White paper

It's all relative

4 reasons why biomarker discovery commonly uses relative quantification

Abstract

Absolute and relative quantification are performed in proteomics to identify and validate protein biomarkers for clinical applications. In this white paper, four reasons why relative quantification remains the prevalent method for biomarker discovery are provided. Moreover, multiplex proteomic platforms from Olink® that offer both absolute and relative quantification options are described.

Introduction

A primary objective of proteomics is to identify and validate protein biomarkers for clinical applications, such as diagnosing, treating, and monitoring patients. Protein levels are measured using absolute or relative quantification and compared across different patient groups, which are frequently based on health status (e.g., diseased and healthy) or treatment type (e.g., treated and untreated).

Absolute quantification extrapolates the protein concentration (e.g., pg/µL) from signal readout (Figure 1). This is often accomplished with a standard curve that is generated using a sample in which the protein concentration is known (i.e., reference standard). Absolute quantification is used in many clinical applications because it facilities the direct comparison of data from different laboratories, organisms, and studies (1).

Relative quantification, on the other hand, does not calculate protein concentration in standard units (e.g., pg/µl). Rather, the relative differences in protein levels across samples are determined using the relative differences in signal readout. Also known as "differential" quantification, relative quantification can be performed in the presence or absence of a reference sample (1, 2). Although uncommon, inter-study sample comparison and data integration are possible with relative quantification when using bridging samples and methods that follow a vigorous validation process.

Both absolute and relative quantification are employed throughout the protein biomarker pipeline, from discovery to clinical use. Here, four reasons why relative quantification remains the predominant quantification method in biomarker discovery are explored. In addition, Olink proteomics technology that measures proteins with absolute and relative quantification will be discussed.

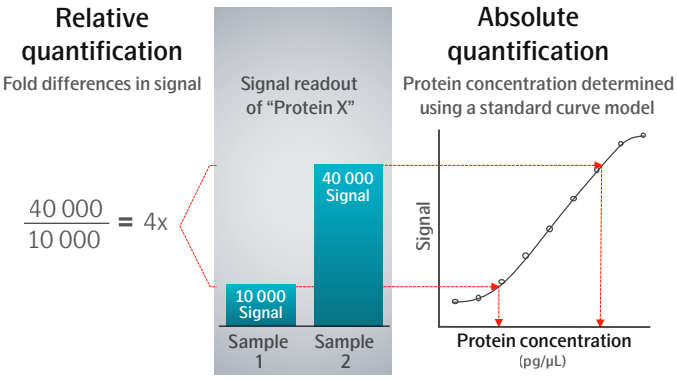

Figure 1. Example comparison of relative and absolute quantification of the same protein-of-interest, Protein "X," across two samples. With relative quantification, the abundance of "Protein X" is 4-fold higher in Sample 2 than Sample 1. With absolute quantification, the protein concentration in Samples 1 and 2 is estimated using a standard curve model.

Quantification is rarely absolute

Biomarker discovery routinely uses protein assays that measure a moderate ("mid-plex") or large ("high-plex") number of proteins simultaneously. Most of these multiplex assays perform relative quantification, rather than absolute quantification, for the following reasons.

1. Limited number of well-defined reference standards.

In most cases, a purified protein "standard" that is expressed in non-native, exogenous systems is employed to produce the standard curve. However, not all proteins can be expressed *in vitro* or purified efficiently, thus limiting the number of proteins that can be quantified (3, 4).

Batch-to-batch variability in protein folding, post-translational modifications (PTMs), and purity also contribute to the limited number of well-defined reference standards. Furthermore, few reference standards for absolute quantification in the proteomics community have been generated using rigorous guidelines like those described by the World Health Organization (WHO) (1, 2). In other words, the measured concentration for the same protein may vary between methods.

To underscore the acknowledged variability of "absolute" quantitative assays (5), two commercial sandwich-based enzyme-linked immunosorbent assays (ELISAs) using standard curves from different vendors were compared. Both measured horse IGF-1 protein using the same set of plasma samples (n=18) (6). However, the concentrations

varied ~100-fold between the two kits for the same samples.

2. Sample matrices can affect the accuracy of standard curves.

The protein used to generate the standard curve should display the same dose-response characteristics as the test samples (7). While biological-based standard matrices address the matrix effect, they may contain the endogenous target protein that can skew accurate quantification (3). Alternative options include costly control "surrogate" matrices or analytes that require a long time to develop. In any case, the dose-response characteristics of a standard curve must be tested thoroughly during development to ensure linearity.

3. Absolute quantification usually increases the risk of experimental bias.

Proteomic workflows with absolute quantification often require more steps compared to relative quantification, such as pipetting, cell culturing, and labeling. However, as sample handling increases, so does the risk of experimental biases that can affect quantitative accuracy (1).

4. Multiplex protein detection is difficult with absolute quantification.

An increasing number of biomarker studies have demonstrated that multi-protein signatures have higher discrimination power than single proteins, including research using Olink® proteomics technology (8). These studies highlight the importance of multiplex protein detection during biomarker discovery. However, absolute quantification is more difficult to perform with increased multiplexing since a standard reference or curve for each protein-of-interest must be developed.

A "gold standard" method for multiplex protein detection is bottom-up mass spectrometry (MS), where relative quantification remains the most common quantification method. This is because absolute quantification is more expensive, the standard curve takes a substantial amount of time to optimize, and some standards are difficult to obtain (3). Incomplete and variable enzymatic digestion also hinders accurate quantification with MS-based methods (9).Finally, precise and robust absolute quantification with "label-free" MS methods remains a challenge, particularly for multiplex protein detection because chromatograms of peptide elution peaks need to be aligned, the appropriate peptide(s) for quantification must be identified in complex samples and in different injections, and there remains no ideal normalization method to remove systematic biases (10).

Protein quantification with Olink technology

Olink® proximity extension assay (PEA) technology enables protein detection across a range of multiplexing levels, providing an end-to-end solution for biomarker discovery while using minimal sample volume (Table 1, Figure 2). Available as a reagent kit or as a fee-for-service, all Olink platforms undergo a rigorous validation process to ensure high specificity, sensitivity, and precision; these reports are freely available on the Olink website.

Olink® Explore 3072 is an ideal solution for biomarker discovery and exploratory studies, measuring nearly 3000 proteins across 10 orders of magnitude while only consuming 6 µL per sample. With a next generation sequencing (NGS) readout and a semi-automated workflow, Olink Explore 3072 is a relative quantification platform with a protein assay library covering all major biological pathways.

Olink® Target 96 is a relative quantification platform with a qPCR readout. Offering 15 different modular panels targeting proteins involved in a specific disease area or key biological process, Olink Target 96 empowers focused analyses for biomarker discovery and validation. It also requires only 1 µL of sample per 96-plex panel.

Olink® Target 48 Cytokine can provide either absolute or relative quantification using qPCR and 1 µL of sample, targeting 45 proteins involved in inflammatory processes that underly many diseases.

Olink® Flex and Olink® Focus measure up to 21 proteins-of-interest in one custom biomarker assay and consume only 1 µL of sample. While Olink Flex provides absolute *and* relative quantification for the same biomarker panel, Olink Focus permits the quantification method and level of validation to be chosen by the researcher. Proteins incorporated into Olink Flex or Olink Focus assays can be selected from a list of over 200 inflammation-related human proteins or the entire Olink protein library of ~3000 proteins, respectively.

To ensure that absolute quantification is accurate in a biological matrix with Olink Target 48 Cytokine, Olink Flex, and Olink Focus, a series of linearity experiments using

Table 1. Overview comparison of Olink® biomarker platforms.

Olink® platform	Instrument	Quantification method	# Modular panels	# Proteins per panel	Protein profiling / Biomarker pipeline †	
					Exploratory / Discovery	Targeted / Validation
Explore 3072	NGS	Relative	8	372	+++	+
Target 96	qPCR	Relative	15	92	++	++
Target 48	qPCR	Absolute & relative	1	45	+	++
Flex	qPCR	Absolute & relative	Custom	21		+++
Focus	qPCR	Absolute & relative	Custom	21		+++

† Applicability: high (+++), moderate (++), low (+)

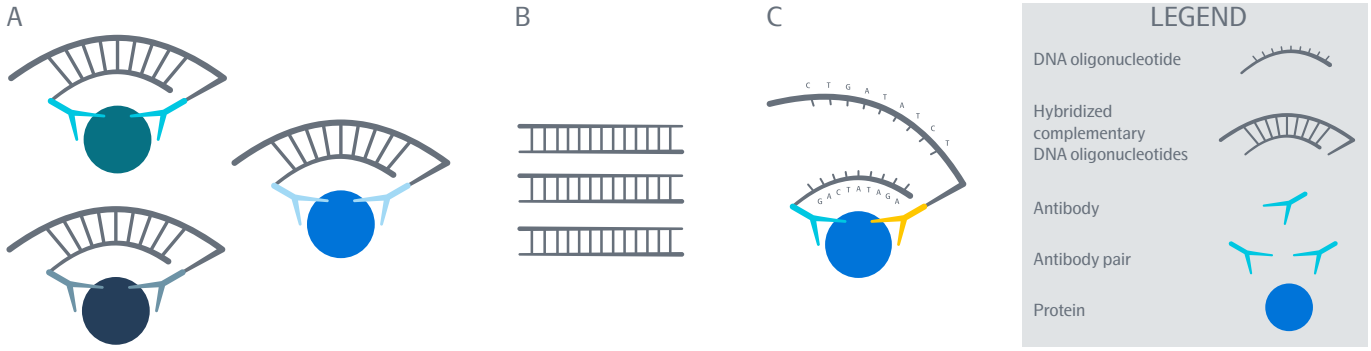

Figure 2. Olink PEA technology uses antibodies labeled with DNA oligonucleotides to quantify proteins. (A) When an antibody pair binds to its target protein in solution, their complementary oligonucleotides hybridize to each other. The double-stranded oligonucleotides act as a barcode to identify the sample and protein since each sample-protein combination has a unique oligonucleotide sequence. (B) The oligonucleotides are then amplified via PCR, and the resulting DNA amplicons are analyzed with qPCR or next-generation sequencing. (C) No signal will be generated if nonspecific binding occurs since the oligonucleotides will not hybridize.

plasma is performed. Moreover, a reference standard in plasma facilitates inter-plate normalization.

Importantly, PEA technology has identical workflows for absolute and relative quantification. As such, experimental biases from additional handling steps that are often necessary to obtain absolute quantification with other proteomic methods are not a concern.

A comparison of absolute and relative quantification obtained with Olink Target 48 Cytokine and Olink Explore, respectively, revealed that the median Spearman correlation coefficient (rs) was 0.95 for 38 detectable proteins (11). For this study, absolute quantification was in pg/µL while relative quantification was performed using an arbitrary unit in log2 (i.e., Olink® NPX, Normalized Protein eXpression). These data underscore that the two products produce data that highly correlate with each other, independently of readout method (NGS or qPCR), level of protein multiplex, and quantification method. The data also demonstrate that differential protein expression can be calculated accurately with relative quantification using Olink technology in the absence of a standard curve. In fact, numerous studies have demonstrated high correlations between the relative concentrations obtained with Olink platforms and the absolute concentrations obtained with other immunoassays. For example, a recent comparative study showed that the relative and absolute concentrations of specific analytes measured with Olink Target 96 and a conventional singleplex immunoassay, respectively, correlated very well (r = 0.89 - 0.93) (12).

Conclusions

Relative quantification remains the predominant method employed by high throughput, multiplex assays in discovery proteomics for two main reasons. First, absolute quantification requires time-consuming assay development and potentially costly reagents for each protein-of-interest. As such, the time and cost scale proportionally to the number of measured proteins. Second, the statistical significance of differentially expressed proteins in case-control studies can be determined with absolute *and* relative quantification if the studies are well-designed and adequately powered.

Once a subset of candidate biomarkers is identified, developing a smaller, focused assay with protein concentrations reported in standard units (e.g., pg/µl) becomes more manageable and affordable. Thus, absolute quantification is commonly performed during biomarker validation and for clinical applications.

There are exceptions to the relative-to-absolute quantification workflow in the biomarker pipeline. Some *in vitro* diagnostic assays are qualitative (e.g., a home pregnancy test) or use reference standards with arbitrary cut-off values. Other *in vitro* diagnostic tests provide relative quantification if clinical cut-off values have been established using reference standards (2, 7). For example, the WHO biological reference standard for hepatitis B surface antigen has an assigned arbitrary value, and was adopted by the medical devices sector of the European Commission for diagnosing hepatitis B (7). Several protein-based therapies are administered in arbitrary units (i.e., international units, IU), with each new reference standard normalized to the previous reference standard (2). These therapies include interferon alpha 2b (IFNα-2b), bevacizumab, trastuzumab, and human chorionic gonadotrophin (hCG).

Measuring proteins accurately is vital to biomarker development. Biomarker candidates identified with robust proteomic technologies are more likely to be validated and approved for clinical use. Accordingly, the benefit-cost ratio of biomarker validation, which is time-consuming and expensive, would increase. These are important considerations since thousands of potential biomarkers are published every year while, on average, only 1.5 new protein biomarkers are approved per year by the U.S. Food and Drug Administration (FDA) (13). Olink platforms deliver accurate and reproducible data across a range of multiplexing levels and quantification methods.

Learn more about Olink technology

PEA technology: olink.com/content/uploads/2021/09/olink-white-paper-pea-a-high-multiplex-immunoassay-technology-with-qpcr-or-ngs-readout-v1.0.pdf

Olink Explore 3072: olink.com/products-services/explore/

Olink Target 96: olink.com/products-services/target/#relative

Olink Target 48 Cytokine: olink.com/products-services/target/48-cytokine-panel/

Olink Flex: olink.com/products-services/flex/

Olink Focus: olink.com/products-services/custom-panels/

Olink NPX: olink.com/faq/what-is-npx/

Development and validation of custom biomarker panels: olink.com/content/uploads/2021/09/olink-development-and-validation-of-customized-pea-biomarker-panels-1083-v2.0.pdf

Full validation data information for Olink Explore and Target panels: olink.com/resources-support/document-download-center/

Contact us

For further information about Olink proteomic platforms, email us at info@olink.com. To find a service provider near you, go to olink.com/products-services/sample-analysis-service/external-service-providers/.

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