation of each protein or PTM of interest. Choosing the most appropriate quantitative proteomics technique depends on experimental demands and instrumental capabilities. This review is intended to assist the decision-making process. Several targeted quantitative techniques have been developed in the past and were viewed as gold standards such as selected reaction monitoring (SRM). The advent of high-resolution MS has enabled techniques such as parallel reaction monitoring (PRM) and SureQuant internal standard (IS) targeted protein quantitation workflow.[12, 13]

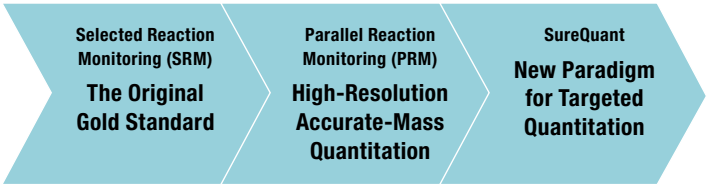


Table 2. Targeted quantitation techniques all have specific applications and advantages

Technique	Type	Number of samples per LC-MS	Precision (%CV)	Accuracy	Reproducibility	Method Development	Benefits	Drawbacks
SRM	Relative or Absolute	1	<5-10	Very Good	Very Good	Medium	• High sensitivity • High dynamic range	• Time consuming assay development • Limited sensitivity in complex matrices
SIM	Relative or Absolute	1	<5-10	Very Good	Very Good	Low	• Uses the same MS system as discovery quantitation • Increases sensitivity 5- to 50-fold compared to full-scan MS	• Requires reproducible LC separations • Limited sensitivity in complex matrices
PRM	Relative or Absolute	1	<5-10	Very Good	Very Good	Low	• High selectivity, eliminates most interferences, providing more accuracy and attomole-level limits of detection and quantification • High sensitivity • Enables confident confirmation of the peptide identity with spectral library matching • Fast method setup, reduces assay development time since no target transitions need to be pre-selected • Suitable for complex matrices	• Requires reproducible LC separations • Limited number of targets
SureQuant	Relative or Absolute	1	<5-10	Very Good	Very Good	Low	• Highest target multiplexing capabilities in a single targeted analysis • Enhanced acquisition efficiency enables increased throughput • Reduced assay development time – spiked in internal standards guide LC-MS acquisition • Internal standard acts as detection positive control and allows absolute quantitation	• Higher up front cost of labeled peptides

Selected reaction monitoring for high-throughput

Historically, targeted quantitation has been performed using SRM with a triple quadrupole mass spectrometer such as Thermo Scientific™ TSQ™ triple quadrupole mass spectrometers (Figure 7). In SRM, a peptide/peptides unique to the protein of interest are selected for targeted quantitation. Specific fragment ions from the target peptide along with its parent mass (referred to as transitions) and retention time are used to monitor the peptide across multiple sample sets. By using very narrow isolation windows to select the fragments, chemical interferences can be reduced to increase both selectivity and sensitivity for transitions of interest. Quantitation is performed by integrating the peak area of the transitions over the chromatographic time scale and comparing them over multiple samples. SRM quantitation is extremely sensitive, reliable, and suitable for analyzing large numbers of samples. SRM can also be used to perform absolute quantitation of targeted proteins by incorporation of appropriate stable isotope-labeled peptides as internal standards.

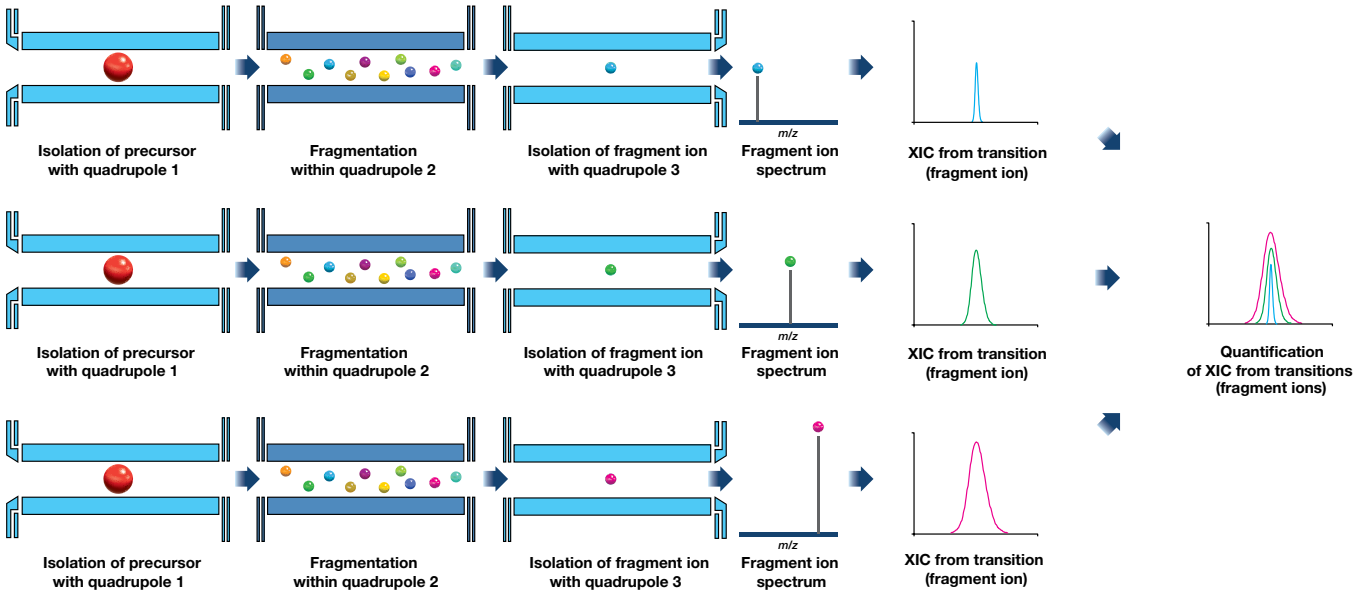
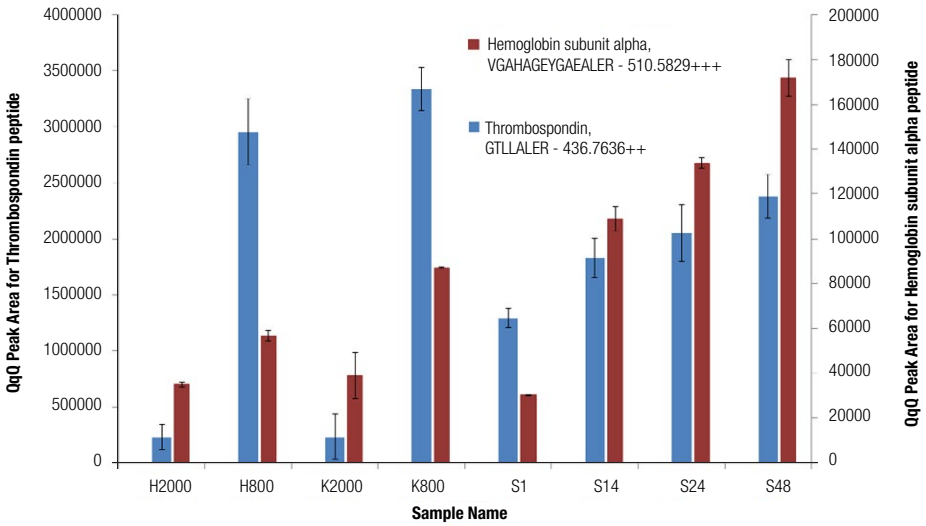


Figure 7. Schematic representation of SRM



Application example: Thrombospondin and Hemoglobin subunit alpha both increase in abundance as clotting occurs in blood. Studies were conducted to examine the effect on these proteins during sample collection and preparation methods.14 These samples were either spun at 2000 RCF for 30 min or 800 RCF for 5 minutes (in Heparin or K2EDTA vacutainers). The serum samples (S1-S48) were left on the benchtop for 1, 14, 24 and 48 hours prior to sample prep, which increases the chance of clotting. This highly multiplexed targeted method on the triple quadrupole MS detected significant increases in the peak area for samples spun at lower RCF, and for serum sitting on the bench top. In addition, Heparin vacutainers appeared to minimize the incidence of clotting, with lower overall peak areas for these 2 proteins.

Selected ion monitoring for flexibility

Selected ion monitoring (SIM) performed on high-resolution accurate-mass instruments such as a Thermo Scientific™ Orbitrap™ mass spectrometer provides the simplest method set up and the most selective and sensitive quantification. It is most suitable for quantifying tens of proteins in samples of medium complexity. SIM also provides higher sensitivity for quantification of labile peptides which do not fragment efficiently. The SIM methodology uses the quadrupole of the MS to isolate the precursor of the target peptide ion. Only the selected target ion is transferred to the mass analyzer for detection. There is no fragmentation. SIM experiments can also be multiplexed (msxSIM). In such experiments up to ten targets can be isolated sequentially, accumulated, and then transferred to the mass analyzer for detection in a single spectrum (Figure 8). Confirmation of the targeted peptide is accomplished using accurate-mass measurements in combination with elution-time information.

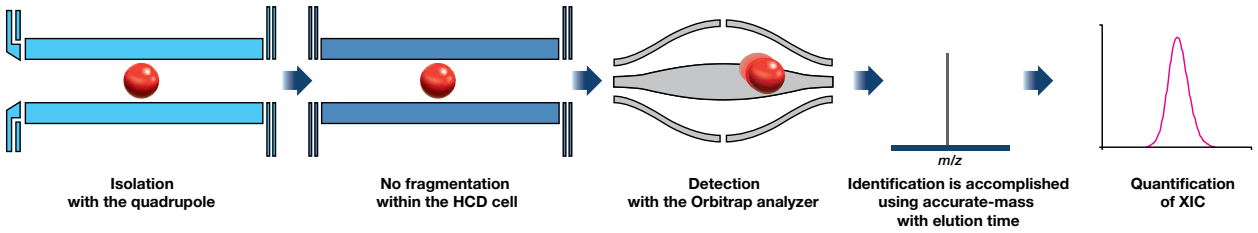
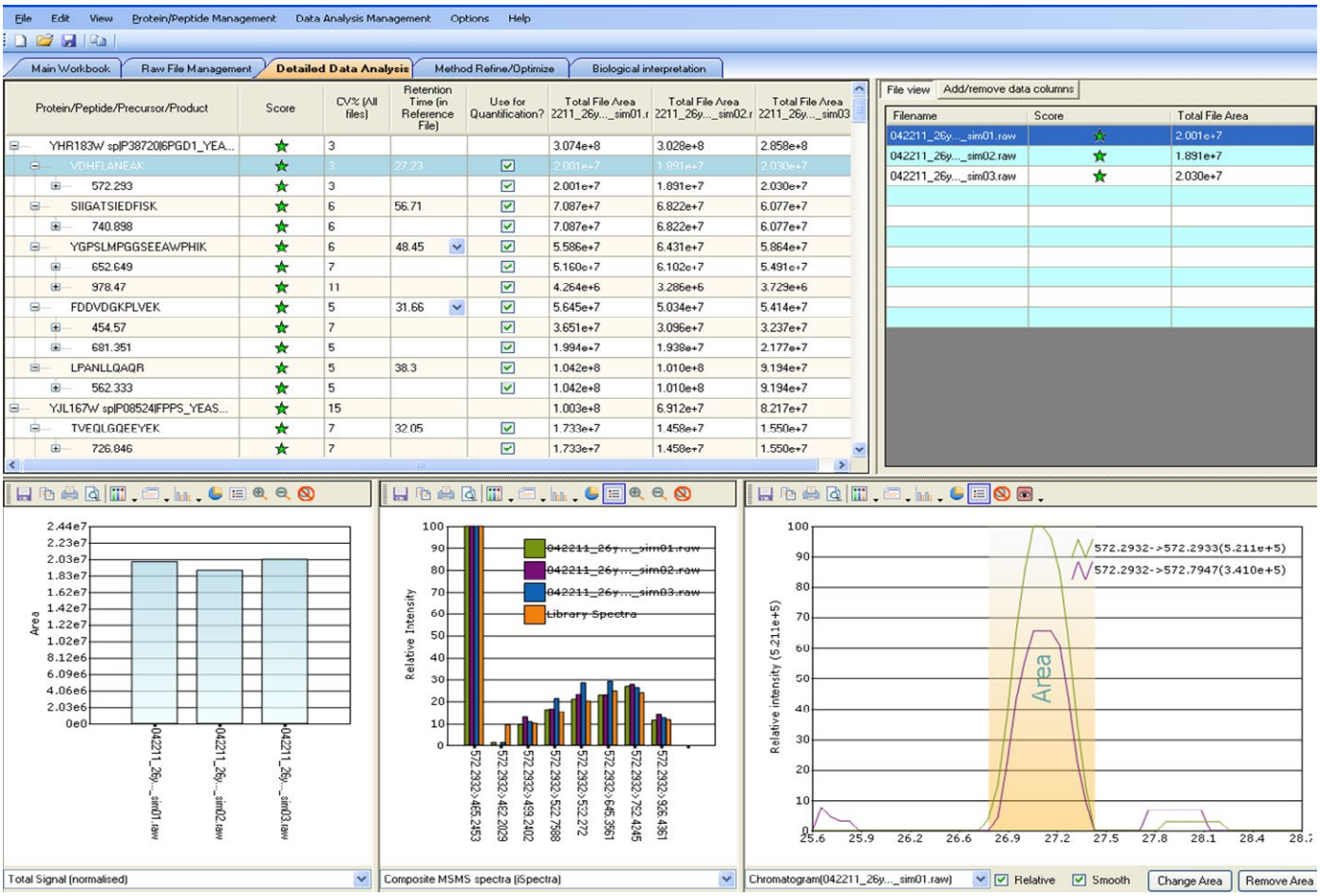


Figure 8. Schematic representation of SIM



Application example: Proteomics software displaying the quantification of 86 low and high abundant yeast peptides using SIM.[15] All of the targeted peptides had CVs below 20% and 86% of the targeted peptides had CVs below 15%.

Parallel reaction monitoring for high selectivity

PRM, also performed on high-resolution accurate-mass instruments, provides high selectivity, high sensitivity, and high-throughput quantification with confident targeted peptide confirmation. It is most suitable for quantifying tens to hundreds of targeted proteins in complex matrices. PRM methodology uses the quadrupole of the mass spectrometer to isolate a target precursor ion, fragments the targeted precursor ion in the collision cell, and then detects the resulting product ions in the mass analyzer (Figure 9). Quantification is carried out by extracting one or more fragment ions' area with a 5–10 ppm mass window and then comparing the information across multiple sample sets.

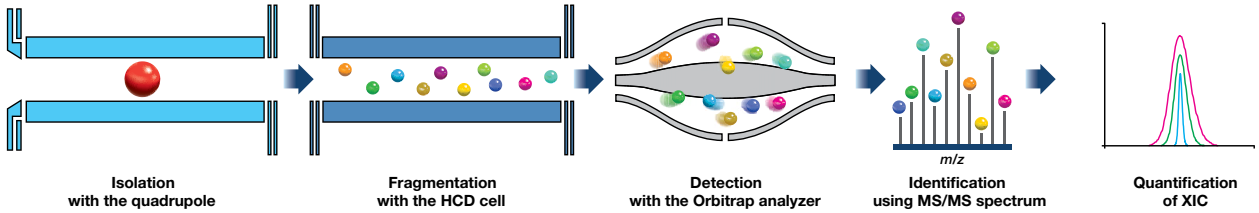
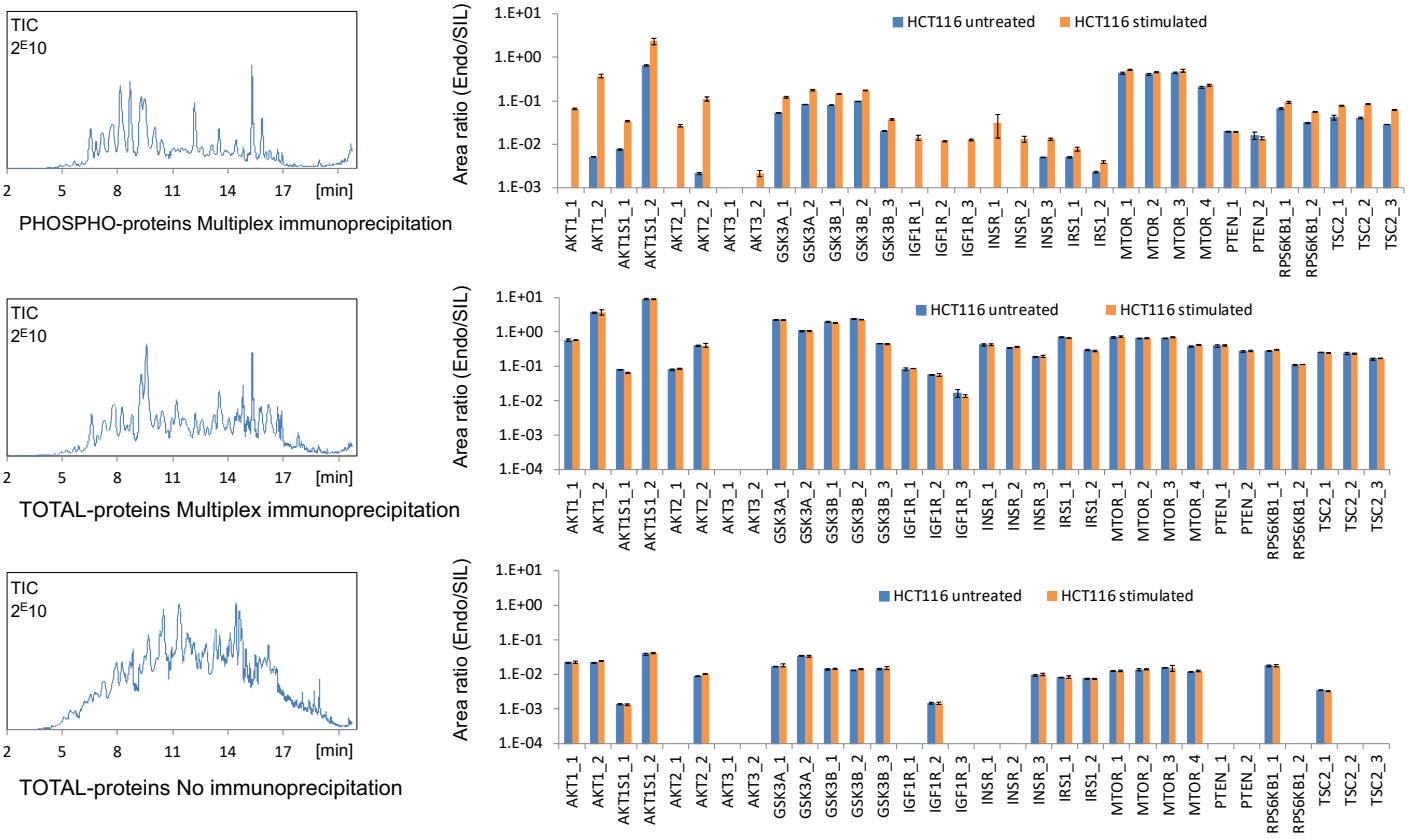


Figure 9. Schematic representation of PRM



Application example: Multiplex immuno-precipitation of proteins from the AKT/mTOR pathway with PRM analyses to measure aberrant activation of this critical signaling pathway in HCT116 cancer cell line. PRM assay for the quantification of AKT/mTOR surrogate peptides was applied to untreated and hIGF-1 stimulated HCT116 digest prepared i) by multiplex immunoprecipitation targeting phosphoproteins of the pathway (upper panel), ii) by multiplex immunoprecipitation targeting "total" proteins of the pathway (middle panel), and iii) without enrichment (lower panel). Peptide surrogates were quantified based on the measurements of pairs of SIL and endogenous in triplicate PRM analyses. While hIFG-1 stimulation did not induce changes in total proteins abundances, it modified the phosphorylation status of most of them, as illustrated by the significant increase in the peptide abundance of phosphoproteins (especially IGF1R, INSR, and AKT proteins). The multiplexed immunoprecipitation steps allowed differentiated quantification of phospho- and total-proteins but also quantification of additional peptides, benefiting from the decrease in sample complexity and the enrichment of targets.[16]

PRM offers several advantages for targeted quantitation. It eliminates most interferences, providing high accuracy and attomole-level limits of detection and quantification. Since PRM generates a fragmentation spectrum for the target peptide, confident confirmation of the peptide identity can be obtained with spectral library matching. Furthermore, it reduces assay development time since target transitions don't need to be preselected.

SureQuant IS targeted protein quantitation workflow – a new paradigm

SureQuant IS targeted protein quantitation workflow builds upon the PRM approach by using spiked-in internal standards to dynamically control MS acquisition parameters and optimize instrument duty cycle, thereby maximizing the number of productive MS scans and improving sensitivity of target detection. This enhanced efficiency enables targeted quantitation of far more targets than PRM while still maintaining high quantitative performance. The overall SureQuant IS targeted protein quantitation workflow is comprised of two steps. First, a survey is run to verify the detectability of the reference internal standards. The internal standards are standards of the peptides that the user of the SureQuant method wants to target and quantify. The survey run analysis only needs to be run one time at the onset of the study, on a user's preferred LC-MS configuration, and no further adjustment is required over time. This analysis verifies the optimal precursor ion of each internal standard peptide and the optimal associated fragment ions that can be detected. The signal intensity of the internal standard and corresponding triggering intensity threshold is also determined from the survey run.

This is followed by the SureQuant analysis, targeting the peptides of interest. Here, using the SureQuant method, the mass spectrometer is programmed to monitor the reference internal standard in the sample using low fill times and resolution. As soon as the internal standard is detected, the instrument switches to using longer fill times and higher resolution to acquire PRM data for the internal standard and the endogenous peptide. The real-time management of acquisition time maximizes the time devoted to analyte quantitation allowing a greater number of targets to be reliably detected and quantified for targeted proteomics experiments. Furthermore, the constant on-the-fly monitoring of the internal standard removes the need for retention time scheduling, allowing for a far more robust and reproducible analytical method (Figure 10).

The built-in positive internal standard control provides a definitive limit of detection (LOD) measure for the presence or absence of proteins in the sample addressing the common need to assess protein copy number expression in many molecular biology experiments. Validated instrument method templates for both Survey Run and SureQuant IS targeted protein quantitation workflow analysis are provided, preset for various Thermo Scientific™ SureQuant™ targeted assay kits, like the AKT/mTOR pathway kit. Generic SureQuant method templates are also available to simplify the development of custom protein panel assays.

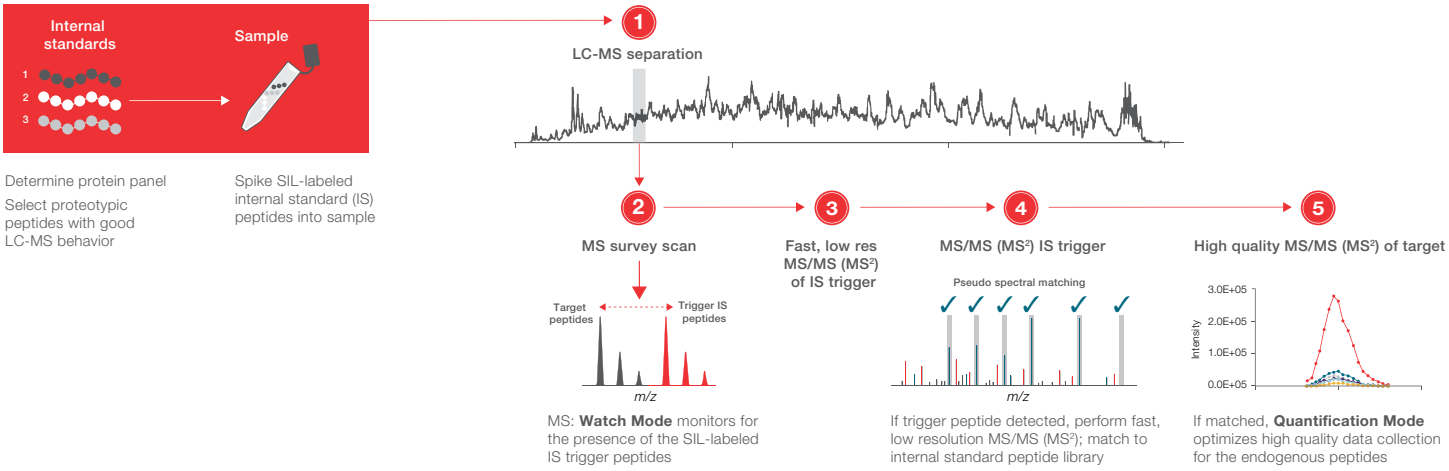
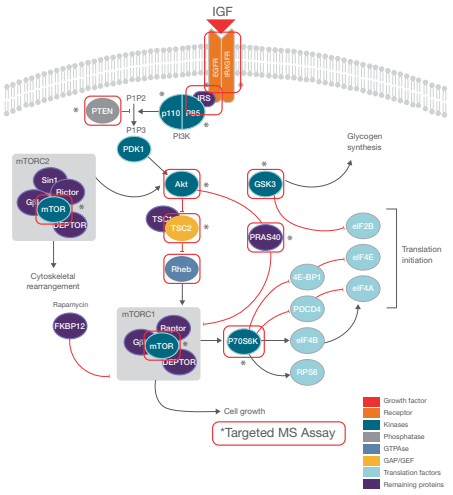


Figure 10. Schematic representation of SureQuant IS targeted protein quantitation workflow

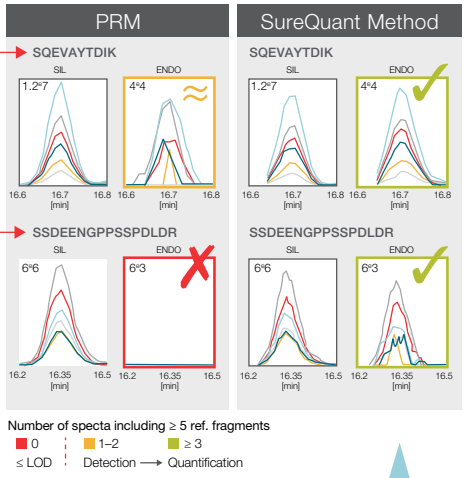
AKT/mTOR

A key cancer biology pathway



From pathway to assay

Peptide	PRM Method	SureQuant Method
AENGPGPGVLVLR	1	15
AQEALDFYGEVR	10	11
DGFYPADPFR	0	1
ETSFNQAYGR	2	6
FEISETSVNR	0	9
GALAEAAR	5	13
GNNLQDTLR	0	6
GQPEGPLPSSSPR	0	8
GYTISDSAPSR	0	5
IDIHSC[Carbamidomethyl]NHEAEK	1	12
IYNLC[Carbamidomethyl]AER	0	15
LC[Carbamidomethyl]DSGELVAIK	5	15
LFDAPEAPLPSR	11	11
LLEYTPTAR	0	15
LPFYNQDHER	0	17
LVPPFKPQVTSETDTR	0	0
NDGTFIGYK	0	13
NNIDDVVR	0	4
SDGSFIGYK	0	13
SNPTDIYPSK	0	9
SQEVAYTDIK	1	9
SSDEENGPPSSPDLDR	0	8
SVSAPQQIINPIR	0	0
TFHVDTPEER	0	0
TGIAAEEVSLPR	0	0
TLDQSPELR	2	10
TPPEAIALC[Carbamidomethyl]SR	1	20
TTINNEYNYR	0	7
VTTVVATLGQGPER	6	15
YSFQTHDR	0	10
# of endogenous peptides detected	11/30	26/30



SureQuant Targeted Protein Analysis

- Easy method set-up
- Sensitive, accurate, precise
- More complete quantitation

Application example: Comparison of the analytical performance of PRM and the SureQuant method. The SureQuant method was applied to the monitoring of AKT/mTOR signaling pathway in HeLa digest. The improved sensitivity afforded by the SureQuant method enabled 26 of the 30 targeted endogenous peptides to be detected and quantified compared to 11 by PRM even in the absence of target immunoprecipitation enrichment.[13]

Choosing the appropriate quantitative technique

The selection of the appropriate quantitative proteomics technique can be challenging due to the plethora of quantitative approaches currently available. The decision-making process must consider a number of factors, including the experimental question at hand, complexity of the sample, the resources available to the researcher such as the types of mass spectrometers, instrument time, reagents and the overall cost of the experiment. Tables 1 and 2 summarize some of the major advantages and disadvantages typically considered when choosing a technique for quantitative proteomics.

Currently, LFQ is one of the more popular quantitative approaches because unlimited numbers of samples can be compared, samples can originate from any source, does not require extensive sample preparation (e.g., labeling) and identification of the peptides is not restricted by the fragmentation technique. If the researcher has access to their own mass spectrometer then factors such as instrument time and labor costs can be negated, enabling such experiments to be performed relatively cheaply. In LFQ, where each sample is analyzed individually and samples are extremely complex, all conditions up to, and including LC analysis, must be highly reproducible. Therefore, meticulous sample handling, sample preparation, reproducible chromatography between technical and biological replicates, and sensitive, high-resolution, accurate-mass mass spectrometers are all essential. Offline fractionation is not recommended with LFQ due to the negative effect on quantitation accuracy resulting from slight variations in sample handling. The latter can affect sample depth of analysis and limit the dynamic range of proteins studied.

If instrument time and access to mass spectrometer is limited and precise data is a requirement, then multiplexing workflows using TMT are desirable. In a single analysis, multiplexing TMT workflows can be used to identify and quantify relative changes in complex protein samples across multiple experimental conditions (up to 16 currently). Since samples are quantified in the same scan, coefficients of variation tend to be quite low. The mixing of the samples following digestion and labeling permits a variety of fractionation and enrichment techniques. These techniques can improve the detection of abundance changes for both low-abundance peptides and PTMs such as phosphorylation and glycosylation. A wide selection of TMT kits are commercially available. These kits contain all of the reagents necessary for comparing two samples in small profiling studies to sixteen samples in complex analyses with multiple conditions (e.g. time courses, dose responses, replicates, and multiple-sample comparisons).

thermo scientific

If the objective of protein quantitation experiments is to determine the protein and/or peptide expression levels of known targets in biological systems, then discovery based relative quantitation experiments can be bypassed for a more focused targeted quantitation strategy.

The experiments may be designed to determine either the relative levels of the target peptide/protein or the absolute levels. The scope of these experiments can range from the analysis of individual samples in a research environment to the assessment of thousands of samples in a clinical research setting. MS-based targeted quantitation requires *a priori* knowledge of the molecular targets, as well as of the general properties of the samples in which they are contained. At a minimum, the scientist must know the molecular weight of the targeted species. Knowledge of additional properties of the targets, such as their LC elution times, expected range of expression levels, dynamic range, as well as knowledge of the characteristics of the background matrix, will all help greatly in designing a successful targeted quantitation experiment.

The recently introduced SureQuant IS targeted protein quantitation workflow has demonstrated acquisition efficiency and robustness, translating into a highly sensitive quantification workflow for large number of peptide targets (hundreds to thousands) in a wide range of samples. Its 'load-and-play' execution, especially for embedded application specific kits, significantly simplifies the user experience for non-mass spectrometry experts.

Summary

Irrespective of the method chosen, quantitative proteomics provides a powerful tool that complements genetic approaches to allow for direct insight in the molecular mechanisms regulating biological processes. In recent years, a steady stream of studies illuminating specific areas of biology have marked the successful transition of these approaches from proof-of-concept applications to essential components of a molecular biologist's toolkit. While the approaches will undoubtedly improve in sensitivity, precision, and ease of use, the current landscape of techniques and technologies allows one to gain rich knowledge about the molecular basis of biology and disease.

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