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Application of Targeted Mass Spectrometry in Bottom-Up Proteomics for Systems Biology Research

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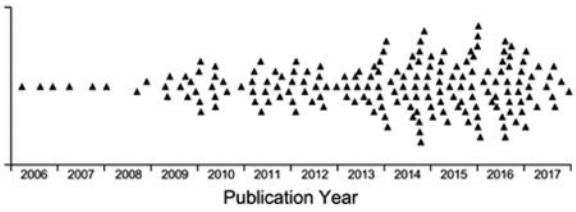
Abstract

The enormous diversity of proteoforms produces tremendous complexity within cellular proteomes, facilitates intricate networks of molecular interactions, and constitutes a formidable analytical challenge for biomedical researchers. Currently, quantitative whole-proteome profiling often relies on non-targeted liquid chromatography – mass spectrometry (LC-MS), which samples proteoforms broadly, but can suffer from lower accuracy, sensitivity, and reproducibility compared with targeted LC-MS. Recent advances in bottom-up proteomics using targeted LC-MS have enabled previously unachievable identification and quantification of target proteins and posttranslational modifications within complex samples. Consequently, targeted LC-MS is rapidly advancing biomedical research, especially systems biology research in diverse areas that include proteogenomics, interactomics, kinomics, and biological pathway modeling. With the recent development of targeted LC-MS assays for nearly the entire human proteome, targeted LC-MS is positioned to enable quantitative proteomic profiling of unprecedented quality and accessibility to support fundamental and clinical research. Here we review recent applications of bottom-up proteomics using targeted LC-MS for systems biology research.

Graphical abstract



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Keywords

bottom-up proteomics; parallel reaction monitoring; quantification; selected reaction monitoring; systems biology; targeted mass spectrometry

1. Introduction

Bottom-up proteomics using high-performance liquid chromatography coupled to mass spectrometry (LC-MS) has developed into a powerful and highly versatile technology that is enabling rapid advances in diverse areas of biomedical research, clinical diagnostics, and biotechnology [1, 2]. This is especially true for the emerging scientific discipline of systems biology [3]. Beyond basic proteomic profiling, proteomics has recently evolved to enable identification and quantification of protein isoforms (*e.g.*, splice isoforms, single amino acid polymorphisms, and other genetic variants), numerous posttranslational modifications (PTMs), protein turnover, protein conformations, protein-protein interactions, protein interactions with other molecules, and protein-protein subcellular proximity [1, 2]. Recent novel applications include the search for "missing proteins" (genes and transcripts that appear to encode proteins but direct experimental evidence is lacking), kinomics, and enzymatic activity assays related to protein modification (described below).

Bottom-up proteomics LC-MS workflows typically use data-dependent acquisition (DDA, often referred to as shotgun MS), data-independent acquisition (DIA), or targeted LC-MS. DDA involves real-time semi-stochastic intensity-based selection of analytes for fragmentation, and is often the method of choice for discovery-level proteomics. DIA is an emerging technology which involves nonstochastic multiplexed fragmentation using relatively wide precursor ion isolation windows [4, 5]. Compared to DDA, DIA spectra are more challenging to analyze, but DIA can identify more peptides with better tandem MS sampling reproducibility, and with better quantification accuracy and precision [6, 7]. As publicly available DDA spectral libraries and DIA software are further developed, the need for experimentalists to perform DDA prior to each DIA experiment will decrease. Importantly, this requires robust control of the false discovery rate of peptide, protein, and PTM identification.

In a targeted LC-MS experiment, a target list of analyte ion descriptors (*e.g.*, precursor and fragment ion m/z, collision energy, LC elution time) is pre-designated to perform MS^1 of the precursor ion and/or tandem MS^n of one or more fragment ions [8, 9]. Targeted LC-MS assays are generally designed with the use of DDA LC-MS data, and therefore targeted LC-MS has greatly benefitted from large-scale proteome-wide DDA LC-MS studies [10], which include studies of the yeast proteome [11], the human proteome [12, 13], and the mouse proteome and phosphoproteome [14]. Bottom-up proteomics using targeted LC-MS is a rapidly developing technology, and numerous methods and protocols have been published [15-21]. Neither DDA nor DIA require pre-MS designation of target analytes; as such, neither is targeted MS per se. However, because data analysis of DIA spectra can involve a target list of analyte descriptors (DIA analyses typically require a library of precursor and

fragment ion m/z values produced using DDA MS), DIA is often classified as a form of targeted proteomics.

Compared with targeted MS, DDA and DIA workflows typically require significantly less preparation and result in far broader proteomic coverage, but both technologies are currently less sensitive, accurate, precise, and robust [7, 22-25]. Consequently, DDA and DIA are often used for discovery-level experimentation, whereas targeted MS is often used for biomarker validation and absolute quantification. Clinical biomarker applications of bottom-up proteomics using targeted LC-MS have been recently described in two detailed reviews [26, 27]. Therefore, here we focus on applications of targeted LC-MS for bottom-up proteomics in systems biology research (Fig. 1).

Quantitative immunoassays (*e.g.*, densitometric western blots and enzyme-linked immunosorbent assays) are widespread, and can be of very high quality. Alongside quantitative immunoassays, targeted LC-MS has developed into a powerful alternative approach that can be much more selective, can have a much wider dynamic range, is often more amenable to multiplexing, and can otherwise be of roughly similar quality [28]. For particularly challenging targets, the two strategies can be integrated by immunoenriching target proteins or peptides, and subsequently performing targeted LC-MS.

The earliest LC-MS quantification procedure involved simply analyzing extracted ion chromatograms of precursor ions, and this technique still performs very well in some applications (described below). Currently, the most common targeted technique is selected reaction monitoring (SRM), which is also referred to as multiple reaction monitoring (MRM) [29-32]. SRM uses MS^2 data for quantification, and is typically performed using a triple quadrupole MS. Due to the recent development of high resolution mass spectrometers (resolution ≥ ~50,000) that have fast scan rates (scan frequency ≥ ~10 Hz), wide dynamic ranges (range ≥ ~1,000), and accurate and precise quantification, parallel reaction monitoring (PRM) has emerged as a powerful alternative to SRM [33]. Like SRM, PRM uses MS^2 data for quantification, but whereas SRM uses fragment ion monitoring, PRM uses high resolution MS^2 full scans to monitor the intensity of multiple fragment ions in parallel (low resolution MS^2 full scans are rarely used; these are classified as PRM below). Occasionally, targeted LC-MS using MS^3 (two stages of fragmentation) is used for qualitative reasons (*e.g.*, identifying a phosphopeptide by fragmenting a neutral loss ion or distinguishing between two highly similar peptides) or for quantitative reasons (*e.g.*, to avoid ratio compression resulting from quantification using isobaric tags). MS^3 is highly selective and can be highly accurate, but it can suffer from lower sensitivity compared with MS^1 and MS^2.

Relative quantification of an analyte across multiple biological samples can be achieved by using the label-free approach (not labeling the analyte using stable isotopes, and performing LC-MS of each sample separately). Alternatively, relative quantification can be performed using a single LC-MS run by simultaneously analyzing a mixture of both unlabeled and stable isotope labeled (SIL) forms of the analyte. For absolute quantification, quantified SIL standards are used [34]. Metabolic labeling strategies include ^{13}C labeling, ^{15}N labeling, stable isotope labeling by amino acids in cell culture (SILAC), and stable isotope labeling of

mammals (SILAM). Alternatively, chemical labels include ^{18}O, SIL dimethylation, isobaric tags for relative and absolute quantitation (iTRAQ), mass differential tags for relative and absolute quantification (mTRAQ), tandem mass tags (TMT), isotope-coded affinity tags (ICAT), and isotope-coded protein labels (ICPL). Standards for qualitative and quantitative proteomics include peptides prepared using solid-phase peptide synthesis (SPPS), peptides prepared using recombinant expression of a quantification concatemer (QconCAT), and intact purified protein standards. The lattermost standards are typically used for protein standard absolute quantification (PSAQ) workflows.

Targeted MS experiments depend heavily on specialized software, and this has been reviewed by others [35-37]. Recently developed software programs for targeted MS assay development include MRMaid [38], PeptideClassifier [39] (notable for its ability to select isoform-specific peptides), PeptidePicker [40], PeptideManager [41] (notable for its support of multi-species experiments), PREGO [42], and Skyline [43]. Prediction of quantotypic peptides, which are peptides that can be assayed to accurately identify and quantify a specific proteoform or a specific set of proteoforms (*e.g.*, a set of splice isoforms) remains challenging due to, for example, alternative splicing, PTMs, and chemical artefacts that result from MS sample preparation [44, 45].

Databases of DDA MS^2 spectra of proteotypic peptides are often helpful during targeted MS assay development [46], and include The Global Proteome Machine [47], The NIST Libraries of Peptide Tandem Mass Spectra [48], and The ProteomeXchange consortium [49] (which integrates the iProX, jPost, MassIVE, PeptideAtlas, and PRIDE databases). In the absence of DDA data, proteotypic peptide prediction can be performed using CONSeQuence [50], ESP predictor [51], PeptideRank [52], PeptideSieve [53], and STEPP [54]. Unique ion signatures can be calculated to avoid MS signal interference using Sigpep [55] and SRMCollider [56]. Targeted MS software programs for peptide identification and quantification include Ariadne [57], Anubis [58], AuDIT [59], mProphet [60], MRMer [61], Pinnacle (Optys Tech Corp. Philadelphia, PA, http://www.optystech.com/), Skyline [43], and SpectroDive (Biognosys Inc., Schlieren, Switzerland, https://biognosys.com/). Downstream data analysis including statistical modeling can be performed using MSstats [62], Qualis-SIS [63], and SRMstats [64]. Online data management and sharing tools include the CPTAC assay portal [65], Panorama [66], and SRMAtlas [67].

2. Biological processes and molecular functions

Targeted proteomics has been used to study numerous biological functions and disorders including autism, cancer, metabolic syndrome, and neuron development (Table 1). For example, SRM was used to develop pluripotency assays of reprogrammed human fibroblasts [68]. In a second example, 188 biological processes in yeast (*e.g.*, osmotic balance, glucose metabolism, autophagy, and DNA damage) were studied using a multiplexed LC-SRM assay [69]. This “sentinel fingerprint assay” was used to quantify 300 target peptides to assay the abundance of 156 proteins, 11 target peptides to assay degradation products from one protein, and 166 target phosphopeptides to assay 80 phosphoproteins.

Cancer-related proteins have been studied using targeted LC-MS. Isobaric tagging combined with targeted LC-MS^3 was used to perform high-throughput relative quantification of 69 cancer-related proteins across the National Cancer Institute NCI-60 panel of sixty cancer cell lines [70]. In this study, the samples as well as the peptide targets were multiplexed, and a correlation between BAZ1B abundance and doxorubicin sensitivity was discovered. Another study used targeted proteomics and transcriptomics to identify breast cancer subtypes [71]. SRM was also used to study estrogen receptor alpha-regulated protein expression in MCF-7 breast cancer cells [72].

Targeted LC-MS has also been used to study sets of proteins related by biochemical function (Table 1). The absolute abundance of 51 chaperone proteins in yeast cells was measured using LC-SRM, and the substrate flux of each chaperone was calculated [73]. Chromatin immunoprecipitation coupled with LC-SRM was used to characterize transcriptional regulation of the environmentally regulated *FLO11* promoter in yeast [74]. Affinity purification coupled with LC-SRM was used to perform activity assays of twelve small GTPases within human platelets stimulated with thrombin and lysophosphatidic acid [75]. LC-SRM was also used to measure the kinetics of caspase-mediated proteolysis of 350 proteins in lysates and living cells [76].

3. Posttranslational modifications

Targeted LC-MS is highly amenable to the study of posttranslationally modified proteins (Table 2). In a recent report, 30 phosphorylation sites within epidermal growth factor receptor (EGFR) were profiled using both DDA and targeted MS of primary tumor explants and 31 lung cancer cell lines [77]. From this data, the authors were able to identify sites related to EGFR activation and erlotinib-mediated inhibition. A separate study found related results [78]. Notably, the authors of the later study successfully developed an LC-MS^3 assay to distinguish between two extremely similar isobaric EGFR phosphopeptides, demonstrating the utility of targeted LC-MS to study extensively modified proteins.

Proteoforms containing multiple PTMs can be especially challenging to study. Histones, for example, can often be heavily modified. In one study, genes known to be active in epigenetic processes were knocked-down in 293T cells, and a novel targeted MS workflow was used to quantify modified histones [79]. In the same report, the authors used the workflow to profile knockdowns, knockouts, and drug treatments of murine stem cells. A similar study used LC-SRM and discovered that histone H2B ubiquitination inversely correlated with H3 methylation in the U937 human leukemia cell line [80].

Among the most challenging PTMs to study are polymeric, branching PTMs such as glycosylation, polyubiquitination, and poly-ADP-ribosylation. Ohtake and colleagues used targeted MS to study polyubiquitin K48-K63 branched chains [81]. The authors found that, in response to interleukin-1β, the E3 ubiquitin ligase HUWE1 produces K48 branches on K63 chains of TRAF6. These K48-K63 branches protected TRAF6 from deubiquitination, resulting in amplification of nuclear factor κB signaling.

4. Protein conformation, protein-protein interaction, and cellular components

Targeted proteomics has been used to measure the stoichiometry of numerous protein complexes including the centrosome, the focal adhesion complex, the nuclear pore, the ribosome, and the spliceosome (Table 3). Shi and colleagues used LC-SRM to determine how ribosomal heterogeneity determines selectivity for subpools of transcripts [82]. Integration of LC-SRM with super-resolution microscopy and cryo-electron tomography was used to determine the structure of the human nuclear pore complex, and to discover that it varies across tissues, cancer cell types, and diseases [83, 84]. Ori and colleagues investigated spatiotemporal variation of human protein complex stoichiometry using numerous transcriptomic and proteomic technologies (including LC-SRM), and the nucleosome remodeling deacetylase (NuRD) complex was discovered to be an example of paralog switching within a moderately-variable protein complex [85].

Limited proteolysis (LiP) integrated with LC-SRM has been used to measure differences in protein conformation across experimental conditions [86, 87]. LiP-SRM was used to measure differences between two conformational states of the amyloid-forming protein α-synuclein (monomeric versus polymeric fibrillar, which are globally different structurally) and of myoglobin (unbound versus bound to heme, which are only structurally different at a single α-helical fold). LiP, DDA, and SRM were integrated to globally profile protein conformation differences between yeast cultured in glucose- versus ethanol-based media. Not surprisingly, conformational differences in the core carbon metabolism pathway were detected. Unexpectedly, the carboxy-terminal region of the 14-3-3 protein BMH1 was dramatically different during glucose- and ethanol-based metabolism. A BMH1-knockout strain displayed a growth defect in the ethanol-based medium, confirming that BMH1 has an as yet undetermined role in yeast ethanol metabolism. In a related study, DDA LC-MS was used to globally measure differences in protein conformation across cancer drug treatments (targeted MS was not used) [88]. Therefore, the use of LC-MS to discover changes in protein conformation across experimental conditions has developed into a novel and powerful methodology to discover changes in protein folding and/or protein-protein interaction.

In addition to studying protein conformation and protein-protein interaction, targeted MS has been used to study cellular components such as the adenovirus, the postsynaptic density, and the Gram-positive bacterial cell surface (Table 3). In the lattermost study, LC-SRM was used to produce a structural model of the *Streptococcus pyogenes* cell surface that included adhered human blood plasma proteins [89]. Targeted LC-MS has also been used to identify and quantify proteins proximal to G protein-coupled receptors (GPCRs) during signaling [90]. The β_2 adrenergic receptor and the δ-opioid receptor were each coupled to an engineered ascorbic acid peroxidase (APEX). This enabled APEX catalyzed proximity labeling, discovery of proximal proteins using DDA MS, and quantification of proximal proteins using targeted MS to reveal spatiotemporal signaling by and trafficking of both GPCRs. WWP2 and TOM1 were identified as novel mediators of δ-opioid receptor

degradation subsequent to prolonged activation (possibly via ubiquitination and trafficking to lysosomes).

5. Kinomics and phosphoproteomics

Targeted phosphoproteomic profiling has been used to investigate drug-induced phosphorylation, EGF-induced tyrosine phosphorylation, and mitochondrial phosphoproteomics (Table 4). It was first used to quantify EGF-induced tyrosine phosphorylation initially discovered using DDA LC-MS [91]. Seven time points following EGF treatment of 184A1 human mammary epithelial cells were analyzed using SRM, and 31 novel EGF-regulated tyrosine phosphorylation sites were discovered. In a second phosphoproteomic study, targeted MS enabled simplified profiling of drug-induced phosphorylation cascades using the P100 abridged set of target phosphopeptides [92]. In this investigation, clusters of correlated phosphosites were identified using DDA LC-MS, and each cluster was assayed using targeted MS of one or two representative phosphopeptide members. Hundreds of drug-treatment samples were rapidly profiled, and it was discovered that each drug produced a highly reproducible and distinct P100 phospho-signature. These two reports demonstrate the utility of targeted phosphoproteomics downstream of DDA LC-MS.

Targeted kinomics has been performed by coupling affinity enrichment of active protein kinases with targeted LC-MS, and this has been used to study breast, colorectal, lung, and skin cancer, as well as diabetes and arsenic poisoning (Table 4). Significant kinome reprogramming was discovered by comparing dasatinib-sensitive and insensitive melanoma cells, and also lung tumor and adjacent normal lung tissue [93]. A comparison of radiation therapy sensitive and resistant breast cancer cells revealed abundance alterations of kinases that control cell cycle progression and DNA repair [94]. Tyrosine kinase profiling was used to investigate EGF stimulation of skin cancer cells, APC mutation within colon cancer cells, ten colorectal cancer cell lines, and erlotinib-sensitive and insensitive lung cancer cells [95]. Colorectal cancer cell kinomics revealed compensatory activation of transforming growth factor beta (TGF-β) receptor superfamily members in response to treatment with three different mitogen-activated protein kinase (MAPK) inhibitors [96]. Fang and colleagues integrated kinomics and tyrosine phosphoproteomics to study lung cancer cell lines and tumors [97]. The activity of many kinases (measured using desthiobiotin-ATP labeling) correlated with their phosphorylation state. This study demonstrated the high value of integrating affinity enrichment of active protein kinases, phosphopeptide-enrichment, and targeted MS to study signaling cascades.

In addition to profiling the abundance and phosphorylation state of the kinome, targeted LC-MS has also been used to assay the enzymatic activity of the kinome using a method termed KAYAK (Kinase ActivitY Assay for Kinome profiling) (Table 4). In a KAYAK assay, the activation state of many kinases within a cell lysate is measured by incubating the lysate with a peptide library and subsequently performing targeted LC-MS of the resulting phosphopeptides. KAYAK was first used to profile the activity of the kinome upon mitogen stimulation, during the cell cycle, and across cancer cell lines [98, 99]. Fast protein liquid chromatography (FPLC) coupled with KAYAK was used to identify phosphorylation

activity, the responsible kinase, and any associated protein complex members. A novel SRC-catalyzed tyrosine phosphorylation site on phosphatidylinositol 3-kinase (PI3K) regulatory subunits was discovered. In addition, the CDC2 – CCNB1 complex was identified as an activated kinase during mitosis. Therefore, KAYAK and FPLC-KAYAK have emerged as powerful methods for quantitative comparative kinome activity profiling and for the discovery of the responsible kinase(s). More recently, KAYAK was used to measure dose response curves of the PKC inhibitor Ro-31-8425 on kinases involved in monocyte differentiation into macrophages [100].

6. Metabolic pathways

A fundamental goal of systems biology is the comprehensive characterization of biological pathways to enable accurate pathway simulation at the molecular interaction level. These simulations are needed for the diagnosis of diseases, to design therapeutic interventions, and for pathway engineering. Numerous targeted proteomics investigations have been focused on the characterization of metabolic pathways (Table 5). Some of these projects used targeted proteomics to support *Escherichia coli* metabolic pathway engineering. These included optimizing the production of a sesquiterpene [101], engineering the mevalonate and tyrosine biosynthesis pathways [102], and increasing tyrosine production [103]. A novel principal component analysis was successfully applied to targeted proteomics and metabolomics data to direct engineering of the mevalonate pathway [104]. Fine-tuning the expression of a polyketide pathway protein was used to optimize the production of metabolites that could function as possible future biofuels [105].

In some investigations, targeted proteomics has been integrated with transcriptomics, metabolomics, and/or phosphoproteomics to study metabolic pathways. Oliveira and colleagues combined targeted proteomics with targeted phosphoproteomics to determine how protein abundance and phosphorylation affect enzymatic fluxes in yeast central metabolic pathways [106]. It was discovered that the absolute abundance of only the non-phosphorylated form of PDA1 correlated significantly with PDA1 enzymatic flux (total PDA1 abundance and phospho-Ser313 PDA1 abundance did not correlate with enzymatic flux). In another study, transcriptomics, targeted proteomics, and metabolomics were combined to produce a full picture of the macrophage prostaglandin biosynthetic pathway over a 24 hour time-course after stimulation with lipid A [107]. Using a similar approach, Wienkoop and colleagues used targeted proteomics and metabolomics to produce a detailed picture of metabolic and photosynthetic pathways within unicellular green algae [108]. In another multi-omic study, transcriptomics, targeted proteomics, and metabolomics were integrated to study the induction of terpene synthesis over a 32 day time-course in tree bark after treatment with an insect defense hormone [109].

In possibly the most extensive investigation of a metabolic pathway thus far, targeted proteomics, metabolomics, enzyme assays, and pathway modeling were integrated to construct and refine a model of the yeast glycolysis pathway [110]. Absolute abundance values of pathway proteins and metabolites were quantified using MS. Enzyme kinetics of purified proteins were assayed using *in vitro* conditions designed to mimic the *in vivo* environment. The protein abundance and kinetics data were input into an initial pathway

model as parameters, pathway simulations were performed, and the resulting predicted metabolite abundances were compared to measured values. Eighteen iterations of model refinement were performed, partly to account for the effects of side reactions (*e.g.*, the glycerol branch on the core glycolytic pathway), reducing the normalized root-mean-square deviation down to ~30%.

7. Signaling pathways

Diverse signaling pathways have been studied using targeted LC-MS (Table 6). Quantification of circadian clock transcript and protein oscillations within wild type and knockout mice revealed the roles of clock proteins and enabled the development of a novel assay for circadian time [111]. Intriguingly, the delay between the circadian transcript and protein abundance peaks spanned from ~0 to ~8 hours via mechanisms that have yet to be discovered. In a recent report, we integrated DDA cellular proteomics, DDA secretomics, targeted secretomics, and transcriptomics to study pattern recognition receptor signaling [112]. This multi-omic approach enabled detailed comparisons of macrophages stimulated using individual pattern recognition receptor ligands (lipopolysaccharide, Pam3CSK4, and resiquimod) and whole bacteria (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Burkholderia cenocepacia*). Sabido and colleagues used targeted proteomics of the insulin signaling pathway merged with seven metabolic pathways to study metabolic syndrome resulting from a high-fat diet [113]. The metabolic pathways were: fatty acid biosynthesis, fatty acid β-oxidation, glycolysis and gluconeogenesis, pentose phosphate pathway, TCA cycle, ketogenesis, and glycogen metabolism. de Graaf and colleagues used targeted phosphoproteomics of the PI3K – mechanistic target of rapamycin (mTOR) – MAPK pathway to discover phosphorylation sites affected by oncogene-induced senescence and pharmacological intervention using BEZ235 (an inhibitor of both PI3K and mTOR) [114].

Some quantitative LC-MS studies of signaling pathways have revealed patterns of conserved stoichiometry. Transcriptomics, targeted proteomics, and targeted phosphoproteomics were used to study the EGFR – MAPK pathway within a variety of normal and cancerous human cell types [115]. The stoichiometry of the pathway transcripts and proteins were found to be very similar across the cell types. The glutamatergic signaling pathway within the auditory cortex of schizophrenic and control subjects was quantitatively compared using LC-SRM, and pathway protein expression and co-expression were significantly correlated with the disease [116]. Dysregulation of co-expression strongly correlated with reduced dendritic spine density (a schizophrenia phenotype), demonstrating the high value of co-expression analysis of targeted proteomics data.

Quantification of pathway proteins and PTMs can be used to enable accurate pathway modeling. Targeted phosphoproteomics was used to study an *in vitro* minimal MAPK pathway consisting of only five proteins: a two stage phosphorylation cascade consisting of three proteins, and the two reverse reactions catalyzed by two phosphatases [117]. The experiments were designed to measure only quasi-steady-state behavior (reaction time = 30 min). Even in this simple system, perturbations caused by altering protein concentrations resulted in reequilibration of phosphorylation that required mass-action kinetics to correctly model (that is, simplistic inferences failed).

Determination of constants related to molecule-molecule interaction, molecular transformation (*e.g.*, in protein conformation), and catalysis is necessary for the simulation of biological pathways at the molecular level. Numerous experimental methods have been developed to measure affinity constants *in vitro* (*e.g.*, surface plasmon resonance) and *in vivo* (*e.g.*, fluorescence cross-correlation spectrometry) [118-121]. In addition, structural modeling software has been developed that can be used to estimate affinity constants (PRODIGY, SDA, TransComp, and related tools) [122-127]. A novel strategy using targeted proteomics was used to measure *in vivo* dissociation constants of the yeast galactose signaling pathway consisting of galactose, four proteins (Gal1p, Gal3p, Gal4p, and Gal80p), and the genes transcriptionally activated by Gal4p (including those encoding Gal1p, Gal3p, and Gal80p) [128]. The abundance of the four proteins was systematically varied genetically and quantified using LC-SRM, and the pathway output (target gene transcription) was quantified. From these data, the protein-protein and protein-DNA dissociation constants were determined.

We used targeted proteomics to enable accurate pathway modeling of the mouse macrophage chemotaxis pathway [129]. RNA-seq was used to identify target protein splice isoforms and to estimate pathway protein absolute abundance values. LC-SRM was used to measure the absolute abundance of pathway proteins to accurately parameterize a pathway model. The Simmune software suite [130, 131] was used for rule-based pathway modeling, microscopy data were used for model training, and GTPase activation assay data were used for model accuracy testing. The model successfully simulated pathway behavior consistent with the GTPase data, which was not used for model training and was highly orthogonal to the microscopy data. In addition, 2,000 perturbed models were generated and used to demonstrate that the pathway model was robust. In this way, targeted MS and other state-of-the-art technologies are enabling the development of accurate and robust pathway models, which are critical to the advancement of systems biology, and which will aid the development of diagnostics, therapeutics, and personalized medicine.

8. Proteome-wide targeted MS and proteogenomics

Targeted MS has an important role in proteogenomics, especially coding sequence annotation (Table 7). Approximately 18% of the human proteome is classified as “missing” because there is not strong experimental evidence of the existence of these proteins [132]. To address this challenge, the Human Proteome Project is employing targeted proteomics and other technologies, and have confidently identified hundreds of formerly missing proteins. A typical strategy is to develop LC-SRM assays using synthesized peptide standards, and then to use these LC-SRM assays to analyze biological samples (selected because they express high levels of the corresponding transcript). Because of the excellent sensitivity and specificity of targeted MS, these efforts have often been very successful. For example, one study used DDA, PRM, and immunohistochemistry to confirm the expression of 206 previously missing proteins [133]. In another example, Omasits and colleagues combined a stringent re-analysis of proteomics and transcriptomics data with validation using LC-PRM to annotate coding sequences of *Bartonella henselae* [134]. Small coding sequences (~50 residues or fewer) are especially challenging to annotate, and have recently been identified in numerous genomes including those of mammals [135, 136], mammalian mitochondria

[137-139], and prokaryotes [140, 141]. For example, LC-MS[1] and LC-SRM were used to identify and quantify small open reading frame-encoded polypeptides within human cancer cells [135, 136].

Targeted proteomics coupled with other technologies has been used for proteome-wide absolute abundance estimation [142]. DDA and targeted LC-MS were combined to estimate protein abundances from precursor ion intensity values [143, 144]. Similarly, because transcript and protein abundance are sometimes significantly correlated [145], targeted proteomics coupled with transcriptomics has been used to estimate protein abundance from transcript abundance [129, 146]. It is important to note that transcript-protein absolute abundance correlations can vary dramatically across different target protein sets. For example, in yeast the transcript-protein correlation of the glycolysis pathway was strong (Spearman r = 0.97), whereas the correlation for ribosomal proteins was weak (Spearman r = ~0) [147]. This disparity may have resulted from the very different ranges in protein abundance across the two target protein sets. The glycolysis proteins ranged from 40,000 – 1,500,000 copies per cell, whereas the ribosomal proteins only ranged from 250,000 – 400,000 copies per cell. Therefore, protein abundance estimation using targeted proteomics integrated with global transcriptomics requires that the target proteins range in abundance across many orders of magnitude.

Recently, targeted LC-MS has developed into a technology capable of proteome-wide investigation, and currently the proteomes of four species have been analyzed almost in entirety: *Mycobacterium tuberculosis*, *Streptococcus pyogenes*, *Saccharomyces cerevisiae*, and *Homo sapiens* (Table 7). The first human proteome-wide investigation used SPPS to generate 166,174 peptide standards, which were used to successfully develop LC-SRM assays for 20,225 proteins (158,015 peptides) [148]. To demonstrate the utility of this resource, the authors investigated the effects of atorvastatin treatment on the cholesterol synthesis pathway in liver cells, and they also investigated a network of proteins associated with docetaxel inhibition of prostate cancer cell division. A second human proteome-wide investigation used 18,081 recombinant proteins to successfully develop LC-SRM assays for 16,108 proteins (138,009 peptides) [149]. The authors used this resource to quantify 634 enzymes to study the effects of oncogenesis on metabolic pathways. Because the development of a targeted LC-MS assay can be demanding and time-consuming, the proteome-wide development of human protein assays has greatly increased the accessibility of this powerful tool to scientists across a wide spectrum of biomedical research fields.

9. Conclusion

Proteomics has evolved far beyond basic proteomic profiling using DDA LC-MS. Targeted proteomics has been used to robustly quantify protein abundance, synthesis, degradation, PTMs, and other chemical modifications. Proteogenomic applications include identification of splice isoforms, identification of single amino acid polymorphisms and other genetic variants, identification of missing proteins, and quantification of DNA- and RNA-level regulation of protein expression (*e.g.*, RNA interference). Functional proteomics applications include kinomics, enzymatic activity assays related to protein modification (*e.g.*, protein phosphorylation, proteolysis), and measurement of protein conformation,

protein-protein interaction, protein interaction with other molecules, and protein-protein subcellular proximity. These technologies are enabling biological pathway mapping, simulation, and engineering, and targeted proteomics integrated with other technologies (*e.g.*, transcriptomics, metabolomics) has been especially productive. With the recent development of LC-SRM assays for nearly the entire human proteome, targeted proteomics has emerged as a powerful technology for biomedical research, clinical applications, and biotechnology research and development.

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Significance

This manuscript is a comprehensive review of the recent advances in bottom-up targeted proteomics research for cell signaling pathways and modeling.

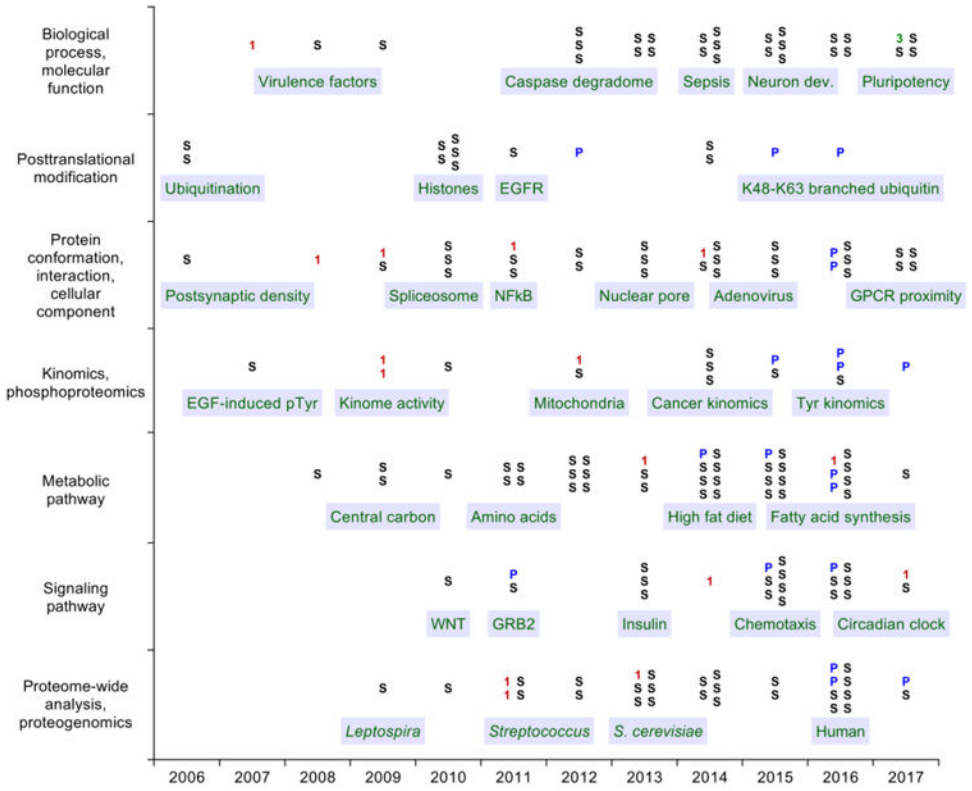

Figure 1. Timeline of selected applications of targeted LC-MS for systems biology research

Research articles were partitioned into seven research categories and plotted by publication year. The symbols (1, 3, P, and S) indicate the principal MS scan type that was used for quantification (MS^1, MS^3, PRM, and SRM, respectively). Selected research topics are noted.

Table 1

Selected studies of biological processes and molecular functions

Topic	Species (specimen)	MS scan	Proteins (peptides)	PTMs	Isotopic labeling (absolute quantification)	Ref.
Adipogenesis transcription factors	Mouse (3T3-L1 preadipocytes)	SRM	10 (25)	None	PSAQ (copies/cell)	[150]
Antibiotic treatment	*Leptospira interrogans*	SRM	39 (151)	None	None, SPPS (copies/cell)	[151]
Autism, schizophrenia	Mouse (frontal cortex, hippocampus)	SRM	38 (86)	None	SPPS	[152]
Biomarkers of cellular processes	*Saccharomyces cerevisiae*	SRM	182 (690)	None, phosphorylation	None, SPPS	[69]
Cancer invasion secretomics	Human (U87 glioblastoma cell line)	SRM	65 (183)	None	SPPS	[153]
Cancer subtypes	Human (30 breast cancer cell lines)	SRM	319 (645)	None	SPPS (pmol/mg protein)	[71]
Cancer-related proteins	Human (60 tumor cell lines)	MS^3	68 (126)	None	SPPS-TMT	[70]
Caspase degradomics	Human (Jurkat cell line)	PRM, SRM	275 (314)	None	None, SPPS	[154]
Caspase degradomics during apoptosis	Human (DB, Jurkat A3, MM.1S cells)	SRM	533 (789)	None	None	[155]
Caspase degradomics during apoptosis	Human (Jurkat cell line)	SRM	350 (431)	None	None	[76]
Chicory leaf infection	*Dickeya dadantii*	SRM	445 (782)	None	None	[156]
Degradomics of wound healing	*Sus scrofa* (skin)	SRM	9 (19)	None	iTRAQ	[157]
Estrogen receptor-mediated expression	Human (MCF-7 breast cancer cell line)	SRM	62 (65)	None	SILAC	[72]
Heat shock response chaperones	*Saccharomyces cerevisiae*	SRM	47 (166)	None	QconCAT (copies/cell)	[158]
Histone H1	Human (3 myeloid cell types)	SRM	8 (13)	None	None	[159]
Ketamine pharmacodynamics	Rat (cerebrum, hippocampus)	SRM	49 (98)	None	SPPS	[160]
Metabolic syndrome	Mouse (liver)	SRM	144 (312)	None	SILAC cells	[7]
Molecular chaperones	*Saccharomyces cerevisiae*	SRM	51 (77)	None	QconCAT (copies/cell)	[73]
Muscle development	*Gallus gallus* (skeletal muscle)	MS^1	20 (20)	None	QconCAT (pmol/mg protein)	[161]
Neuronal development	Human (3 neural stem cell lines)	SRM	175 (279)	None	SPPS	[162]
Oncogenesis transcription factors	Human (8 lung cancer cell lines)	SRM	28 (36)	None	SPPS (pmol/mg protein)	[163]
Parkinson's disease	Rat (PC12 cell line)	SRM	2 (20)	None, nitration	None	[164]
PhoP-PhoQ virulence gene expression	*Salmonella typhimurium*	SRM	92 (152)	None	SIL dimethylation	[165]
Pluripotency evaluation	Human (fibroblasts, stem cells, embryoi bodies)	d SRM	15 (33)	None	SPPS (pmol/pmol GAPDH)	[68]
Sepsis	Human (neutrophils, plasma)	SRM	49 (136)	None	None	[166]

Topic	Species (specimen)	MS scan	Proteins (peptides)	PTMs	Isotopic labeling (absolute quantification)	Ref.
Small GTPase activity assays	Human (platelets)	SRM	12 (17)	None	SPPS	[75]
Transcription regulation	*Saccharomyces cerevisiae*	SRM	209 (355)	None	SPPS (copies/cell), SILAC	[74]
Virulence factors	*Streptococcus pyogenes*	SRM	21 (48)	None	ICPL	[167]

n.i., not indicated

Table 2

Selected studies of posttranslational modifications

Topic	Species (specimen)	MS scan	Proteins (peptides)	PTMs	Isotopic labeling (absolute quantification)	Ref.
Cell-division cycle	Human (Jurkat cell line)	SRM	1 (4)	None, phosphorylation	SPPS (stoichiometry)	[168]
Cyclin-B1 ubiquitination	Human (recombinantly expressed)	SRM	1 (11)	Ubiquitination	SPPS (stoichiometry)	[169]
EphA2 autophosphorylation	Human (recombinantly expressed)	SRM	1 (23)	None, phosphorylation	None, SPPS (stoichiometry)	[170]
Epidermal growth factor receptor	Human (31 lung cancer cell lines)	SRM	1 (7)	None, phosphorylation	SPPS	[77]
Epidermal growth factor receptor	Human (A431 carcinoma cell line)	MS^3, PRM	1 (15)	None, phosphorylation	None, SPPS (stoichiometry)	[78]
Heavy metal ubiquitinomics	Human, *Saccharomyces cerevisiae*	SRM	1 (24)	None, phosphorylation, ubiquitination	SPPS (pmol/mg protein)	[171]
Histone H3	Human, mouse (40 cell lines)	PRM	1 (66)	Acetylation, methylation, dimethylation, trimethylation, phosphorylation, ubiquitination	SPPS (stoichiometry), SILAC	[79]
Histones	Human (U-937, HL-60 cell lines)	SRM	4 (21)	Acetylation, methylation, dimethylation, trimethylation, ubiquitination	SPPS (stoichiometry)	[80]
HSP27 isobaric phosphopeptides	Human (breast cancer cells, tumor)	SRM	1 (4)	None, phosphorylation	SPPS (stoichiometry)	[172]
Intracellular Cys oxidation	Human (MCF-7, HCA2 cell lines)	SRM	2 (14)	Oxidation	SIL alkylation	[173]
K48-K63 branched ubiquitin	Human (U2OS, HEK293-F cell lines)	PRM	1 (13)	Ubiquitination	SPPS (pmol/injection)	[81]
Lyn phosphorylation stoichiometry	Human (myeloma cells, xenograft tumor)	SRM	1 (14)	None, phosphorylation	None	[174]
Mercury toxicology	Mouse (WEHI-231 B lymphoma cell line)	SRM	1 (5)	None, phosphorylation	None	[175]

Table 3

Selected studies of protein conformation, protein-protein interaction, and cellular components

Topic	Species (specimen)	MS scan	Proteins (peptides)	PTMs	Isotopic labeling (absolute quantification)	Ref.
60S pre-ribosome	*Saccharomyces cerevisiae*	SRM	51 (149)	None	None	[176]
Adenovirus	Adenovirus particles	SRM	13 (33)	None	SPPS (stoichiometry)	[177]
ASK signalosome	Human (HEK293 cell line)	PRM	99 (265)	None	None, SPPS (stoichiometry)	[178]
Blood-*Streptococcus* interactomics	Human (plasma)	SRM	152 (406)	None	None	[179]
Blood-*Streptococcus* interactomics	Human (plasma), *Streptococcus pyogenes*	SRM	56 (76)	None	SPPS (stoichiometry)	[89]
Centrosome	Human (5 cell lines)	SRM	9 (18)	None	SPPS (copies/centrosome)	[180]
Cohesin complex	Human (MCF-7, HeLa cell lines)	MS[1]	11 (22)	None	QconCAT (stoichiometry)	[181]
Cullin-RING complex	Human (HEK293T cell line)	SRM	25 (38)	None	SPPS (stoichiometry)	[182]
Focal adhesion complex	Human (breast cancer cells, tumor)	MS[1], SRM	17 (27)	None	None, SILAC	[183]
G protein subcellular localization	Mouse (4 brain tissues)	SRM	12 (33)	None	None	[184]
GPCR proximity labeling	Human (HEK293 cell line)	SRM	65 (187)	None	None	[90]
Hsp90-associated proteins	Human (HeLa cell line)	PRM	4 (36)	None	SILAC	[185]
MP1-p14 complex	Mouse (recombinantly expressed)	MS[1], SRM	2 (10)	None	SPPS-mTRAQ (stoichiometry)	[186]
NFκB complex	Human (A549 lung epithelial cell line)	SRM	3 (3)	None	SPPS (copies/cell)	[187]
NFκB complex	Human (SK-N-AS cell line)	SRM	4 (11)	None	QconCAT (copies/cell)	[188]
Nuclear pore	Human (45 cells, tissues)	SRM	47 (157)	None	SPPS (stoichiometry)	[83]
Nuclear pore	Human (HEK293 cell line)	SRM	32 (76)	None	SPPS (stoichiometry)	[84]
Numb-associated endocytosis	Human (HEK293T cell line)	SRM	14 (40)	None	None	[189]
NuRD complex	Human (HEK293, HeLa cell lines)	SRM	10 (24)	None	SPPS	[85]
Podocyte slit diaphragm nephrin	Rat (renal glomeruli)	SRM	1 (1)	None	SPPS (copies/cell)	[190]
Postsynaptic density	Rat (brain)	SRM	112 (337)	None	None, SPPS	[191]
Postsynaptic density	Rat (brain)	SRM	32 (32)	None	SPPS (pmol/mg protein)	[192]
Postsynaptic density	Rat (cerebral cortex)	SRM	42 (89)	None	QconCAT (copies/postsynaptic density)	[193]
Protein complexes	Human (recombinantly expressed)	SRM	6 (2)	None	SPPS (stoichiometry)	[194]
Protein conformation dynamics	*Saccharomyces cerevisiae*	SRM	135 (697)	None	None	[86]
Protein phosphatase 2A complexes	Human (HEK293 cell line)	MS[1]	10 (n.i.)	None	SPPS (stoichiometry)	[195]

Topic	Species (specimen)	MS scan	Proteins (peptides)	PTMs	Isotopic labeling (absolute quantification)	Ref.
Ribosome	*Escherichia coli*	MS^1	24 (41)	None	QconCAT (stoichiometry)	[196]
Ribosome	Mouse (ES-E14 embryonic stem cells)	SRM	15 (28)	None	SPPS (stoichiometry)	[82]
RNA polymerase complex	*Bacillus subtilis*	SRM	8 (16)	None	SPPS-mTRAQ (stoichiometry)	[197]
Rod photoreceptor outer segment	Rat (retinas)	SRM	2 (2)	None	SPPS	[198]
Spliceosome	Human (HeLa cell line)	SRM	5 (10)	None	SPPS (stoichiometry)	[199]
Transducin heterotrimeric G-protein	*Bos taurus* (retina)	MS^1	3 (3)	None	QconCAT (stoichiometry)	[200]

n.i., not indicated

Table 4

Selected kinomics and phosphoproteomics studies

Topic	Species (specimen)	MS scan	Proteins (peptides)	PTMs	Isotopic labeling (absolute quantification)	Ref.
Breast cancer kinomics	Human (MCF-7 breast cancer cell lines)	SRM	150 (179)	None	SIL chemical tag	[94]
Cancer Tyr kinomics	Human (12 cancer cell lines)	PRM	86 (307)	None	None	[95]
Colorectal cancer kinomics	Human (HCT116 colorectal cancer cells)	PRM	173 (822)	None	None	[96]
Drug-induced phosphorylation	Human (5 cell lines)	PRM	86 (96)	Phosphorylation	SPPS	[92]
EGF-induced pTyr	Human (184A1 mammary epithelial cells)	SRM	144 (226)	Phosphorylation	iTRAQ	[91]
Kinome activity during differentiation	Human (HeLa, THP-1 cell lines)	MS^1	47 (60)	Phosphorylation	SPPS (pmol/(min*mg protein))	[100]
Kinome activity in cancer	Human (14 cell lines, tumor)	MS^1	73 (90)	Phosphorylation	SPPS (pmol/(min*mg protein))	[99]
Kinome activity in cancer	Human (9 cell lines)	MS^1	73 (90)	Phosphorylation	SPPS (pmol/(min*mg protein))	[98]
Kinomics	Human (12 cell lines)	SRM	83 (204)	None	None	[44]
Kinomics	*Saccharomyces cerevisiae*	SRM	118 (214)	None	None	[201]
Kinomics of arsenic poisoning	Human (GM00637 skin fibroblast cell line)	SRM	234 (245)	None	SILAC	[202]
Kinomics of diabetes	Human (HEK293T, GM00637 cell lines)	SRM	328 (402)	None	SIL chemical tag	[203]
Lung cancer kinomics	Human (lung cancer tumor, cells)	PRM, SRM	329 (789)	None, phosphorylation	None	[97]
Lung, skin cancer kinomics	Human (cancer cells, tumor)	SRM	270 (301)	None	SIL chemical tag	[93]
Mitochondrial phosphoproteomics	Mouse (heart)	SRM	7 (23)	None, phosphorylation	SPPS	[204]

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Table 5

Selected studies of metabolic pathways

Topic	Species (specimen)	MS scan	Proteins (peptides)	PTMs	Isotopic labeling (absolute quantification)	Ref.
Acetyl-CoA biosynthesis	*Escherichia coli*, *Saccharomyces cerevisiae*	PRM	177 (901)	None	None	[205]
Central carbon metabolism	*Bacillus subtilis*	SRM	41 (85)	None	QconCAT	[206]
Central carbon metabolism	*Corynebacterium glutamicum*	SRM	10 (30)	None	QconCAT (copies/cell)	[207]
Central carbon metabolism	*Corynebacterium glutamicum*	SRM	19 (57)	None	^{15}N cells	[208]
Central carbon metabolism	*Escherichia coli*	SRM	22 (99)	None	PSAQ (pmol/ml cytoplasm)	[209]
Central carbon metabolism	Human (colorectal cancer cells, tumors)	PRM, SRM	75 (208)	None	None, SPPS (pmol/mg protein)	[210]
Central carbon metabolism	Human (MCF-7 breast cancer cell line)	SRM	76 (134)	None	None	[211]
Central carbon metabolism	*Saccharomyces cerevisiae*	SRM	137 (260)	None	SPPS (copies/cell)	[212]
Central carbon, amino acid metabolism	*Saccharomyces cerevisiae*	SRM	135 (300)	None	^{13}C cells	[213]
Central carbon, amino acid metabolism	*Saccharomyces cerevisiae*	SRM	137 (303)	None	^{13}C cells	[214]
Central carbon, amino acid metabolism	*Saccharomyces cerevisiae*	SRM	228 (428)	None	^{15}N cells	[215]
Central carbon, amino acid metabolism	*Saccharomyces cerevisiae*	SRM	4 (11)	None, phosphorylation	SPPS (pmol/ml lysate)	[106]
Central metabolic pathways	*Escherichia coli*	SRM	392 (665)	None	QconCAT (pmol/injection)	[216]
Citric acid cycle	Mouse (liver)	SRM	4 (58)	None	SILAM	[217]
Drug metabolism	Human (liver)	SRM	25 (51)	None	QconCAT (pmol/mg protein)	[218]
Drug metabolism	Human (liver)	SRM	14 (30)	None	SPPS (pmol/mg protein)	[219]
Drug metabolism	Human (liver)	SRM	22 (38)	None	^{18}O-QconCAT (pmol/mg protein)	[220]
Drug metabolism	Human (liver, intestine)	SRM	13 (13)	None	SPPS (pmol/mg protein)	[221]
Drug metabolism	Mouse (6 tissues)	SRM	27 (27)	None	None, SPPS (pmol/mg protein)	[222]
Drug metabolism and transport	Human (intestine, liver, kidney)	PRM, SRM	43 (44)	None	SPPS (pmol/mg protein)	[223]
Drug transport	Human (jejunum, ileum)	SRM	10 (10)	None	SPPS (pmol/mg protein)	[224]
Drug transport	Human (jejunum, ileum)	SRM	6 (6)	None	QconCAT (pmol/mg protein)	[225]
Drug transport	Human (renal cortex)	SRM	17 (17)	None	SPPS (pmol/mg protein)	[226]
Drug transport	Mouse (brain, liver, kidney)	SRM	36 (38)	None	SPPS (pmol/mg protein)	[227]

Topic	Species (specimen)	MS scan	Proteins (peptides)	PTMs	Isotopic labeling (absolute quantification)	Ref.
Eicosanoid synthesis pathway	Mouse (RAW 264.7 macrophage cell line)	SRM	29 (41)	None	SILAC	[107]
Fatty acid synthesis pathway	*Escherichia coli*	SRM	12 (22)	None	PSAQ (pmol/ml lysate)	[228]
Glycolytic pathway	Human (6 cell lines)	SRM	24 (80)	None	SIL dimethylation	[229]
Glycolytic pathway	*Saccharomyces cerevisiae*	SRM	27 (59)	None	QconCAT (copies/cell)	[230]
Glycolytic pathway	*Saccharomyces cerevisiae*	MS[1]	27 (59)	None	QconCAT (copies/cell)	[110]
High fat diet metabolic pathways	Mouse (liver)	SRM	192 (309)	None	SILAC cells, SPPS	[231]
Metabolic and photosynthetic	*Chlamydomonas reinhardtii*	SRM	88 (105)	None	SPPS (copies/cell)	[108]
pathways Mevalonate pathway	*Escherichia coli*	SRM	17 (18)	None	QconCAT (pmol/ml cytoplasm)	[232]
Mevalonate pathway	*Escherichia coli*	SRM	9 (n.i.)	None	None	[104]
Mevalonate, tyrosine pathways	*Escherichia coli*	SRM	24 (48)	None	None	[102]
Mitochondrial metabolism	Human, mouse, rat (liver)	SRM	57 (118)	None	QconCAT (pmol/mg protein)	[233]
Organohalide respiration	*Dehalococcoides mccartyi*	PRM, SRM	10 (25)	None	SPPS (copies/cell)	[234]
Polyketide pathway	*Escherichia coli*	SRM	2 (6)	None	None	[105]
Ribosome, glycolytic pathway	*Saccharomyces cerevisiae*	MS[1]	78 (102)	None	QconCAT (copies/cell)	[147]
Terpene pathway	*Escherichia coli*	SRM	9 (9)	None	None	[101]
Terpene pathway	*Picea abies* (bark)	SRM	16 (19)	None	SPPS	[109]
Tyr metabolic pathway	*Escherichia coli*	SRM	11 (11)	None	None	[103]

n.i., not indicated

Table 6

Selected studies of signaling pathways

Topic	Species (specimen)	MS scan	Proteins (peptides)	PTMs	Isotopic labeling (absolute quantification)	Ref.
Chemotaxis signaling pathway	Mouse (RAW 264.7 macrophage cell line)	SRM	41 (60)	None	SPPS (copies/cell)	[129]
Circadian clock	Mouse (liver)	SRM	20 (124)	None, phosphorylation	QconCAT (pmol/mg protein), SIL dimethylation	[111]
DNA damage response network	Human (cells, tumor)	SRM	26 (69)	None, phosphorylation	SPPS (pmol/mg protein)	[235]
DNA damage response network	Human (MCF-10A, blood cells)	SRM	93 (107)	Phosphorylation	SPPS (pmol/mg protein)	[236]
EGF signaling pathway	Human (MCF-10A, tumor xenografts)	PRM, SRM	10 (36)	Phosphorylation	SPPS-iTRAQ (copies/cell)	[237]
EGF-mediated Erk1 phosphorylation	Mouse (smooth muscle)	SRM	1 (4)	None, phosphorylation	SPPS	[238]
EGFR-MAPK pathway	Human (8 cell lines)	SRM	26 (53)	None, phosphorylation	SPPS (copies/cell)	[115]
ERK signaling pathway	Human (184A1 mammary epithelial cells)	SRM	2 (8)	None, phosphorylation	SPPS (stoichiometry)	[239]
Galactose signaling pathway	*Saccharomyces cerevisiae*	SRM	5 (11)	None	SPPS (copies/cell)	[128]
GRB2 signaling	Human (HEK293T cell line)	SRM	90 (326)	None, phosphorylation	None	[240]
IGF-1 signaling pathway	Human (MCF-7 breast cancer cell line)	PRM	75 (101)	Phosphorylation	None	[241]
Insulin, central metabolic pathways	Mouse (liver)	SRM	144 (316)	None	SILAC cells	[113]
Neurotransmitter signaling	Mouse (6 brain tissues)	SRM	260 (3501)	None	Recombinant protein	[242]
Pattern recognition receptor signaling	*Arabidopsis thaliana* (leaves)	SRM	8 (13)	Phosphorylation	SPPS	[243]
Pattern recognition receptor signaling	Human (A549 lung epithelial cell line)	SRM	10 (10)	None	SPPS	[244]
Pattern recognition receptor signaling	Mouse (RAW 264.7 macrophage cell line)	MS^1	24 (178)	None	SILAC	[112]
Pattern recognition receptor signaling	Mouse (RAW 264.7 macrophage cell line)	PRM	14 (14)	None	SPPS-mTRAQ (pmol/injection)	[245]
PI3K-mTOR-MAPK pathway	Human (2 lung cancer cell lines)	MS^3, SRM	30 (42)	Phosphorylation	SPPS (pmol/mg protein)	[246]
PI3K-mTOR-MAPK pathway	Human (TIG-3 fibroblast cell line)	SRM	27 (51)	Phosphorylation	SPPS	[114]
RAF-MEK-ERK in vitro dynamics	Human, *Xenopus laevis* (recombinantly expressed)	MS^1	2 (2)	Phosphorylation	SPPS	[117]
Synaptic glutamate signaling	Human (auditory cortex)	SRM	155 (223)	None	SILAM mouse	[116]
WNT signaling pathway	Human (colon cancer cells, tissue)	SRM	22 (85)	None	SPPS (copies/cell)	[247]

Table 7

Selected proteome-wide and proteogenomics studies

Topic	Species (specimen)	MS scan	Proteins (peptides)	PTMs	Isotopic labeling (absolute quantification)	Ref.
Coding sequence annotation	*Bartonella henselae*	PRM	73 (107)	None	SPPS	[134]
Coding sequence annotation	Human (3 cancer cell lines)	SRM	36 (62)	None	None	[135]
Coding sequence annotation	Human (glioblastoma, HepaRG cells)	SRM	3 (6)	None	SPPS	[248]
Coding sequence annotation	Human (HeLa cell line)	SRM	8 (9)	None	SPPS	[249]
Coding sequence annotation	Human (K-562 leukemia cell line)	MS^1	8 (8)	None	SPPS (copies/cell)	[136]
Coding sequence annotation	Human (liver cells, tissue)	SRM	81 (81)	None	SPPS (copies/cell)	[250]
Coding sequence annotation	Human (liver)	SRM	57 (57)	None	None	[251]
Coding sequence annotation	Human (plasma)	SRM	84 (84)	None	SPPS (pmol/ml plasma)	[252]
Coding sequence annotation	Human (plasma, liver)	SRM	249 (516)	None	None	[253]
Coding sequence annotation	Human (plasma, liver)	SRM	267 (267)	None	SPPS (copies/cell)	[254]
Coding sequence annotation	Human (plasma, liver, HepG2 cells)	SRM	250 (693)	None	SPPS (copies/ml plasma, copies/cell)	[255]
Coding sequence annotation	Human (spermatozoa)	PRM	31 (51)	None	SPPS	[133]
Glycoproteome-wide analysis	Human, mouse (plasma, serum)	SRM	3360 (5568)	Glycosylation	SPPS (pmol/ml biofluid)	[256]
microRNA-mediated regulation	*Caenorhabditis elegans*	SRM	215 (470)	None	^{15}N cells	[257]
microRNA-mediated regulation	*Caenorhabditis elegans*	SRM	307 (591)	None	ICAT, ^{15}N cells	[258]
Neurexin isoform profiling	Mouse (8 brain tissues)	SRM	20 (44)	None	PSAQ (pmol/mg protein)	[259]
Proteome-wide abundance estimation	*Bacillus subtilis*	SRM	7 (16)	None	QconCAT (copies/cell)	[260]
Proteome-wide abundance estimation	*Bacillus subtilis, Staphylococcus aureus*	SRM	23 (55)	None	QconCAT (copies/cell)	[261]
Proteome-wide abundance estimation	*Escherichia coli*	SRM	41 (41)	None	SPPS (copies/cell)	[262]
Proteome-wide abundance estimation	Human (9 cell lines, 11 tissues)	PRM	55 (113)	None	QconCAT (copies/cell)	[146]
Proteome-wide abundance estimation	Human (U2OS cell line)	MS^1	53 (71)	None	SPPS (copies/cell)	[144]
Proteome-wide abundance estimation	*Leptospira interrogans*	MS^1	19 (29)	None	SPPS (copies/cell)	[263]
Proteome-wide abundance estimation	*Leptospira interrogans*	SRM	19 (32)	None	SPPS (copies/cell)	[143]
Proteome-wide analysis	Human (5 cell lines)	SRM	20225 (158015)	None	None	[148]
Proteome-wide analysis	Human (TIG-3 lung fibroblasts)	SRM	16108 (138009)	None	PSAQ-mTRAQ (copies/cell), PSAQ (copies/cell), SPPS (copies/cell)	[149]

Topic	Species (specimen)	MS scan	Proteins (peptides)	PTMs	Isotopic labeling (absolute quantification)	Ref.
Proteome-wide analysis	*Mycobacterium tuberculosis*	SRM	3894 (15679)	None	None, SPPS (pmol/mg protein)	[264]
Proteome-wide analysis	*Saccharomyces cerevisiae*	SRM	1167 (1700)	None	QconCAT (copies/cell)	[265]
Proteome-wide analysis	*Saccharomyces cerevisiae*	SRM	6399 (28216)	None	^{15}N cells	[266]
Proteome-wide analysis	*Streptococcus pyogenes*	SRM	1332 (2594)	None	None	[267]
RNAi knockdown efficacy	Human (GM00639, HEK293T cells), *Drosophila melanogaster*	SRM	6 (11)	None	None, SPPS	[268]
Single amino acid polymorphisms	Human (plasma)	SRM	3 (6)	None	SPPS (pmol/ml plasma)	[269]

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