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Automated proteomic sample preparation: The key component for high throughput and quantitative mass spectrometry analysis

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Abstract

Sample preparation for mass spectrometry-based proteomics has many tedious and time-consuming steps that can introduce analytical errors. In particular, the steps around the proteolytic digestion of protein samples are prone to inconsistency. One route for reliable sample processing is the development and optimization of a workflow utilizing an automated liquid handling workstation. Diligent assessment of the sample type, protocol design, reagents, and incubation conditions can significantly improve the speed and consistency of preparation. When combining robust liquid chromatography-mass spectrometry with either discovery or targeted methods, automated sample preparation facilitates increased throughput and reproducible quantitation of biomarker candidates. These improvements in analysis are also essential to process the large patient cohorts necessary to validate a candidate biomarker for potential clinical use. This article reviews the steps in the workflow, optimization strategies, and known applications in clinical, pharmaceutical, and research fields that demonstrate the broad utility for improved automation of sample preparation in the proteomic field.

Keywords

automation; high throughput; optimization; proteomics; quantitative mass spectrometry; robot; sample preparation

1 | INTRODUCTION

The recent rapid improvements in liquid chromatography mass spectrometry (LC-MS) instrumentation and informatics have empowered new advancements in MS-based protein and peptide quantification methodology (Aslam et al., 2017). As an applied bioanalytic tool in basic and clinical research, MS-based approaches offer the ability to quantify 1000s of

proteins in a single MS run (Geyer et al., 2016; Pappireddi et al., 2019). Biofluids, such as blood or urine, provide an easily obtained source of biomolecules that can offer insight into a patient's health or disease status. The analytical power of MS can be harnessed to identify biomarkers from tissues or biofluids that diagnose, stage, monitor or predict pathology and/or clinical outcomes in a wide variety of diseases.

Developing a biomarker for clinical use requires two distinct phases: discovery and validation. The discovery phase is characterized by comprehensive analysis of patient samples to develop candidate biomarkers. This is often performed using data-dependent or data-independent MS approaches (discussed below). In this phase, reliable quantitation of specific proteoforms is essential to be able to discriminate potentially diagnostic signals from noise. When one or more candidate biomarkers have been identified, each needs to be validated using much larger patient cohorts to establish the specificity and sensitivity of the markers for potential clinical use. This utilizes targeted MS approaches like multiple/selective reaction monitoring (M/SRM), parallel reaction monitoring (PRM) or sure quant-mass spectrometry (SQ-MS) (discussed below). These approaches provide thorough and accurate quantitation of peptides in complex biological samples.

Regardless of the technique in discovery-based or targeted-MS workflows (Li et al., 2020; Vidova & Spacil, 2017; Zhang et al., 2019), the common fundamental step before MS analysis is sample preparation. Protein samples are digested into peptides before MS analysis following a series of labor-intensive steps. However, reproducibility, time, and cost remain longstanding barriers to large-scale and high-throughput MS sample processing (Rogers & Bomgarden, 2016). To address this, the development of fast and accurate MS protein sample preparation workflows is necessary to improve the throughput of MS analysis and help speed the translation from biomarker discovery to validated clinical assay. An emerging innovation to improve sample preparation has been the utilization of robotic liquid handling workstations that can perform the most labor-intensive aspects of sample preparation accurately and reliably. In the following sections, we outline considerations for adapting and optimizing steps in the MS sample preparation protocol for automation and review examples of how automation has been implemented in discovery, validation, and clinical proteomic workflows.

2 | ESSENTIAL STEPS AND OPTIMIZATION IN LC-MS SAMPLE PREPARATION

To prepare samples for proteomic analysis there are several core steps that need to be performed on each sample and, depending on the needs of the experiment, additional specialized processing steps that may be required. The core steps include (1) protein concentration measurement; (2) protein denaturation; (3) reduction of disulfide bonds; (4) alkylation with a cysteine-blocking agent; (5) enzymatic digestions, for example, trypsin; (6) quenching; (7) desalting by solid phase extraction to remove interfering components (Figure 1). Optimization of each step during sample preparation is essential to obtain a robust, reproducible workflow for quantitative proteomics. For clinical grade validation of biomarkers, the total imprecision should be <20% including inter- and intra-day

assessments (Grant & Hoofnagle, 2014). Table 1 outlines general recommendations required to achieve the desired performance and reproducibility. Characteristics like denaturation agents, trypsin- (or other enzyme)-to-substrate ratio, buffer composition, incubation time, temperature, should each be assessed to understand the implications on MS sensitivity and reliability across the proteome in question. This is frequently performed in a series of sequential evaluations comparing various aspects of the protocol either manually or using an automated liquid handling workstation. Using an automated workstation, the user can achieve much greater control over liquid handling, mixing, incubation times and temperatures for uniform reactions.

2.1 | Protein denaturation

Protein denaturation is an initial and important step in preparing the sample for digestion. Denaturation unfolds the secondary structure of the protein to permit preferential access by downstream reagents and proteases. Effective denaturation will increase proteolytic efficiency and ultimately reproducibility of the sample preparation (Proc et al., 2010). The use of detergents, most notably sodium dodecyl sulfate (SDS), is widely reported for efficient digestion of proteins to peptides in the context of complex biological matrices. However, detergents can have serious interference issues for subsequent LC-MS/MS analysis (Funk et al., 2005; Loo et al., 1996). Other chaotropic agents, such as urea or guanidine-HCl, can also be used to denature proteins but also interfere with the MS analysis and require specific removal from the sample after proteolysis. As an alternative, MS compatible acid-labile surfactants (ALS), such as Rapigest SF, have been introduced (Meng et al., 2002). Rapigest SF is an SDS analog that is an effective surfactant at physiological pH for proteolysis but is cleaved to noninterfering components when the peptides are acidified for MS analysis (Yu et al., 2003). Several versions of ALSs have been introduced and found to be effective (Chen et al., 2007) although the cost of using these reagents can be prohibitive for large scale cohorts.

A landmark study by Proc et al. (2010) demonstrated that, while indeed SDS yields the most efficient digestion of the plasma proteome, 2-2-2 trifluoroethanol (TFE) 50% (v/v), a volatile solvent, can be used in its place and give comparable data (Proc et al., 2010). Many studies describe the effect of TFE on protein structure and it has been shown to melt the hydrophobic core of globular proteins suggesting it is effective in denaturing membrane-bound proteins. Further, Reiersen et al. demonstrated that TFE concentration 30% (v/v) and temperature of 58.6°C induced desirable denaturing conditions (Reiersen & Rees, 2000). Finally, the use of TFE has also been described in the context of many matrices including blood, plasma, urine, tissue and cells (Adachi et al., 2006; Coscia et al., 2020; Yu et al., 2003). Given the breadth of applications, instrument compatibility, and the ease of automation due to its volatile nature, there are significant advantages for using TFE to denature proteins in MS sample preparation however, the effect of any denaturant should be assessed to determine the optimal reagent for a particular workflow.

2.2 | Reduction and alkylation

Following denaturation, proteins are further reduced and alkylated to break the covalent disulfide bonds that maintain a protein's tertiary structure and cap the free cysteines

to prevent disulfides from reforming. The unlinking of a protein's cysteine residues improves access for proteolysis and simplifies database searching by eliminating any disulfide linked peptide complexes (Sechi & Chait, 1998). For this process, several reagents have been used. Reduction can be achieved by dithiothreitol (DTT), 2-mercaptoethanol (2-ME), tris(2-carboxyethyl)phosphine (TCEP) or tris(3-hydroxypropyl)phosphine (THPP). This reaction is usually done at elevated temperatures. Alkylation has been performed using iodoacetamide (IAA), acrylamide (AA), methyl methanethiosulfonate (MMTS), *N*-ethylmaleimide (N-EM), or 4-vinylpyridine (4-VP) (Crankshaw & Grant, 2001; Gundry et al., 2009; Hamdan et al., 2001; Hill et al., 2009; Righetti, 2006). The sequential pairing of two of these reagent types can provide reduction and alkylation however, each reaction needs to be evaluated for completeness or the accumulation of side reactions that will alter the anticipated mass of resulting peptides. Comprehensive assessments of reduction and alkylation conditions by Suttapitugsakul et al. (2017) and Muller and Winter (2017) suggest that optimization is necessary to achieve effective reactions in this aspect of sample processing.

2.3 | Enzymatic digestion

The most common application of bottom-up proteomics requires that proteins in a sample be digested with a specific protease to facilitate database searching. Trypsin has been the most popular protease in proteomic experiments because of its cleavage specificity (after lysine and arginine residues) and high proteolytic activity. Digestion with trypsin produces many peptides within an analyzable range, 500–5000 Da, and has been found to give preferential sequence coverage. There are several other proteases that can be used such as chymotrypsin, Glu-C, Asp-N or proteinase K, each with their own specificity. These have been used on their own or in combination with trypsin to increase sequence coverage of trypsin incompatible regions (Fan et al., 2014). With any proteolysis, effective digestion depends on optimizing the ratio of enzyme to protein, temperature, and time to ensure complete and reliable digestion conditions.

Previous studies have shown that the proteolysis step is a major source for variability in the LC-MS analysis (van den Broek et al., 2015; Fu et al., 2018; Martin et al., 2013). One of the primary drivers of variability is the inherent complexity of most proteomes. The human proteome arises from >20,000 genes and exhibits additional chemical diversity via the ability of each gene to produce multiple isoforms along with the potential of numerous posttranslational modifications (PTMs). Collectively, the proteoforms are highly regulated in time and space in response to an individual's physiologic or pathophysiologic states (Figure 2). Beyond the regulation of individual proteins there can also be an enormous dynamic range in protein concentration, where highly abundant proteins can be up to 10 orders of magnitude greater than lowest abundance proteins in a sample (Alpi et al., 2015). In one report, 14 different protein denaturation conditions were investigated for an optimal human plasma digestion efficiency (Proc et al., 2010). Proteolytic cleavage reactions in different matrices (plasma, serum, cerebral spinal fluid [CSF] and other proteomic samples) are complicated as each protein has its own tertiary and quaternary structure as well as unique cleavage site accessibility. For example, in our hands the manual processing of 171 human plasma samples had a coefficient of variation (CV) between 18% and 25% when monitored

by spiked exogenous bacterial protein beta-galactosidase (β -gal). For comparison, we used an automated workstation to perform all the steps in the preparation of 5 μ l of plasma without optimization. Using two proteins (exogenous β -gal and endogenous human serum albumin) as representative analytes in monitoring the automated digestion, the variability of the peak area ratios (native/stable isotope labeled [SIL] peptides signal) for albumin and β -gal were 30% CV and 20% CV before optimization, respectively. After the optimization process for each step in the automated workflow, CVs of 3% and 4%–8% were achieved for endogenous human serum albumin and spiked β -gal protein (Figure 3).

2.4 | Quenching

After digestion, the reaction is stopped by quenching in an acidic solution. This step halts the proteolysis and acidifies the peptide solution for desalting. This can be done using several volatile acids: formic, acetic, or trifluoroacetic acids. In case of ^{18}O labeling, quenching is particularly important to prevent trypsin mediated back-exchange of the heavy label (Petritis et al., 2009).

2.5 | Desalting

Peptides analyzed by LC-MS with a high-resolution mass spectrometer (Orbitrap, TripleTOF or TimsTOF, e.g.) require an extra desalting step postdigestion to remove contaminants and concentrate the peptides. This is usually achieved using a C18 resin to specifically bind peptides while contaminants are removed by wash steps. Solid phase extraction can be achieved with either manual vacuum extraction or a positive pressure apparatus (manually or automated). Administration of a vacuum can be difficult to regulate which would affect the recovery and reproducibility (Psillakis, 2017); use of a positive pressure apparatus is recommended (Johnson et al., 2020). It is possible to integrate a positive-pressure manifold for peptide desalting on an automation workstation. With an integrated and automated positive pressure apparatus, the pressure is regulated by clean nitrogen gas, and the gas flow and time applied can be pre-programmed based on sample and solvent type. Therefore, more uniform recovery and reproducible solid phase extraction can be achieved.

Of note, there have been recent innovations to integrate the discrete steps of sample preparation into a single self-contained column or tip. The S-trap protocol utilizes a porous material to trap denatured protein solutions permitting removal of contaminants, reduction, alkylation and digestion (HaileMariam et al., 2018; Ludwig et al., 2018). This approach has been found to be particularly effective at removing contaminants and denaturing surfactants during sample preparation (Zacchi et al., 2020). A similar approach has been proposed and extended by Chen et al. (2016). Their simple and integrated spin-tip proteomics technology (SISPROT) utilizes strong cation exchange beads and C18 resin to fully integrate all the steps of sample digestion, including the final desalting, into a single tip that can be rapidly processed achieving high sensitivity with little sample loss (Chen et al., 2016). This approach is also compatible with some PTM enrichment protocols (Chen et al., 2018). The integration and simplification of sample preparation is an important aspect in the potential to automate these protocols.

A further consideration for the development of an automated workflow is the requirement to select reagents which can be stored as aliquots to minimize the reagent batch effects. Exogenous protein β -gal can be used for quality control (Fu et al., 2018; Grant & Hoofnagle, 2014) to monitor the reproducibility and accuracy of the automated proteomics sample preparation workflow within a 96-well plate format. The β -gal protein and a mixture of its corresponding SIL peptide standards can be added before the reduction and alkylation reactions before enzymatic digestion. Once an optimized automation workflow is established, it is important to evaluate the reproducibility across multiple days. For a standardized automated workflow, multisite validation to ensure the validity of the results is good laboratory practice (Fu et al., 2018; Martin et al., 2013).

3 | QUANTITATIVE MASS SPECTROMETRY

Once samples have been prepared, peptides can be analyzed using a variety of high-performance mass spectrometry techniques to perform discovery or validation studies of disease biomarkers. Discovery-based proteomics is commonly performed using either data-dependent acquisition mass spectrometry (DDA-MS) (Zhang et al., 2013) or data-independent acquisition mass spectrometry (DIA-MS) (Gillet et al., 2012; Krasny & Huang, 2021) to provide a comprehensive and quantitative evaluation of biological samples. In DDA-MS, all precursor ions are scanned during the survey scan (MS1), then a subset of ions are individually selected for fragmentation, producing a series of tandem (MS/MS or MS2) mass spectra. The collected mass spectra are searched against a database to be matched and assigned to peptides and then proteins. This approach is highly powerful but suffers from sampling only the n most abundant precursors (predefined numbers of precursor with signals above “noise”) for subsequent MS2 fragmentation. This sampling bias can limit the reliable identification and quantification of low abundance peptides and proteins. In DIA-MS, all precursor ions within a defined mass to charge (m/z) window observed in a MS1 survey scan are subjected to fragmentation. The analysis is repeated, and the fragment ions are accumulated in a fixed number of isolation windows that span entire mass-to-charge (m/z) range. Because of all detected precursors are fragmented, the advantage of DIA-MS is that the accurate peptide quantification without limitation of selecting predefined peptides as in DDA-MS. DIA-MS method requires a mass spectrometer capable of high-resolution MS/MS spectra.

Moving beyond the discovery phase requires targeted validation, usually involving large scale cohorts of patient samples. Currently, the methods of choice for the evaluation of these samples are multiple reaction monitoring mass spectrometry (MRM-MS), also known as selected reaction monitoring mass spectrometry (SRM-MS) (Lange et al., 2008), parallel reaction monitoring mass spectrometry (PRM-MS) (Gallien et al., 2015), and Sure Quant-Mass Spectrometry (SQ-MS) (Liebler & Zimmerman, 2013) which provides precise quantitation of proteotypic peptides (unique surrogates for the corresponding protein) in complex biological samples derived from bodily fluids, biopsies, or cultured cells. SRM-MS is a targeted approach which utilizes the highly reproducible triple quadrupole (QqQ) mass analyzer to select known peptides of choice for quantitation. The advantages of SRM-MS are selectivity, sensitivity, and multiplexity in peptide/protein quantitation. PRM-MS is a SRM-like targeted approach but performed in a high resolution and high mass

accuracy (HR/AM) mode on a mass spectrometer. In PRM-MS, full MS/MS spectra of the selected peptides are acquired with high resolution and high mass accuracy. Peptide and protein quantification is highly selective and specific. SQ-MS is a recently developed acquisition method for internal standards targeted approach by using HR/AM Orbitrap mass spectrometer. The mass spectrometer programed to monitor referential internal standards in samples using dynamically changed MS parameters. This method enhances endogenous peptides quantitative data quality and may quantify a large number of peptide targets (Stopfer et al., 2021).

4 | IMPLEMENTED AUTOMATED PROTEOMIC SAMPLE PREPARATION IN LC-MS ANALYSIS

The success of any discovery or validation proteomics experiment depends greatly on the speed and quality of the sample preparation. An emerging option to increase throughput and accuracy is the use of liquid handling stations to perform many of the steps in the sample preparation protocol. Any automated workstation suitable for full hands-off sample preparation for MS requires the following functions: (1) time-controlled liquid transferring with accuracy, (2) liquid transferring from reagents vials/plates to digestion plate, (3) 96/384 pin and/or 8-span head, (4) grippers to move the digestion plate, (5) temperature and time-controlled incubator with shaking ability, and (6) an independent plate shaker to be used as a mixer. These features remove human hands from the most repetitive and labor-intensive steps and increase accuracy and reproducibility for large numbers of samples. There are a growing number of examples in the literature where automated workstations are utilized for proteomic sample preparation for the quantitative MS analysis applications for high-throughput clinical, pathological, pharmaceutical, and research domains (Table 2). The following section describes several key studies, and the relative performance of the automated workflow.

4.1 | Direct digestion-MS

Whole biofluids such as plasma, serum, CSF, urine, as well as blood, dry blood including Whatman paper spots or volumetric absorptive microsampling (VAMS) are complex matrices with rich proteome information. Several studies have utilized automated workstations to develop hands-off workflows for direct protein digestion coupled with quantitative MS analysis in preclinical and clinical research studies (Table 2). Direct digestion is the simplest form of sample preparation, it does not require any specific extraction, from cells or tissues, or any specific enrichment/depletion steps. For example, an automated workflow with Biomek NX^P (Beckman Coulter Life Science) to directly digest dried blood from a Mitra (a volumetric absorptive microsampling device) has been used with high flow LC with a sensitive triple-quadrupole mass spectrometer acquisition method to achieve reasonable precision (CV < 20%) in quantifying high abundant proteins with MRM analysis (van den Broek et al., 2017). In another study, a Bravo (Agilent) 96-channel liquid handler was used to process dried blood spots (DBS) which was then combined with high flow LC-MS analysis to quantify each peptide representing Apolipoproteins A-I, B, C-II, C-III and E with the total observed CV < 6% using MRM (van den Broek et al., 2016). A further study used a Tecan Freedom liquid handler automation workstation to quantify 97

blood proteins also from DBS samples achieved an average intra- and inter-assay CV < 12% (including MS CV) for the various peptides (Chambers et al., 2015).

4.2 | Solid phase extraction

This workflow has been implemented in the clinical laboratory domain, with Quest Diagnostics reporting a high-throughput MS assay to determine the ratio of β -amyloid 1–42 (A42) to β -amyloid 1–40 (A40) in CSF. This assay is able to diagnose and differentiate patients with Alzheimer's disease from nondiseased healthy controls. This clinical assay utilizes solid phase extraction-based automated workflow on a Hamilton automation liquid handler following trypsin digestion of CSF (DeMarco et al., 2020; Weber et al., 2019). Our group have integrated a positive pressure apparatus for desalting on HLB plates using the i7 hybrid liquid handling workstation (Beckman Coulter) (Johnson et al., 2020; McArdle et al., 2021). In our lab, the liquid handling %CV was calculated to be around 6% (when the desalting step was included, the total %CV was ~10%; and without desalting the total %CV was ~4%) (Figure 3 bottom right panel).

4.3 | Quality control

Monoclonal antibody (mABs) therapy has rapidly emerged as an important therapeutic agent for the treatment of various diseases, including cancer, chronic inflammation and pathological infections (Chung, 2017). Recently, the US Food and Drug Administration (FDA) has authorized the use of monoclonal antibodies for the treatment of COVID-19 (Drozdal et al., 2020). For the biopharmaceutical industry, it is critical to monitor mAB quality control, including recombinant sequence verification (An et al., 2020). One inter-laboratory study on mAB quality control utilized an automated sample preparation method using a KingFisher liquid handling workstation (Thermo Fisher Scientific) to map global mAB peptides by MS (Millan-Martin et al., 2020). This team has also performed an inter-laboratory study using the same automated protocol to demonstrate consistency in mAB product quality control (Millan-Martin et al., 2020).

4.4 | Immunoaffinity enrichment and depletion

The blood proteome (and resulting subproteomes of plasma and serum) has a substantial range of concentrations among its various proteins; $\sim 10^{10}$ proteins from high mg/ml to sub-pg/ml concentrations. The highly abundant resident proteins can overwhelm the MS analysis, obscuring the observation of less abundant proteins. To increase the analytic sensitivity of the less abundant proteins, immunoaffinity (IA) approaches have been successfully adapted to automated sample preparation. For example, Lassman et al. used specific antibodies to enrich low abundant plasma cholesteryl ester transfer protein (CEPT) and proprotein convertase subtilisin/kexin type 9 (PSCK9) from 1400 plasma samples in under 3 weeks using an optimized and automated IA procedure (Freedom EVO, Tecan Trading) to achieve an intra- and inter-assay CVs below 15% (Lassman et al., 2014).

Peptides have also been targeted in immune-capture workflows using stable isotope standards and capture by anti-peptide antibodies (SISCAPA or immuno-MRM [iMRM]). This workflow performs the proteolytic digestion first, then captures surrogate peptides with specific antibodies to detect and quantify via MS. iMRM assays have been used to

accurately quantify clinical protein panels (>10 analytes) such as Cystatin C, C-reactive protein, apolipoprotein E, apolipoprotein C III and other proteins (Campino et al., 1987). Plasma, serum, and DBSs have all been found suitable for iMRM analysis (Anderson et al., 2020). Automated iMRM immuno-MS workflows have been extensively validated. Multiple automated workstations and platforms have been used successfully in iMRM assays for plasma biomarker quantification, such as the Bravo automated liquid handler (Agilent Technologies) (Razavi et al., 2016, 2019), KingFisher magnetic particle processor (Thermo Fisher Scientific) (Whiteaker et al., 2010), and Hamilton liquid handling workstations (Microlab STAR Hamilton) (van den Broek et al., 2015). Each of these liquid handling workstations have demonstrated that automation can achieve rapid tryptic digestion and immunocapture with inter- and intra-day CVs of <10%.

In addition, one advantage of iMRM antipeptide antibody capture is that the proteolysis before capture exposes previously inaccessible epitopes. Therefore, iMRM can be used to quantify clinically relevant plasma biomarkers with minimal autoantibody interference. For example, studies using thyroglobulin, a circulating tumor marker that has been used to evaluate the effectiveness of thyroid cancer treatment, have portrayed false-negative results in antibody-based immunoassays due to the presence of antithyroglobulin autoantibodies, which blocked the epitope of interest (Hoofnagle et al., 2008). In response, an automated iMRM assay sample preparation workflow was developed to measure specific thyroglobulin tryptic peptides (Kushnir et al., 2013). After trypsin digestion of the plasma proteins, selective thyroglobulin specific peptides were enriched using a peptide-specific antibody via an automation liquid handler workstation (epMotion from Eppendorf). After enrichment, the peptides were submitted for LC-MS for the quantification of endogenous thyroglobulin peptides along with internal peptide standards, and this method was able to satisfy performance criteria required for clinical diagnosis (CV < 20%) (Kushnir et al., 2013).

4.5 | Protein extraction from cells or tissues

Any automation workstations that include proteolytic digestion of cells, solid tumors, and/or tissues often require several additional processing steps to be included when compared to sample processing of body fluids. This includes steps required for tissues or tumors to be disrupted, followed by cell lysis, DNA shearing, and protein extraction before protein denaturation and subsequent digestion steps as discussed above. Recently, Müller et al. developed a method to integrate an adaptive-focused acoustics (AFA) Technology and single-pot solid-phase enhanced sample preparation (SP3) on liquid handling workstations for automated processing of tissue lysates in a 96-well format (Muller et al., 2020). AFA ultrasonication is an advanced acoustic technology which enables the mechanical processing of samples through focused ultrasonication in a 96-well plate format. SP3 utilizes paramagnetic beads in the presence of an organic solvent (>50% ACN or EtOH) to promote protein binding to the beads and allows extensive washes to eliminate contaminants and harsh detergents. The resulting workflow is an end-to-end automation of the sample preparation for cells or tissues (Muller et al., 2020). A recently reported SP2 workflow (an alternative adaptation of carboxylate-modified paramagnetic particles for peptide isolation utilizing a liquid handler (epMotion workstation, Eppendorf)) was efficient in removing

contaminants (e.g., detergents and salts) and additionally cleaning up peptides before mass spectrometry injection (Waas et al., 2019).

4.6 | COVID-19

The COVID-19 pandemic highlights the need and urgency for the integration of the automated sample preparation into the clinical laboratory. The pandemic has accelerated the need for rapid and accurate automated RNA based assays in the clinical laboratory (Tauschmann & Hovorka, 2018). Most recently, a few reports were published in using mass spectrometry-based for COVID-19 related screening in clinical laboratories (Cardozo et al., 2020; Jayawardena et al., 2021; Rajczewski et al., 2021; Renuse et al., 2021). One study by Cardozo et al. developed an automated sample preparation method using a robotic liquid handler combined with a high throughput targeted LC-MS/MS assay to detect SARS-Cov-2 nucleoprotein peptides from nasopharyngeal and oropharyngeal swabs (Cardozo et al., 2020). The automated digestion of 96 samples could be completed in 4 h and by multiplexing four samples per 10 min LC-MS/MS run, 500 samples per day could be analyzed. Another study utilized PRM-MS to develop a test for COVID-19 spike glycoprotein and nucleoprotein, the sample preparation was accomplished by an automated workflow with immunoaffinity-based enrichment and in-solution digestion (Renuse et al., 2021). The mass spectrometry analysis was done with a high resolution PRM-MS-based workflow to develop a test for COVID-19 spike glycoprotein and nucleoprotein.

5 | AUTOMATION IN CLINICAL PROTEOMICS-FUTURE PROSPECTIVE

Beyond liquid handling of the proteolytic digestion, there are many other steps in the sample preparation process that could benefit from automation. For example, automated sample receiving processes (bar coding and verification) that could aliquot, store, and maintain an archive would streamline the management of large clinical cohorts. Automated solutions for transport and loading specimens into the sample preparation workstation would also eliminate many time-consuming (capping and recapping tubes) and error prone steps. Furthermore, the evolution of a fully automated process would include the standardization of high quality, prepackaged, ready-to-use reagents. The continued development of robotics, liquid handling, mass spectrometry analyzers, data handling, and reporting in proteomics labs will ultimately reduce time and cost of preparation while improving reliability and precision of the subsequent analysis.

Rapid advances in MS technology combined with various automated sample preparation workflows suggests these techniques will become more prominent in routine clinical laboratories. A prime example is the relatively recent availability of high-resolution, accurate MS instruments that can accommodate multiplex analysis. Currently, there are multiple automated sample preparation methods available to supply the various discovery (DDA-MS, Zhang et al., 2013; DIA-MS, Gillet et al., 2012) and targeted (PRM-MS, Gallien et al., 2015; MRM/SRM-MS, SQ-MS, Liebler & Zimmerman, 2013) MS acquisition methods. However, automated tools and workflows will need to remain flexible to meet the challenges presented by emerging MS methods. Developing assays that combine automated sample preparation and LC-MS/MS that utilize high multiplexing capacity, high analytical

specificity, and high sensitivity are key for moving proteomic technologies from the basic research realm to the clinical laboratory and into precision medicine.

A fundamental requirement in the evaluation of biological material is its refinement to an analysis ready state. In proteomics, automation of the sample preparation will increase the uniformity and precision of that process, which is critical for reliable quantification of proteins for biomarker discovery, validation, preclinical, clinical, and pharmaceutical studies. Developing and optimizing sample preparation workflows with precision, reproducibility and robustness is essential in the implementation in routine clinical laboratories. The constant improvement of automation technologies for proteomic sample preparation in a true high-throughput manner is key in integrating mass spectrometry and proteomics into personalized medicine.

Biographies

Qin Fu earned her PhD degree in Genetics from the University of Minnesota and received postdoctoral training in Immunology at the University of California, San Francisco. She has research experience in human genetics, autoimmunity, drug resistance, cardiovascular diseases, and biomarker discovery and validation. Her current research interests include automated MS sample preparation, multiplexed targeted MS assays, and biomarker discovery. Dr. Fu is the Director of the High-Throughput Laboratory in the Advanced Clinical Biosystems Research Institute at Cedars Sinai Medical Center.



Christopher Murray received a PhD in Biological Chemistry from Johns Hopkins in 2012, where he investigated quantitative measures for oxidative posttranslational modifications in heart failure. He did his postdoctoral work in biophysics examining dynamic protein structure in cardiac voltage gated ion channels using unnatural amino acids. His current research interests include MS-based structural proteomics and automation.



Oleg A. Karpov has received his BSc and MBiol degrees in Medical Biochemistry at the University of Leeds working at the Astbury Centre for Structural Molecular Biology studying novel approaches for the inhibition of cancer angiogenesis. Oleg then went on to receive his PhD in Chemistry from Imperial College London working on the identification of novel kinase substrates using non-canonical amino acids in tandem with mass spectrometry. Currently, he is based at Jennifer Van Eyk's group at Cedars-Sinai Medical Center, specializing in the application of chemical biology approaches paired

with mass spectrometry for the elucidation of molecular mechanisms of COVID19 and cardiovascular disease.



Jennifer (Jenny) Van Eyk, PhD is a Professor of Medicine within the Smidt Heart Institute at Cedars-Sinai Medical Center, in Los Angeles, CA. She is the Director of Advanced Clinical Biosystems Research Institute and of the Precision Biomarker Laboratories and is the inaugural Erika Glazer Endowed Chair in Women's Heart Health. Dr. Van Eyk is an international leader in the area of proteomics and her lab has focused the developing technical pipelines to precisely quantify proteins and their posttranslational modifications to better define and understand the disease-induced interplay between cellular pathways. Collectively, these tools are used in many disease contexts to provide better therapeutic interventions and robust biomarkers for the diagnosis, prognosis and risk stratification.



ACRONYMS

ACN	acetonitrile
AFA	adaptive focused acoustics
CSF	cerebrospinal fluid
CV	coefficient of variation
DBS	dried blood spot
DDA-MS	data-dependent mass spectrometry
DIA-MS	data-independent mass spectrometry
EtOH	ethanol
FDA	US Food and Drug Administration
IA	immunoaffinity
iMRM	immuno-multiple reaction monitoring
LC	liquid chromatography
LC-MS	liquid chromatography mass spectrometry

mABs	monoclonal antibodies
MRM	multiple reaction monitoring
MRM-MS	multiple reaction monitoring mass spectrometry
MS	mass spectrometry
PTMs	posttranslational modifications
SIL peptides	stable isotope labeled peptides
SISCAPA	stable isotope standards and capture by antipeptide antibodies
SISPROT	simple and integrated spin-tip proteomics technology
SQ-MS	sure quant mass spectrometry
SRM-MS	selected reaction monitoring mass spectrometry
TOF	time-of-flight
VAMS	volumetric absorptive microsampling

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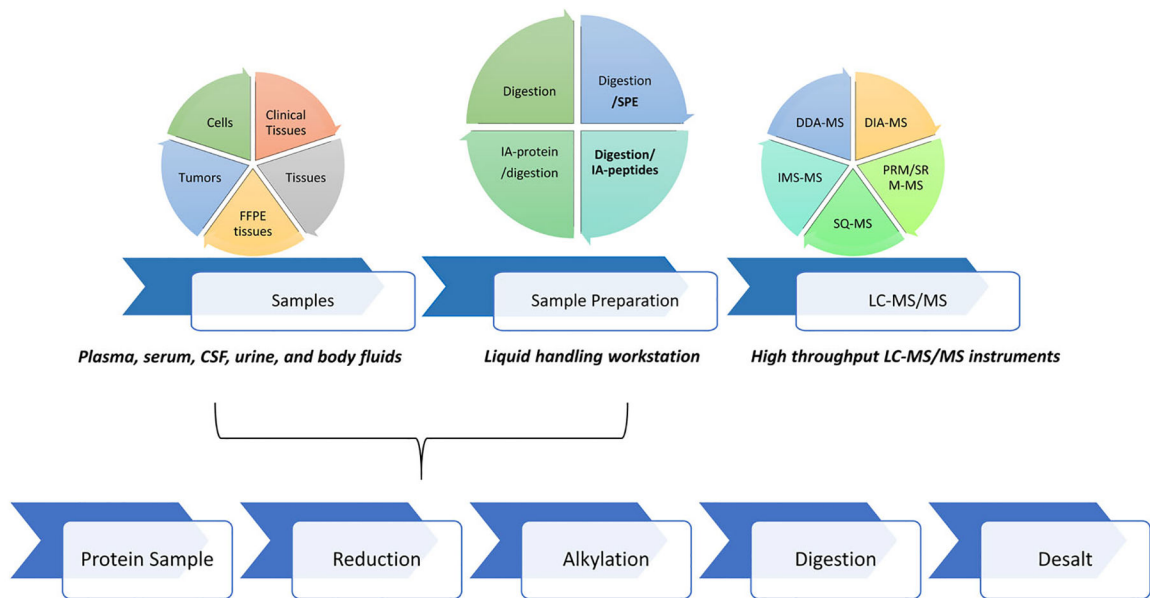
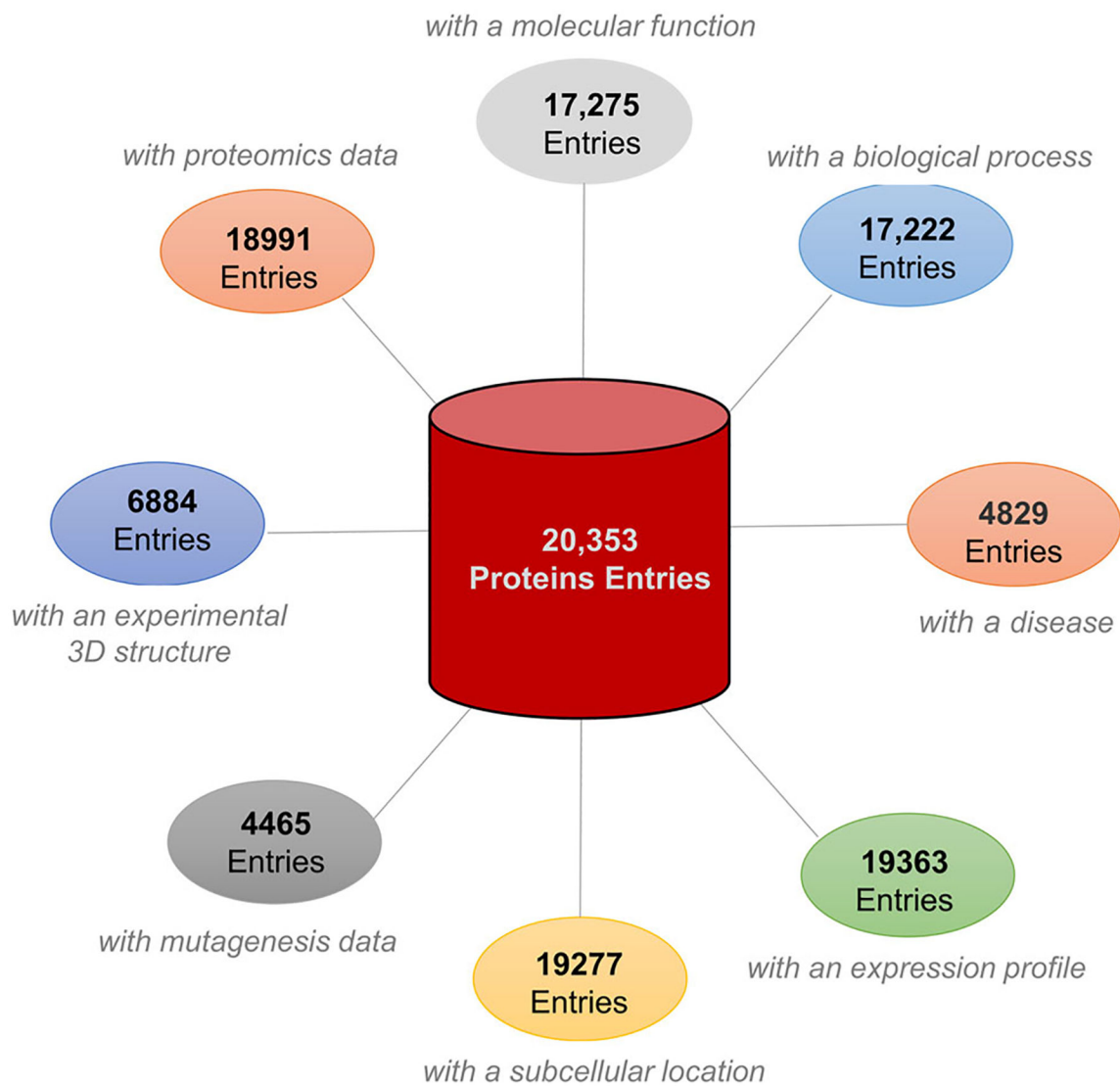
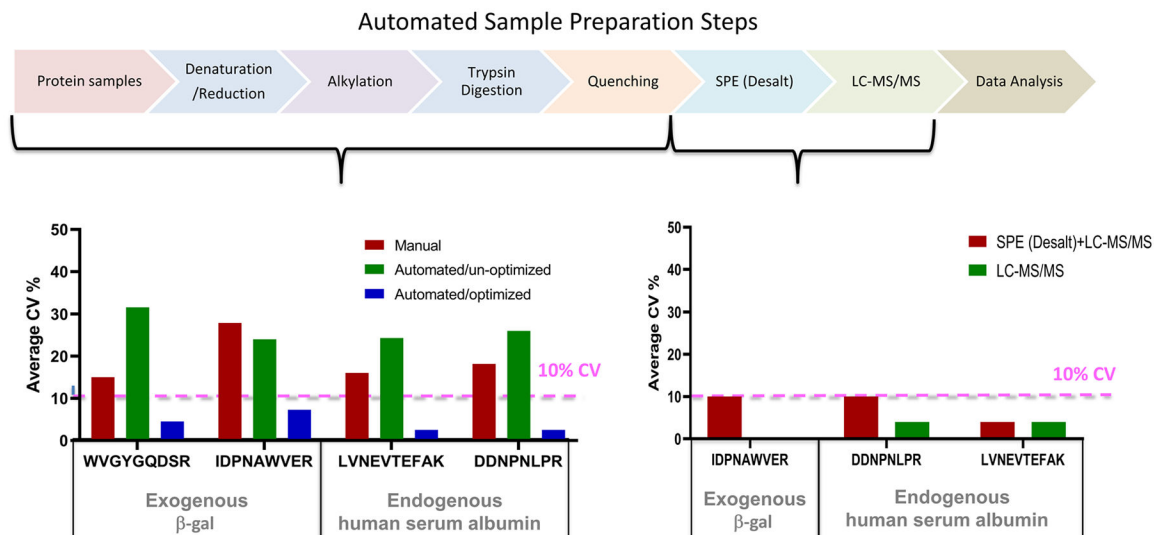


FIGURE 1. Schematic of the proteomic sample preparation process. Top panel summarizes sample type, different automated processes, and MS acquisition methods. Bottom panel illustrates the basic steps in proteomic sample preparation

**FIGURE 2.**

The human proteome illustrated by biological, functional, structural, and sequence complexity. The total number of proteins and their isoforms reported by Swiss-Prot database; and total proteins associated with eight large categories of biological function (<https://www.nextprot.org/about/statistics>)

**FIGURE 3.**

Automated proteomic sample preparation evaluation. Left panel, CV% assessment of three distinct digestion conditions are shown: manual digestion (red), unoptimized automated digestion (green), and optimized automated digestion (blue). The reproducibility was assessed by area ratio (light native/heavy SIL) and the CV% was calculated with 4–8 digestion replicates of β -gal spiked into pooled healthy human plasma. Plasma samples were denatured, reduced, alkylated, and digested with trypsin. Average area ratio CV% β -gal and human serum albumin were calculated from peak area light (native/heavy SIL). MRM data was acquired by an 6500QTRAP. Right panel, the CV% assessment of automated solid phase extraction (24-well) is shown. β -gal and human serum albumin SIL peptides spiked into predigestion healthy human plasma pool, then desalted by a positive pressure apparatus integrated into a controlled i7 automated workstation. SIL heavy peptide signals after desalting were used to calculate CV%. MRM data was acquired similarly by LC-MS. CV% was calculated from seven repeated injections. SIL, stable isotope labeled

TABLE 1

Optimization of automated sample preparation techniques

Automation method	Step	Parameters that may be optimized	Recommendations
Aliquoting/plating	From protein samples to a 96-well plate aliquoting	Pipetting techniques (e.g., plasma and serum are viscous); dispensing of samples into the bottom of wells	Slowly aspirate and dispense protein samples
		Labware (tubes and plates)	Accurately define the plate (X, Y, Z axials, spacing and well offset)
Digestion	Buffer	Final concentration	Tris-HCl (pH 8.3), NH ₄ HCO ₃ (pH 8.5)
	Denaturation/reduction	Chemistry, temperature, incubation time and final concentration of selectants and/or denaturants	Denaturants (OGS, TFE, Rapigest, urea, guanidine HCl; and reducing agents (TCEP, DTT)
	Cysteine blocking	Chemistry and concentration	MMTS, iodoacetamide
	Trypsin	Substrate to enzyme ratio, incubation time, choice of vendor and grade. Note, other enzymes can be used (e.g., AspN, GluC, etc.)	Trypsin from Promega, Scitex, Thermo Fisher Scientific, Sigma
	Quenching	Acid choice and concentration	Formic, acetic, or trifluoroacetic acid
Solid phase extraction	Positive pressure apparatus integrated into and controlled by the automated workstation	Pressure, time, SPE (desalting) chemistry, sorbent to digested peptide ratio), organics	Oasis HLB

TABLE 2

Examples of automated hands-off workflows for quantitative preclinical and clinical research

Liquid handling system/workstation property									
Analytes	Throughput	Field application	Sample process	Liquid handling system/workstation	Company	Robotic arm (volume)	Channels	Note	MS (Reference)
Protein markers panels (Health Surveillance and Apolipoproteins)	High-throughput preclinical laboratory and research assay	Preclinical and research	Whole plasma/serum/blood Mitra direct tryptic digestion	Biomek NX ^P Automated Liquid Handler	Beckman Coulter Life Sciences	0.5–1000 µl (Multichannel); 0.5–5000 µl (Span-8)	Multichannel head (96 or 384) or Span-8 pipetting with gripper	Biomek NX ^P is no longer available and is replaced by Biomek i-Series, such as i5 and i7	MRM-MS (Fu et al., 2018; van den Broek, 2017)
Apolipoproteins panel	High-throughput preclinical laboratory and research assay	Preclinical and research	Volumetric microsampling (Mitra)	Biomek NX ^P Automated Liquid Handler					MRM-MS (van den Broek et al., 2020)
Apolipoproteins/plasma	High-throughput clinical laboratory assay	Cardiovascular Diseases	Whole plasma/serum direct tryptic digestion	Bravo 96-channel liquid handling platform	Agilent Technologies	100 nl–200 µl	8, 16, 96, and 384 Channel Heads		DDA-MS (van den Broek et al., 2016)
Dried blood spots (DBS)	High-throughput/research	Research	Direct tryptic digestion	Triversa Nanomate robotic platform	Advion Ltd	<10 µl	The Nanomate probe	Chip-based electrospray device with the Nanomate probe	DDA-MS (Martin et al., 2013)
Amyloid 40 and 42	High-throughput clinical laboratory assay	Alzheimer's disease/Clinical Diagnostics	Solid phase extraction	MicroLab Star system integrated with a SPEware IP8 positive-pressure manifold	Hamilton	Custom (Inquire)	Custom (Inquire)		MRM-MS (Weber et al., 2019)
Peptide mapping/monoclonal monitoring	Biopharmaceutical industry	Recombinant mAB quality control	Direct tryptic digestion and SPE	KingFisher	Thermo Scientific	50–1000 nl for 12-pin head 200–5000 µl/6-pin head	6- or 12-pin head		DDA-MS (Millan-Martin et al., 2020)
Insulin and C-peptide	High-throughput clinical laboratory assay	Diabetes/Clinical Diagnostics	Immuno-capture of intact protein	MicroLab Star system (Hamilton)	Hamilton Company	custom (Inquire)	Custom (Inquire)		MRM-MS (Taylor et al., 2016)
Thyroglobulin/plasma	High-throughput clinical laboratory assay	Treatment follow-ups/clinical pathology	Trypsin digestion and peptide immune-capture	epMotion system	Eppendorf	1–1000 µl	Up to 8 channels		MRM-MS (Kushnir et al., 2013)