Bioanalysis ZONE

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Effective biomarker measurement is key for biotherapeutic development

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Contents

Technology Digest: effective biomarker measurement is key for biotherapeutic development

by Vivian Xie
(Digital Editor, Bioanalysis Zone)

Biomarkers and their role in drug development

A hybrid of the word 'biological marker', biomarkers are a biologic or physiological characteristic that can act as a measurement of either normal or pathogenic biological processes or responses to a therapeutic/intervention [1]. Biomarkers can thus be utilized as important tools for disease diagnosis, prognosis and prediction, as well as an indicator of drug safety and efficacy [1,2].

When utilizing biomarkers in the drug development process, their fit-for-purpose validation is essential in ensuring that the biomarker effectively reveals the targeted outcome and that the characteristics used to assess biomarker assay performance are purposeful. Biomarkers have been identified as a critical player in the progress towards personalized or patient-centered medicine, tailoring therapeutics and drugs to a specific patient's needs [2]. Proper clinical and analytical validation of biomarkers relies on carefully considering aspects such as reference standard sourcing, instrumentation, sample preparation strategies and matrix interference in biological matrices [2,3].

The biomarkers pipeline

A comprehensive biomarker pipeline has recently been made possible from advances in processes and technologies, with six essential components: candidate discovery, qualification, verification, research assay optimization, biomarker validation and commercialization [4]. To better evaluate and support this pipeline, it is important to develop accurate and specific analytical platforms and assays. However, assay developers and regulators face various challenges throughout the process of identifying targeted drugs, along with their respective predictive and prognostic biomarkers [3]. These difficulties range from the allocation of resources, to the difficulty of extracting meaningful molecular signatures from complex datasets of biological processes as a result of high-throughput analysis [3,4]. While biomarker discovery and characterization has witnessed growing interest and opportunities for sophisticated analytical approaches, challenges remain, particularly in the need for robust assays and their development for single-analyte quantification [5].

Biomarker validation: where are we and where do we go?

To utilize biomarkers as outcomes in clinical trials, assessment of a biomarker's relevance and validity is required. While relevance is determining whether a biomarker is capable of providing clinically relevant information, a biomarker's validity refers to the characterization of its effectiveness or utility. Validation ensures that the biomarker proposed is capable of being measured objectively and reproducibly, and if it measures a characteristic successfully [1].

In recent years, there has been a focus on separately considering biomarker assay development and validation in their own context [6]. While ligand-binding assays (LBA) have developed considerably, what has persisted in fundamental assay principles is the generation of multiple readings of processed samples to produce a single reportable result. Despite these advancements in LBA methods, the standard practice has remained – aliquoting each sample in an analytical batch, the addition and treatment of buffers, capture and detection antibodies and incubation before the sample is divided into aliquots on an assay plate. Duplicate analysis in LBA has been a mainstay in LBA approaches due to the imprecision of these practices, resulting in processes that can be time-consuming and costly. While replicate analyses can reduce uncertainty, any remaining variance resulting from a late sample split is difficult to limit [7].

High immunoassay robustness and precision are thus required for the continued development and validation of biomarker assays [4]. In a workshop that addressed lessons learnt and ongoing biomarker development challenges (sponsored by the National Cancer Institute and the US FDA), it was stated that:

"A robust technology for testing in clinical samples must be available or it must be technically and economically feasible to develop an analytically reliable testing system on a timeline consistent with the development timeline for the drug [3]."

Developments in assays for biomarker development

As the ideal single biomarker assay development would typically occur before the preclinical study of an identified drug candidate, precise and robust immunoassays that are fit-for-purpose to their target biomarker are required for preclinical and clinical biotherapeutic development [8]. With the development of platforms such as those from Gyros Protein Technologies, singlicate LBA analyses are now being recommended through platforms such as Gyrolab®, which also support the automation of immunoassays and can provide low sample volume requirements, a wide dynamic range and custom assay development to support analysis in regulated laboratories [7–9]. Recently, the Gyrolab Bioaffy™ 4000 CD has expanded the sensitivity provided by Gyrolab assays, allowing for powerful biomarker analysis for low sample concentrations [8].

> *Developing biomarker assays in support of drug development is a challenge due to the fit-for-purpose validation requirements, which will depend on the context of use. The Gyrolab as a fully automated immunoassay open platform is well-suited to support biomarker analysis as part of drug development due to the advantages of reproducibility, robustness, sensitivity and acceptance in regulated environments – John Chappell, Director of Scientific Support, EMEA and Asia Pacific, Gyros Protein Technologies (Manchester, UK).*

A range of assay protocols, or ready-to-use kits, enable open, customizable systems and in-house development and evaluation of custom immunoassays [9]. For most LBAs, improvements have been achieved through the advancement of reagents, consumables and instrumentation but workflows have largely remained unchanged [7].

Confidence in the clinical implications of a set of data relies on confidence in the concentration values of the target analyte/analytes, including biomarkers [2]. Exploring how singlicate measurements can be applied into an efficient workflow without compromising data precision maintains this confidence in the translation of data to a clinical setting while reducing the various disadvantages of replicate analyses. Gyrolab immunoassays have been demonstrated to be reliably robust for singlet analysis, suitable for preclinical and clinical phases of biomarker analysis and supported by PD studies also measured with Gyrolab immunoassays [7]. Advantages such as minimal hands-on time and rapid output of data result in productive biomarker surrogate endpoint quantification and validation for drug safety, efficacy and PK/PD [8].

In addition, the development of compact discs (CDs) for immunoassays has aided in covering a range of assay formats and concentrations [10]. Technologies such as the Gyrolab Bioaffy™ CDs have been demonstrated to process samples at the nano-liter scale, reducing sample and reagent consumption and delivering 96 or 112 datapoints in less than 1 hour [10]. Such high-throughput analysis enables time- and cost-efficient quantification and validation of biomarkers and biomarker surrogate endpoints [10].

Software design and development have also been areas of interest for when considering biomarker immunoassay improvement. The US FDA administers the 21 CFR Part 11 regulations for electronic records and electronic signatures, which most software programs and modules are designed to satisfy in support of biomarker immunoassay support [11].

Summary

Biomarker immunoassays are quickly playing a greater role in the drug development process [2,7]. As they continue to offer the growing ability to quantify and validate biomarkers as endpoints to test for drug safety and efficacy, the need for validated immunoassays also increases [5]. Developments in biomarker analysis with accurate immunoassays has seen its fair share of challenges but recent advances have provided precise and robust fit-for-purpose methods, which support preclinical and clinical drug and biotherapeutic development [8].

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Editorial
10th Anniversary Issue of *Bioanalysis*

Bioanalysis

Evolving our thinking on biomarker assay validation: are we ready for the next leap?

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> “Biomarker science is not black and white, but countless shades of gray. We must therefore align on the fundamental principles of scientific rigor and accountability while accepting ambiguity.”

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Keywords: assay • biomarker • validation

Over the last decade, there has been an explosion of interest in the discovery and development of biomarkers to facilitate drug development and improve patient care. The potential utility of biomarkers in drug development is broad in scope, with biomarkers being leveraged right from the earliest phases to demonstrate target engagement and proof of biology, thereby reducing the risk of investment in later stages. Indeed, biomarkers can accelerate overall drug development, defining appropriate patient subsets and providing early indicators of response. It is therefore not surprising that the US FDA Critical Path Initiative identified development of new biomarkers as the highest priority for focused scientific effort [1].

A critical element to successful deployment of a biomarker is, of course, the ability to reliably measure it. Accordingly, over the past few years, the topic of analytical validation of methods to measure biomarkers has been hotly debated, with scientists and regulators alike wrestling with how best to define expected performance criteria for biomarker assays. An interesting scientific excursion occurred during this time whereby, with the best of intentions and presumed desire for scientific rigor, it was suggested that biomarker assays be held to the standards of PK assays. In retrospect, this was an intriguing development, given that the seminal paper by Lee *et al.,* entitled ‘Fit-for-purpose method development and validation for successful biomarker measurement’ [2], preceded these discussions by several years. Regrettably, as biomarker discussions had gained steam, the concept of fit-for-purpose (FFP) began to be interpreted by some as meaning lacking in rigor, which was quite opposite to its true meaning, which is application of appropriate scientific rigor.

With the aim of beginning to build common understanding, the AAPS Crystal City VI (CCVI) Workshop: ‘Bioanalytical Method Validation for Biomarkers’, was held in Baltimore, MD, USA, on 28–29 September 2015 to discuss the critical issues. At that meeting the simple refrain ‘Biomarker assays are not PK assays’ was first uttered and repeated – again and again [3,4]. That repetition served a purpose. It solidified the concept as foundational for the discussion, disrupted some preconceived patterns of thinking and catalyzed an evolutionary leap in the conversation on development and validation of biomarker assays. Critical takeaways from that meeting included: clarification that biomarker assays should be approached scientifically in their own right, with evaluations that focus on the measurement of endogenous analyte; highlighting the limitations of spike recovery experiments and the foundational nature of parallelism assessments; and building an understanding that some biomarker applications would require analytical criteria that would be even more stringent than those applied to PK assays [4]. It was also emphasized that in order to set appropriate performance criteria for a given biomarker assay, focus must be on the specific question being asked and the decision the biomarker data must support. Furthermore, in addition to evaluation of the analytical performance of the assay, an understanding of the pertinent biology and biological variability of the biomarker is necessary to determine whether the assay is suitable to meet the needs of the question. In short, biomarker assays should be assessed in context.

Another key outcome from CCVI was that it raised the volume on the discussion and thereafter virtually every scientific forum that brought together industry professionals and regulators continued the dialogue. Subsequently,

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the Public Workshop on 'Scientific and Regulatory Considerations for the Analytical Validation of Assays Used in the Qualification of Biomarkers in Biological Matrices' was held in Washington, DC, USA, on 14–15 June 2017 where these critical concepts were revisited in depth as the content of the draft white paper by the same name (final version in preparation) was discussed and debated [5]. Through that forum an even broader audience was reached and appreciation for the not-PK nature of biomarker assays was expanded. Since then, through various forums, consensus has continued to build, with scientific alignment on several key concepts occurring by the close of 2018. Importantly, the concept of Context of Use (COU), an elegant restatement of CCVI's "focus on the specific question being asked" has entrenched itself within the biomarker community. Common understanding has evolved beyond broad acknowledgment that biomarker assays are not PK assays to include specific conceptual leaps, such as:

- Recombinant/purified standard calibrator material ≠ endogenous analyte;
- Accurate recovery of spiked recombinant/purified material ≠ endogenous analyte accuracy;
- Recombinant/purified material stability ≠ endogenous analyte stability;
- Dilutional linearity ≠ parallelism.

It seems that we have succeeded in viewing biomarker assays through their own lens and successfully shed the ill-fitting mantle of PK assays.

Before we become too self-congratulatory or complacent, we must acknowledge that our work is far from over. The question that currently seems to get the most air time is how to provide clear guidance for biomarker assay validation. Despite the desire expressed by many to have concrete rules to follow, we must accept that the breadth of questions to be addressed by biomarkers is not amenable to such an approach. Recommendations can certainly be made, but they must provide adequate plasticity to enable broad applicability. So, are we ready for another leap in the evolution of our thinking? Can we embrace a level of ambiguity that has not generally been associated with bioanalysis, a field built on accuracy and precision? I not only believe we can, but that we must. To this end, I propose three simple recommendations: (i) be a scientist; (ii) embrace and own FFP and (iii) demand COU. No doubt, the vague nature of these recommendations will disappoint many. So, what do they really mean?

'Be a scientist' highlights the need for biomarker scientists to accept a new level of accountability in determining the right assessments to perform to ensure that their assays meet the needs of the biomarker. To do so requires that biomarker scientists embrace and own FFP, appreciating that FFP does not mean lower quality or less rigor, but instead means 'do good science' and 'get it right'. Furthermore, the purpose in FFP is in fact, COU. The biomarker scientist must assume responsibility for being the critical judge of what level of assay performance is required to meet a given COU. Therefore, to be a successful biomarker scientist, one must demand COU. A challenge faced in many organizations, however, is a disconnect between those requesting a biomarker assay and those who are expected to deliver it, whereby the proposed COU is not made evident to the latter. An approach frequently proposed to enable a path forward in such cases has been to develop the biomarker assay to the highest possible standard, so the assay will be capable of meeting all potential COUs. However, in my experience, proposed COUs evolve with the biomarker and clinical development program and are not amenable to *a priori* prediction. Even if such predictions were possible, the time and cost of developing every biomarker assay to meet all future conceivable COUs, many of which would never come to fruition, is not wise investment of resources. In a world of finite resources, the consequence of overinvestment is the lost opportunity to do other, genuinely impactful work. We should never be guilty of overinvesting because we were unwilling to take on, and fight for, scientific accountability for our own work. Demand COU.

Biomarker science is not for everyone. These broad stroke recommendations are necessary because flexibility is requisite. Biomarker assays serve unique and varied COUs, which cannot be addressed by one-size-fits-all guidance. When it comes to guidance for biomarker assays, we must continue to apply the not-PK-assay mindset. PK assays share the same COU, so a single guidance may reasonably be expected to ensure that they are fit for their purpose. Such is not the case for biomarker assays.

Those who remember why they became scientists – to engage in critical thinking, solve problems, create new knowledge and change the lives of patients – will relish the challenge. Biomarker science is not black and white, but countless shades of gray. We must therefore align on the fundamental principles of scientific rigor and accountability while accepting ambiguity. There can be no specific rule book. We must think critically, generate data, share our learnings and continue robust discourse that includes industry professionals, regulators, academics and clinicians

and considers the best interests of the patients who are counting on biomarker scientists to do the right assay at the right time to generate the right data to improve their lives. It is time to take the next leap.

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GYROS PROTEIN Technologies

Extended sensitivity of microfluidic immunoassays for pharmacokinetic (PK) and biomarker analysis

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Background

The use of ligand-binding assays in biotherapeutic development is a mainstay of analytical groups in biopharma companies. Since its introduction, the compact disk (CD) based, nanoliter-scale Gyrolab® microfluidic immunoassay platform has been widely accepted as an essential analytical technology due to its time-saving, automated, and robust performance. The success of the assay format centers around the microfluidic CD labware where the immunoassay takes place. Recently, the Gyrolab Bioaffy™ 4000 CD has been introduced to increase assay sensitivity 2- to 6-fold beyond the current 6-log dynamic range. In this poster, we present data pharmacokinetic (PK) and biomarker analysis down to low pg/mL levels with the Gyrolab Bioaffy 4000 CD demonstrating the sensitivity expansion of Gyrolab immunoassays facilitated with the new CD labware.

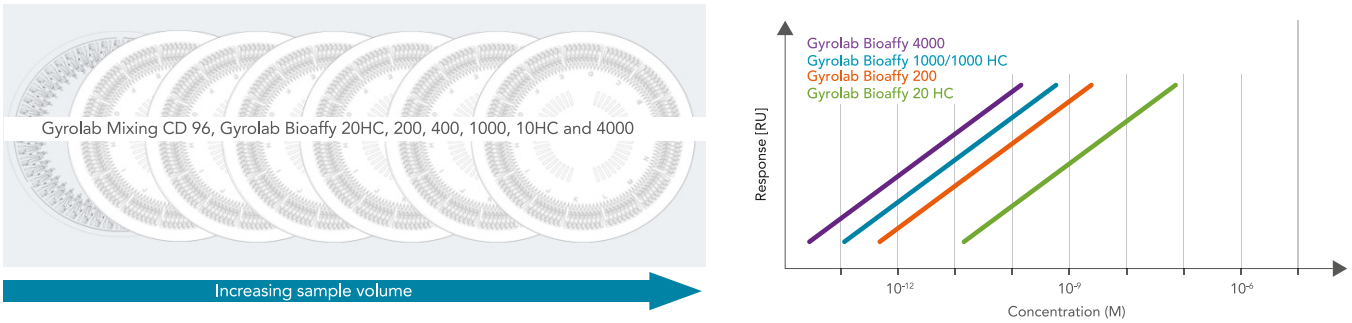
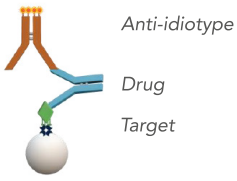


Figure 1. ***Gyrolab Bioaffy CD sample volume capacity correlation to assay sensitivity.*** *By altering the size of the volume definition chamber, or the affinity of the column using high-capacity beads, the assay range provided by the Gyrolab Bioaffy CD family is over 6 logs. This range has been extended with the introduction of Gyrolab Bioaffy 4000 CD.*

Sensitivity of Actemra® and Keytruda® PK assay is extended using Gyrolab Bioaffy 4000 CD

a)



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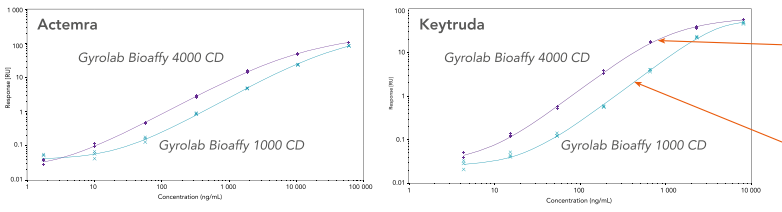

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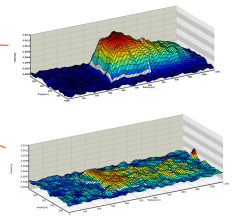

d)

CD Type	ACTEMRA		KEYTRUDA	
	LLOQ (ng/mL)	ULOQ (ng/mL)	LLOQ (ng/mL)	ULOQ (ng/mL)
1000	60	20,000	18	6,000
4000	20	20,000	6	6,000

Figure 2. ***Actemra® (tocilizumab) and Keytruda® (pembrolizumab) Gyrolab PK assay standard curves.***

a) *Bridging three-step sandwich assay with biotinylated human PD-1 as capture reagent and recombinant human anti-idiotype (pembrolizumab or tocilizumab) labeled with Alexa Fluor® 647 as detection reagent,* ***b)*** *PK assay dynamic range for Actemra (left) and Keytruda (right) using Gyrolab Bioaffy 4000 CD and 1000 CD,* ***c)*** *Viewer profiles of representative data points showing column fluorescence representing analyte binding,* ***d)*** *LLoQ and ULoQ assay results for Actemra and Keytruda. The three-step bridging Gyrolab PK assay was run using Rexxip H with 5% human serum and humanized IgG1 monoclonal antibody tocilizumab or humanized IgG4 monoclonal antibody pembrolizumab as the standard.*

Increased assay sensitivity for cytokine biomarker analysis using Gyrolab Bioaffy 4000 CD

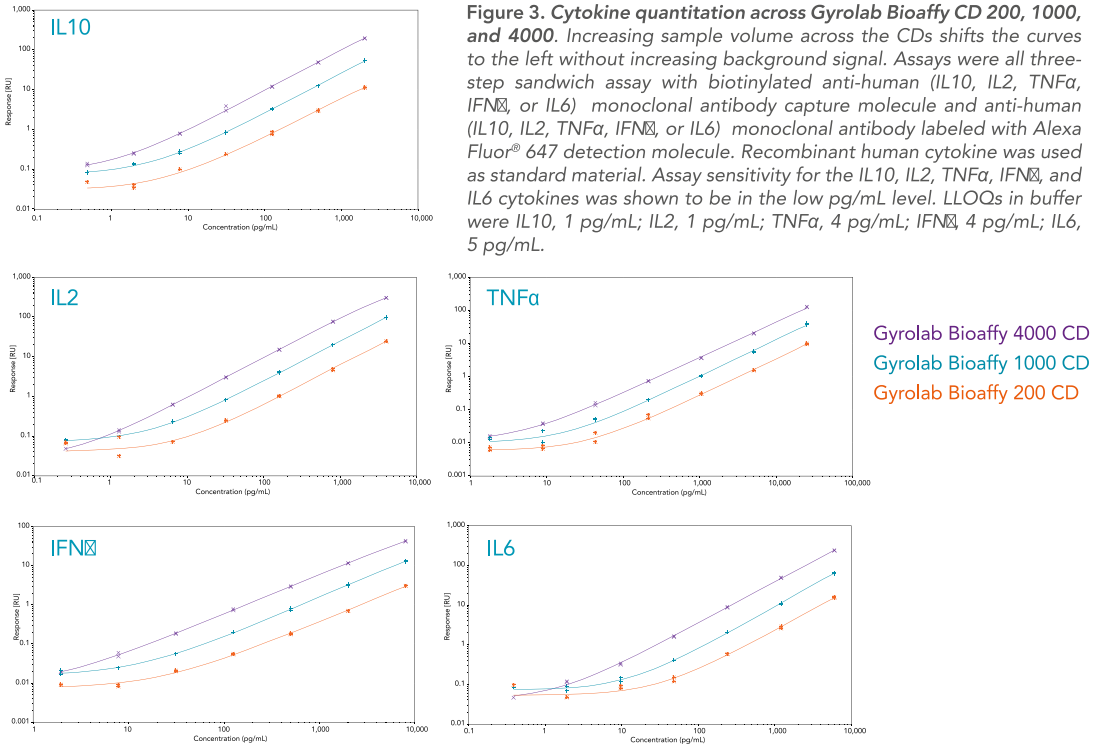

Figure 3. ***Cytokine quantitation across Gyrolab Bioaffy CD 200, 1000, and 4000****. Increasing sample volume across the CDs shifts the curves to the left without increasing background signal. Assays were all three-step sandwich assay with biotinylated anti-human (IL10, IL2, TNFα, IFNγ, or IL6) monoclonal antibody capture molecule and anti-human (IL10, IL2, TNFα, IFNγ, or IL6) monoclonal antibody labeled with Alexa Fluor® 647 detection molecule. Recombinant human cytokine was used as standard material. Assay sensitivity for the IL10, IL2, TNFα, IFNγ, and IL6 cytokines was shown to be in the low pg/mL level. LLOQs in buffer were IL10, 1 pg/mL; IL2, 1 pg/mL; TNFα, 4 pg/mL; IFNγ, 4 pg/mL; IL6, 5 pg/mL.*

Summary

Gyrolab Bioaffy 4000 CD has been recently introduced to extend the sensitivity of the Gyrolab Bioaffy CD family by increasing the sample volume in the CD to 4000 nL. PK and biomarker assay results support this extended sensitivity:

- Use of the Bioaffy 4000 CD increased LLoQ of Actemra and Keytruda PK assays from 60 ng/mL to 20 ng/mL and 18 to 6 ng/mL, respectively, demonstrating a 3-fold increase.
- Viewer profiles support increased assay sensitivity with greater fluorescent peaks of analyte bound to the Gyrolab Bioaffy 4000 versus Gyrolab Bioaffy 1000 CD columns.
- Similarly, curves for analysis of IL10, IL2, TNFα, IFNγ, and IL6 cytokines were shifted to the left, indicating increased assay sensitivity, with LLoQ values in the low ng/mL range.
- Background signal was not increased with the larger sample volumes of the Gyrolab Bioaffy 4000 CD in any of the PK and biomarker assays shown.

This extended sensitivity will be useful for PK and biomarker studies requiring higher sensitivity using Gyrolab platform, while maintaining the high reproducibility and broad dynamic range that Gyrolab microfluidic assays provide.

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Perspective

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Bioanalysis

Accelerating protein biomarker discovery and translation from proteomics research for clinical utility

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Discovery proteomics research has made significant progress in the past several years; however, the number of protein biomarkers deployed in clinical practice remains rather limited. There are several scientific and procedural gaps between discovery proteomics research and clinical implementation, which have contributed to poor biomarker validity and few clinical applications. The complexity and low throughput of proteomics approaches have added additional barriers for biomarker assay translation to clinical applications. Recently, targeted proteomics have become a powerful tool to bridge the biomarker discovery to clinical validation. In this perspective, we discuss the challenges and strategies in proteomics research from a clinical perspective, and propose several recommendations for discovery proteomics research to accelerate protein biomarker discovery and translation for future clinical applications.

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Keywords: biomarker qualification • clinical biomarker utility • mass spectrometry • protein biomarker discovery • proteomics research • targeted proteomics

The application of biomarkers has a long history in the clinic. Physicians in ancient India observed that urine collections of diabetic patients attracted ants, and they called the pathological condition 'madhumeha', or honey urine [1]. Sugar levels in urine served as a diagnostic biomarker for the disease. In modern medicine, the critical role biomarkers play in drug discovery and development has long been appreciated in the scientific community. For example, HER2 overexpression was utilized for patient selection during clinical trials of trastuzumab, the first monoclonal antibody approved for solid tumors [2]. Similarly, PD-L1, one of the best-characterized biomarkers in oncology, was incorporated in the early clinical development plan of the first anti-PD-1 therapy in the mid-2000s [3]. Moreover, in recent years there have been an explosion of interest in biomarkers [4,5]. They are driven, in no small part, by the low response rates of many cancer therapies. Immunotherapy, for instance, has revolutionized cancer treatments. However, only a small fraction (~12% of the total cancer patient population in the USA) is expected to respond to these therapies [6]. Identifying patients who will benefit from these transformative treatments remains a daunting task. The discovery, qualification and application of biomarkers is a key pillar for precision medicine [7], which aims to deliver the right treatment at the right dose to the right patient at the right time.

Development of a protein biomarker starts with hypothesis generation. Proteomics, where expression levels of thousands of proteins are compared between the test and control group, is one of the key approaches in generating hypothesis. Once biomarker candidates are generated from these discovery experiments in discovery proteomics, it takes a long and uncertain process to verify and validate them, which includes four additional essential process components: biomarker verification, analytical validation, clinical validation and biomarker qualification [8–10]. The process and challenges in biomarker translation from discovery proteomics to biomarker qualification are shown in Figure 1. Some of these biomarker candidates may be used as potential drug targets being evaluated during drug discovery. Other biomarker candidates continue to be evaluated retrospectively in clinical research. Before the biomarkers are qualified for clinical utilities, accurate, precise and high-throughput analytical assays need to be developed and validated to ascertain that they can be reliably measured. These biomarkers are then tested in a

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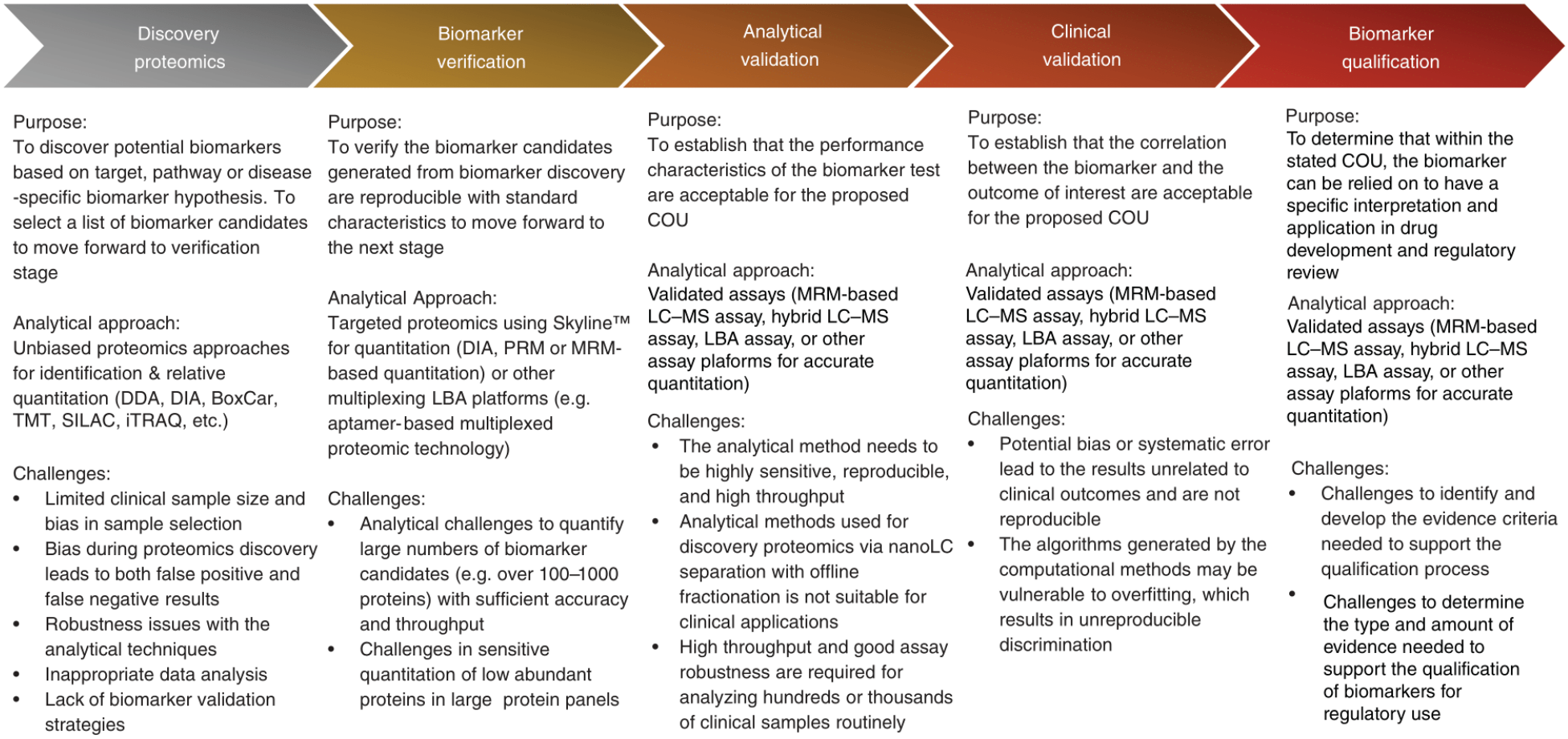

Figure 1. Process and challenges in biomarker translation from discovery proteomics to biomarker qualification.
COU: Context of use.
Information gathered from [8–10].

Table 1. List of biomarkers approved for companion diagnostic devices or as qualified biomarkers†.

Category	Assay target	Biomarker type	Indication/context of use	Requestor
Companion diagnostic	HER2	Predictive marker	Breast cancer, gastric and gastroesophageal cancer	Various
Companion diagnostic	c-KIT	Predictive marker	Gastrointestinal stromal tumors	Various
Companion diagnostic	PD-L1	Predictive biomarker	Various cancers	Dako, Ventana Medical Systems
Qualified biomarker	Albumin, β2-Microglobulin, Clusterin, Cystatin C, KIM-1, Total Protein and Trefoil Factor-3	Safety biomarker	To be used with traditional indicators to indicate renal injury in rat	Predictive Safety and Testing Consortium, Nephrotoxicity Working Group
Qualified biomarker	Clusterin, Renal Papillary Antigen	Safety biomarker	To be used with traditional indicators to indicate renal injury in rat	International Life Sciences Institute/ Health and Environmental Sciences Institute, Nephrotoxicity Working Group
Qualified biomarker	Cardiac troponins T and I	Safety biomarker	To indicate cardiotoxicity in rats, dogs or monkeys when testing known cardiotoxic drugs and may be used to help estimate non toxic human dose	PJ O'Brien, WJ Reagan, MJ York and MC Jacobsen
Qualified biomarker	Galactomannan	Diagnostic biomarker	To be used with other clinical and host factors to identify patients with invasive aspergillosis	Mycoses Study Group
Qualified biomarker	Fibrinogen	Prognostic biomarker	To be used with other characteristics to enrich for COPD exacerbations	Chronic Obstructive Pulmonary Disease Biomarker Qualification Consortium
Qualified biomarker	Clusterin, Cystatin-C, Kidney Injury Molecule-1, N-acetyl-beta-D-glucosaminidase, Neutrophil Gelatinase-Associated Lipocalin and Osteopontin	Safety biomarker	To aid in the detection of kidney tubular injury in Phase 1 trials in healthy volunteers	Critical Path Institute's Predictive Safety Testing Consortium Nephrotoxicity Working Group, and Foundation for the National Institutes of Health's Biomarker Consortium Kidney Safety Biomarker Project Team

†Data compiled from data from US FDA websites [14,15].

prospective clinical study to demonstrate that within the stated context of use (COU), they can be relied upon to support a diagnostic, drug development or regulatory decision.

Breakthroughs in massively parallel DNA sequencing technology (next-generation sequencing) [11] have evolutionized many aspects of biological and medical sciences. Similarly, there is a convergence of technological advances in mass spectrometry, such that the discovery community is at an inflection point to conduct a much larger number of experiments in a shorter amount of time, with higher data quality. Advances in mass spectrometry technology has led to an explosion of proteomics studies, and this trend is expected to accelerate with new methods outlined above. In our laboratory, each mass spectrometer generates over 75 GB of protein profiling data every 24 h. This astonishing amount of information across the research community will undoubtedly fuel the discovery of many more biomarkers. However, compared with the number of publications in biomarker discovery with the proteomics research, the number of protein biomarkers deployed in clinical practice remains rather limited. At this writing, a PubMed search for 'proteomics' and 'biomarker' returned over 23,000 articles. Even though some protein biomarkers generated from discovery proteomics have been used for internal decision-making in the pharmaceutical industry, protein biomarkers that eventually become companion diagnostics or surrogate endpoints are still very rare. For example, there are only three protein biomarkers that are approved by the US FDA as companion diagnostics (Table 1). In 2008, the FDA introduced the Biomarker Qualification Program to streamline drug development [12,13]. Based on the FDA guideline, a qualified biomarker can be used in multiple drug development programs without the agency's reconfirmation of the suitability of the biomarker's specific COU in regulatory submissions. Right now, there are only a total of six panels of protein biomarkers that have been qualified (Table 1) with an additional nine panels being under review in Biomarker Qualification Program.

In this perspective, we will review current advances, strategies and challenges in discovery proteomics research, and their impact on biomarker discovery, development and qualification, while pointing out current gaps in moving candidate biomarkers into clinical practice. We proposed several recommendations to bridge the gaps between discovery proteomics research and clinical biomarker utility for future research in this exciting field. We will focus our discussion on protein biomarkers. Even though the technologies are specific for proteins, the general principles

outlined in this article in executing an effective biomarker discovery and development strategy are equally applicable to other biomarkers.

Biomarker discovery, verification & qualification: current advances & strategies in proteomics research from the clinical perspective

Biomarker discovery: simultaneous improvements in proteome depth & throughput

Bottom-up proteomics uses proteases to digest proteins into short peptides, which are then detected and sequenced by mass spectrometry. The use of isotopes has a long and distinguished history in mass spectrometry. In the early days of proteomics, isotope labeling techniques such as Stable Isotope Labeling with Amino acids in Cell culture (SILAC) [16] were utilized to account for variations in different steps of the analysis, such as sample purification and mass spectrometer response. In the past 10 years, label-free quantification, which simplifies study design, has gained significant prominence with improvements in both instrumentation and software [17,18]. One particular isotope labeling technique, tandem mass tag (TMT), remains popular some 15 years after its commercial introduction, likely due to its capacity to multiplex 16 samples or more [19]. It should be noted, however, that a typical TMT experiment requires off-line fractionation before LC–MS analysis to increase the proteome depth and alleviate the issue of ratio compression [20]. Therefore, the throughput enhancement from sample multiplexing is usually modest. Specifically, the ability to run only 16 samples in parallel (16 TMT tags) is a major bottleneck for further throughput enhancement. Similarly, isobaric tags for relative and absolute quantitation (iTRAQ)-based quantitative proteomics was also used for protein quantitation [21,22]. However, multiplexing in iTRAQ is usually limited to four or eight samples.

In analytical chemistry, there is often a trade-off in the performance attributes. For example, in LC, throughput increase typically results in reduction in separation efficiency, when the mobile phase linear velocity exceeds the optimal flow rate. Several new techniques in proteomics, however, promise to enhance throughput and the proteome depth at the same time. We will discuss three of these new techniques as illustrative examples.

The first technique is BoxCar data acquisition. Orbitrap-based instruments are extremely popular in proteomics studies. In each survey scan, a fixed number of ions are trapped inside the mass analyzer. Due to the very high dynamic range of the analytes, peptides of low abundance are poorly detected. In this novel method, ions are trapped in narrow mass windows, and analyzed together in the Orbitrap over the entire mass range, resulting in a dramatic enhancement in sensitivity for low abundant peptides. It has been shown that 10,000 proteins can be quantified with a throughput of over 20 samples a day [23].

The second technique is data-independent acquisition (DIA). In a traditional bottom-up proteomics experiment, a subset of the most abundant peptides in each survey scan (MS1) are selected for MS/MS sequencing, this is called data-dependent acquisition (DDA). Since hundreds of peptides can be eluted in any survey scan and the MS/MS scan is performed in a sequential manner, only a small fraction of the proteome is sampled in an LC–MS run [24]. In DIA, all co-eluting peptides are fragmented in a given precursor isolation window, and the resulting highly complex MS/MS spectra are deconvoluted using sophisticated algorithms. Although conceptually simple, it took more than 10 years of intense hardware and more importantly, software development to bring this technique to a level of maturity that can be widely deployed for proteomics experiments [25]. Bekker-Jensen *et al.* recently demonstrated that with a throughput of 60 samples per day (approximately a tenfold improvement over a typical proteomics experiment), over 5000 proteins could be reliably quantified [26].

The third technique is the application of ion mobility as an additional dimension of separation for complex samples, which significantly increases sensitivity and throughput, as well as reduces spectral complexity. In the same example described by Bekker-Jensen *et al.*, the throughput of the analysis was significantly improved by connecting an Orbitrap Exploris 480 platform with a front-end high field asymmetric waveform ion mobility spectrometry interface [26]. Combination of DIA with field asymmetric waveform ion mobility spectrometry (FAIMS) with single compensation voltages allows for the analysis of up to 2000 peptides per LC gradient minute and more than 5000 protein groups in 20 min [26]. Using the ion mobility function on the Bruker's timsTOF Pro™ platform, Meier *et al.* extended the parallel accumulation – serial fragmentation (PASEF) with DIA (DIA-PASEF) as a new data acquisition mode [27,28]. DIA-PASEF technology allows the high-throughput in-depth proteome analysis of up to 300 samples per day, which is extremely useful for true clinical proteomics where robust analysis of several hundreds of samples per day is highly desirable [29]. It is worth noting that a newly developed nanoLC system, called Evosep One, was connected to the timsTOF Pro™ system enabling high throughput analysis with high robustness [29,30]. Unlike traditional nanoLC systems, Evosep One uses four low-pressure pumps in parallel to

selectively elute analytes, while leaving highly hydrophobic contaminants on a single-use trap column [30]. Separation in the analytical column is accomplished by a pre-formed gradient using a single high-pressure pump, delivering the robustness and throughput that are essential for clinical research applications.

Designing a rigorous biomarker discovery study

State-of-the-art analytical methods do not by themselves guarantee high-quality candidate biomarkers. More often than not, failure to identify valid biomarkers is the result of poor experimental design and sample procurement. Bias in a discovery study can lead to both false-positive and false-negative results. It can be introduced through different stages of discovery research: pre-analytical, analytical and post analytical. The pre-analytical stage refers to how samples are procured, collected and stored before analysis, and can be a significant source of bias. It is also very important to consider any factors influencing biological variability or any confounding factors. A classic example [31] is a biomarker study comparing the serum of cancer patients with that of normal healthy volunteers. Differences between the protein profiles of these two groups of subjects could very well come from the stress level or the effect of medications in the patient group, and have nothing to do with the underlying pathology. A stress biomarker from this study, for instance, might robustly classify cancer patients undergoing chemotherapy from healthy controls, but offers little value if the goal is to screen for asymptomatic patients or use it as a pharmacodynamics biomarker for a new therapy. More recently, Geyer *et al.* reported a striking example of pre-analytical bias in plasma proteome profiling [32]. They found that plasma samples are often contaminated with erythrocyte and platelet proteins. An extensive survey of the literature of 113 plasma discovery studies revealed that over half of the studies reported at least one of these contaminant proteins as highly significant biomarkers.

Bias in the analytical stage can often be controlled by having an analysis workflow with consistent performance. In the laboratory setting, fluctuations in ambient temperature may lead to errors in mass measurement, and accumulation of contaminants in the samples may lead to changes in LC retention time and detector signal in the mass spectrometer. These inconsistencies are especially problematic in a large experiment when the data acquisition lasts for weeks and months. Optimization of both the sample preparation and the LC–MS method is therefore crucial in obtaining high-quality data. In addition, sample randomization can help remove systemic bias in the analysis, for example, gradual decline of analyte signal intensity from the first sample to the last sample. When samples are analyzed in different batches due to the large size of the study, it is important to have bridging samples so that samples from different batches can be compared. In addition, it was previously reported that a universal and synthetic protein standard, called DIGESTIF, was added to the biological samples during sample preparation [33]. After sample processing, the tryptic peptides in the samples can be used as the quality control samples to assess the quality of sample preparation, digestion efficiency and the performance of the LC–MS system [33].

Bias in the post analytical stage is usually introduced by inappropriate data analysis, the most common being over-fitting of the data. If 10,000 proteins are identified in an experiment, and the scientist performs a *t*-test on each one of them between the test and control groups, by random chance some of the proteins will be identified as statistically significant, even if the null-hypothesis holds true for every protein. This multiple comparisons problem ('if you torture the data enough, nature will always confess') can be readily corrected using a variety of statistical methods [34]. In addition, unlike in clinical trials, the data analysis plan is usually not pre-specified in an exploratory experiment, and the bioinformatician may be tempted to tweak the analysis scheme so that the number of interesting hits is maximized. Regardless of the origin of data over-fitting, one can check for its presence by evaluating the reproducibility of the findings in a completely independent set of samples [35].

In an effort to minimize bias in biomarker research, investigators from the Early Detection and Research Network (EDRN) formulated [36] the principle of prospective-specimen collection, retrospective-blinded-evaluation (PRoBE). Although this standard was proposed for validation studies, various groups advocated that the same principle should be applied to discovery experiments [31,37]. Key elements of the PRoBE design principle include: conduct the study in the target population to improve the applicability of the findings; utilize samples from a prospective, clinical relevant cohort; select case and control randomly from the cohort; use blinding for sample collection and evaluation.

Most biomarker discovery scientists are lab-based researchers who strive to maintain analytical and statistical rigor in their work. An important lesson from over two decades of proteomics research for biomarker discovery is that having the appropriate samples and having enough of them are just as important as what happens after the samples arrive in the lab. Before embarking on a discovery experiment, the scientists should ascertain that the

samples are representative of the intended clinical use and are collected appropriately and uniformly between the case and control arms, and the study is sufficiently powered.

Selecting biomarker candidates for qualification

As many thousands of proteins are detected in a typical proteomics experiment, it is not uncommon to identify more than a handful of proteins showing significant changes between the case and control groups. There are a number of factors influencing the selection of these candidates for validation studies. Robustness in the change of protein level and the effect size are often the most important selection criteria. In addition to changes in protein levels, correlation of biomarker levels with clinical outcome is also an essential factor to identify prognostic or predictive biomarkers for patient selection or stratification. Having a biological explanation for the biomarker change adds a substantial amount of weight for further investigation. However, absence of clear biological relevance should not preclude selection of a biomarker. For example, carcinoembryonic antigen, one of the most widely used clinical cancer biomarkers, was discovered in the 1960s and its role in tumorigenesis was poorly defined at the time, and is still the subject of study decades later [38]. On the other hand, known general inflammation and stress biomarkers are typically not specific to the disease of interest and should usually be excluded. There are some exceptions depending on the biomarker context of use (COU). If the inflammation is a hallmark of the disease, it can be still used as part of composite biomarkers.

Recent advances in analytical validation techniques

Qualification of a biomarker for use in drug development consists of a clinical validation and an analytical validation. Adequate validation in both the clinical validation and analytical validation is very important for applying a biomarker for any purpose to elucidate disease etiology or pathophysiology, to predict clinical course, to identify potential responders for predictive enrichment or to represent clinical benefit [39]. Since the biomarkers can be used for different purposes, a fit-for-purpose validation approach should be used to determine the proper extent and acceptable criteria of analytical method validation [40]. The analytical method should be fully validated if the biomarker data generated will be used to support a decision-making for regulatory purpose, such as the pivotal safety and/or effectiveness determination or to support dosing instructions related to the product labeling [40]. The requirements for biomarker assay validation have been included in the FDA's 2018 Bioanalytical Method Validation Guidance [40]. In general, method validation for biomarker assays should evaluate accuracy, precision, sensitivity, selectivity, parallelism, range, reproducibility and stability of a biomarker [40].

The analytical methods for biomarkers can be based on different technology platforms and must take into account the COU [41]. Extensive progress has been made in the field of biomarkers that was enabled by recent advances in the analytical technologies. Currently, LC–MS assays and ligand-binding assays (LBAs) are still two of the most popular platforms routinely used for quantitative and semi-quantitative measurement of endogenous biomarkers in soluble matrices such as blood (serum or plasma), urine and cerebrospinal fluid. LBA technology has evolved from plate or bead-based enzyme-linked immunosorbent assay (ELISA) to multiple ultrasensitive platforms such as Simoa® and SMCxPro® [42]. LC–MS platforms can be used for both small molecule and large molecule biomarker assays; however, LC–MS approaches for protein biomarker assays will be the focus of this discussion. The choice of techniques for validation studies places some constraints on biomarker selection. Each technology platform has its own pros and cons. Not all biomarkers can be measured by antibody-based methods, such as ELISA or immunohistochemistry (IHC) due to reagent availability. In addition, high-level multiplexing introduces many challenges, and large number of analytes in the same assay can over-complicate method development. In this regard, MS has the unique advantage with its inherent multiplexing capacity and largely reagent-agnostic nature.

In contrast to proteomics research for biomarker discovery which mainly utilizes high resolution mass spectrometers (HRMS; e.g., Thermo's Q Exactive or Bruker's timsTOF™ HRMS systems), protein biomarker assay for biomarker verification or validations are often performed by multiple reaction monitoring (MRM) with stable isotope dilution LC–MS/MS methods on triple quadrupole mass spectrometers (TQ-MS). Recent advances in MS-based technologies (ion sources, optics and detectors) have resulted in several highly sensitive TQ-MS systems from several vendors, such as Sciex, Thermo Fisher Scientific, Waters, Agilent and Shimadzu, etc. In combination with micro flow LC–MS interface, the sensitivity of MRM-based targeted proteomic approach is further improved for quantitative analysis. Unlike proteomics research, sensitivity improvement via nanoLC separation with off-line sample fractionation approaches is not suitable for many clinical applications when dealing with large cohorts of patients in clinical routines due to low throughput and poor assay robustness. Automated sample extraction

using 96-well format for LC–MS/MS analysis using regular flow or micro flow LC is preferred for biomarker assay validation [43,44]. In recent years, hybrid LBA-LC–MS/MS methods that combine automated high throughput immunocapture with LC–MS/MS detection has become a powerful platform to measure protein biomarkers with superb detection sensitivity and selectivity [45–47]. However, not all biomarkers can be measured by hybrid LBA-LC–MS/MS assays, especially by multiplex assays due to reagent availability.

Challenges in biomarker assay translation from discovery proteomics to clinical applications

Despite its immense potential to transform drug development and clinical practice, biomarker research, similar to many other emerging scientific disciplines, has had several high-profile setbacks. One example is related to proteomics research on ovarian cancer. Since most ovarian cancer patients are asymptomatic in the early stages of the disease, the earlier the detection, the more effective the intervention becomes. In an effort to identify serum biomarkers for preventive screening, several laboratories discovered putative candidates that were highly promising. A panel of six biomarkers, which had a sensitivity of 95.3% and a specificity of 99.4%, significantly outperformed CA-125, a biomarker that had been in clinical use for 20 years [48]. The excitement for this test was so palpable that a diagnostic company started to produce and market this test in the same year [49]. However, when this panel, along with 29 other novel biomarkers, were tested in a large and prospective trial, none was superior to CA-125 [50]. This sobering experience underscores the importance of rigorous validation studies to ascertain the validity of any biomarker candidates.

As shown in Figure 1, after the initial generation of biomarker candidates from biomarker discovery stage, verification and validation of the biomarkers through analysis of hundreds and even thousands of biological samples is usually required [51]. The analytical method used to verify the biomarkers needs to be highly sensitive, reproducible, quantitative and high throughput [8,52,53]. Although hundreds to thousands of biomarker candidates were identified in discovery proteomics research, only a small number of biomarkers were validated in the clinical utilities. In many cases, this could be due to the mismatch between the large numbers of biomarker candidates and the lack of reliable assays for biomarker verification and validation [54,55]. For example, the isotope-labeling-based TMT technique is not well suited for the analysis of large number of specimens because this approach is limited by the number of samples that can be quantified in one batch, and its inherent variability resulting from the batch effect. On the other hand, the DIA-based label-free quantitative still remains challenging for large-cohort analysis, such as compromised quantitative accuracy and precision due to significant experimental variations without using any stable isotope-labeled peptides for internal calibration [18,56,57].

After reviewing current advances and strategies in discovery proteomics research, we have identified several scientific and procedural gaps between discovery proteomics research and clinical implementation. There are several factors in discovery proteomics stage that can challenge a successful outcome. One of the challenges is the selection of the starting material for discovery proteomics research. Each biological sample is unique and there are potential inter-individual variabilities due to human biology nature or sample-to-sample variabilities that could be generated during sample collection [58]. These variations could lead to an unsuccessful qualification of a biomarker and therefore, it is important to determine the cohort size that can help to distinguish between protein molecules that merely reflect changes due to biological or individual variability and 'true biomarkers'. There are several other issues associated with the discovery proteomics research, including poor experimental design, lack of biomarker validation strategies and robustness issues with the analytical techniques for clinical tests (Figure 1) [31,54,55,59,60]. These gaps have contributed to poor biomarker validity and lack of clinical relevance. In addition, the complexity and low throughput of the proteomics research approaches, such as peptide fractionation and nanoLC workflow, make it very difficult for biomarker assay translation to clinical applications.

Strategies to bridge the gap between discovery proteomics research & clinical implementation

As discussed earlier, several factors can affect the outcome of the biomarker discovery during proteomics research, and the first and foremost important challenge in any biomarker discovery program is the availability of large number of good quality biospecimens. Most of the biomarker studies are hampered by the poor supply of required biospecimens in sufficient quantity. Since biomarker discovery is based on the target, disease or pathway-specific hypotheses [61,62], it is important to select the right samples from the right patients in sufficient quantity. Due to the difficulty to obtain samples from certain and specific population of diseases, it is even harder to obtain samples at different stages of certain diseases. Therefore, setting up biobanks is one approach of addressing this issue.

As shown in Figure 1, biomarker discovery starts from proteomics research; however, there are several additional critical steps leading to clinical utilization, including verification, analytical validation, clinical validation and biomarker qualification before finally commercialization [8–10]. A better understanding of the overall biomarker discovery and validation process would be helpful to improve the experimental study design, to increase the efficiency of biomarker development and to implement clinical applications. On the other hand, majority of the biomarker candidates generated from discovery proteomics studies do not progress to the validation stage and hence they will not be translated to clinical biomarkers. Alternatively, this problem can be alleviated by establishing the regional database center for deposition of the proteomics data at the discovery stage. Upon maturation of database, the biomarker candidates generated from discovery proteomics studies could be validated by retrospective studies. However, having a database center is not enough without establishing a robust proteomics database infrastructure for biomarker discovery program.

Recently, a number of reports showed that the determination of a single or a couple of proteins may not be sufficient to predict the therapeutic outcome because most therapeutics could alter the activities of many proteins during the treatment [63]. However, the verification and validation of these potential biomarkers is very challenging due to the need for targeting multiple specific candidates and the requirement of high-throughput quantitative assays. To overcome these challenges, a targeted proteomics technique using Skyline™ software for MRM method development and data processing was developed as an essential tool for biomarker panel verification and validation [17,25,64,65]. Unlike unbiased proteomics in which thousands of proteins are quantified without any prior knowledge, the targeted proteomics approach focuses on specific hypotheses for a subset of proteins. Targeted proteomics is usually achieved by spiking unlabeled samples with known concentrations of stable isotopically labeled (SIL) synthetic peptides to quantify target peptides via MRM-based LC–MS analysis. Addition of SIL-peptides is critical to account for sample preparation errors and signal variations in MS. Targeted proteomics has gained increasing popularity in protein quantification because it is a robust approach to detect proteins of interest with high sensitivity, accuracy and reproducibility [17,25,64,65]. In general, discovery proteomics focuses on method optimization to maximize protein identification by having long data acquisition time per sample, limiting the number of samples in a study in some cases. In contrast, targeted proteomics approaches focus on the limited number of the proteins of interest and then optimize the LC–MS conditions, including MS tuning and data acquisition methods to achieve the highest sensitivity and accuracy. In addition, the throughput of the assays has been also improved to allow for analyzing hundreds or thousands of samples. As a result, targeted proteomics has become a powerful tool to accelerate biomarker translation from biomarker discovery to clinical validation [55,66,67]. In general, the biomarker candidates generated from proteomics discovery should be confirmed by targeted proteomics or multiplexing LBA platforms (e.g., aptamer-based multiplexed proteomic technology [68,69]) before being selected for biomarker qualification. Beside biomarker candidate verification, the list of surrogate peptides and their MRM transitions generated by targeted proteomics are also very useful for bioanalytical assay development for biomarker qualification.

Most recently, Skyline™ software has been expanded to data acquisition using parallel reaction monitoring (PRM) and DIA or SWATH-MS (sequential windowed acquisition of all theoretical fragment ion mass spectra) techniques on HRMS systems, which allow the quantitative analyses of peptides covering over 100s or 1000s of proteins with good data quality [25,65]. Due to the popularity of Skyline™ software and availability of HRMS systems in many clinical labs, targeted proteomics can be conducted in clinical labs as a new analytical validation tool for protein panel verification and validation [66,67].

In general, the complexity and low throughput of the mass spectrometry-based proteomic technologies are due to the need to achieve sufficient proteome depth using off-line peptide fractionation and nanoLC with very long gradients. Recent advances in highly sensitive TQ-MSs, to some extent, allows the simplification of LC–MS/MS analysis using regular flow or microLC systems. In addition, HRMS platforms have become increasingly available in many clinical labs [70,71]. It is expected that it can be easier for biomarker assay translation from discovery proteomics to clinical validation when there are any needs for using HRMS platforms.

On the other hand, assay development of robust LC–MS assays to quantify protein biomarkers in the clinical settings remains a major challenge mainly due to the difficulty to account for matrix effects. Due to the heterogeneity of the clinical samples, it is very difficult to identify alternative biological matrices to mimic the matrix effect-causing components similar to that of authentic matrix for the preparation of calibration curves. Recently, an accurate and robust assay was developed for formalin fixed paraffin embedded (FFPE) tissue bioanalysis using a calibration curve prepared from five different stable isotope-labeled peptide analytes spiked into each study sample for the

quantitation of a targeted peptide [72]. This approach successfully eliminated the need for authentic FFPE matrix for the preparation of external calibration curves; however, it required the synthesis of multiple differently labeled peptide analytes. More recently, Gu *et al.* reported an in-sample calibration curve strategy with multiple isotopolog reaction monitoring (MIRM) detection of the SIL peptides, which allowed accurate measurement of protein biomarkers in each individual sample requiring only one SIL-peptide for each peptide analyte [73].

Another challenge for biomarker discovery and development is the statistical analysis. The types of analysis can vary from biomarker discovery to validation stage, and these tests are critical for the development of a quality panel of biomarkers. In particular, high throughput LC–MS machines generate large amount of data. Big data, data diversity and complexity have become a significant challenge in biomarker discovery and development. Even though software packages capable of processing extremely large datasets have been utilized by the proteomics community for some time [74], more efficient algorithms will help reduce computational resource requirements. Recently, contemporary statistical tools such as ANN (artificial neural networks), AI (artificial intelligence) and machine learning have been on the horizon in biomarker discovery [75–77]. They will play an increasingly important role in signal discovery in unbiased protein profiling, especially for studies with complex design. On the other hand, we expect rigorous and established statistical techniques to remain the gold standard for biomarker validation and qualification studies.

Despite technological advances in recent years, many challenges remain in terms of biomarker assay transfer from discovery to clinical utility. In an attempt to further increase the productivity in biomarker research, we propose the following recommendations to address these issues:

- We call for more rigorous experimental designs in biomarker discovery studies. While reproducibility by itself does not guarantee the removal of bias in discovery research ("Bias times 12 is still a bias" [78]), at a minimum internal consistency should be demonstrated. This could take the form of dividing the study samples into a training set and a validation set, or procuring an independent set of samples after the discovery study;
- Researchers engaging in discovery efforts should consider the clinical utility of any biomarkers resulting from their efforts at project initiation. For example, with a disease prevalence of 1 in 2500, a screening biomarker with a 75% clinical sensitivity and 99.6% specificity has a positive predictive value of 10%, meaning that there are nine-times more false positives than true positives [79]. The performance requirement is exceedingly demanding in this case, and a lot of resources and efforts could be wasted in discovery research without consideration to the eventual goal. Target sensitivity and specificity can be readily calculated given a projected clinical use [60];
- Vertical integration of discovery and validation efforts should be incentivized. Currently, most of the discovery research is performed in individual laboratories, and their efforts typically stop with the publication of the findings. Very few candidate biomarkers entered validation studies, and even fewer were confirmed in the validation. Advances in analytical methodology continue to reduce sample amount requirements [80]. As a result, in many cases clinical samples can be split between discovery studies and analytical validation work when coordination between the groups is put in place ahead of time;
- Biomarkers Consortium [81] has been a major contributor to approved biomarkers in FDA's Qualified Biomarker Program (Table 1). Given the resource requirements for biomarker qualification, we expect them to continue to play a leading role in the discovery and qualification of novel biomarkers. More broadly, collaborative research between specimen collecting institutions, laboratories with advanced analytical instrumentations and clinical centers with access to a sufficiently large number of patients will be necessary to have the scale and scope of expertise to advance many of the candidate biomarkers.

To some extent, the paucity of qualified biomarkers is a reflection of the incredible complexity of biological systems and the considerable heterogeneity of many human diseases. Just as synthesizing a molecule with high affinity to a biological target is very far from being a therapy in the clinic, discovering a novel biomarker is just the very first step in an often long and arduous journey. With high quality samples, rigorous experimental designs, continuous improvements in analytical methodology and more emphasis on biomarker qualification, we are at the cusp of a golden age of biomarkers for delivering transformational therapies for our patients.

Conclusion & future perspective

Clinical biomarkers are playing an increasingly important role in drug discovery and development. Recent advances in MS technologies and proteomics research have led to the identification of hundreds to thousands of protein

biomarker candidates; however, disappointedly only a small number of biomarkers have been qualified for clinical applications. In this perspective, we review current biomarker steps from biomarker discovery, verification and qualification, highlight current advances, strategies and potential issues in proteomics research from the clinical perspective, discuss challenges in biomarker assay translation from discovery proteomics to clinical applications, and propose strategies to bridge the gap between biomarker discovery and clinical implementation. Targeted proteomics provides a powerful tool to bridge the gaps between biomarker discovery and clinical validation. Some additional thoughts and opinions to accelerate and improve the productivity in biomarker research are also presented. Several recommendations are provided to the proteomics community for discussion and considerations: conduct biomarker discovery studies with more rigorous experimental designs; consider potential clinical utility of any biomarkers at discovery project initiation; integrate biomarker discovery with clinical validation efforts; continue to support biomarkers consortium for leading the discovery and qualification efforts of novel biomarkers.

Future proteomics research will focus not only on the number of protein biomarkers identified, but also on the reproducibility of the biomarker findings. Recent improvements in proteomics technology and experiment design will help reduce the gaps between the discovery proteomics research and clinical biomarker utility. Targeted proteomics will be more widely used as an essential tool to bridge the discovery proteomics to clinical implementation. In the near future, it is highly expected that more and more biomarkers will be qualified for clinical applications.

Executive summary

- Despite significant progress having been made in the proteomics research areas in biomarker discovery proteomics research, the number of protein biomarkers deployed in clinical practice remains rather limited.
- There are several scientific and procedural gaps identified between biomarker discovery and clinical implementation, which include: poor experimental design; limited clinical sample size; bias in sample selection; lack of biomarker validation strategies; and robustness issues with the analytical techniques used in clinical trials.
- These gaps have contributed to poor biomarker validity and lack of clinical relevance.
- The complexity and low throughput of the proteomics research approaches make it difficult for assay translation to clinical applications.
- Recent advances in targeted proteomics provide a powerful tool to bridge the gaps between biomarker discovery and clinical validation.
- Several recommendations are provided to the proteomics community for discussion and considerations:
  - Conduct biomarker discovery studies with more rigorous experimental designs.
  - Consider potential clinical utility at the beginning of discovery project initiation.
  - Integrate biomarker discovery with biomarker qualification efforts.
  - Support biomarkers consortium for leading the discovery and qualification efforts of novel biomarkers.
- With a clinical application mindset, better proteomics experimental design and better analytical qualification strategy, there is a potential to discover more clinically relevant biomarkers.

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