White paper

What is a good biomarker?

Introduction

Biomarkers such as ribonucleic acid (RNA), deoxyribonucleic acid (DNA), proteins, lipids and other biomolecules are playing an increasingly vital role in advancing our understanding of health, driving drug development and improving clinical outcomes.

Among these, protein biomarkers are particularly significant, as they regulate many of the body's biological functions and act as targets for most existing drugs, positioning them as central players in the advancement of personalized medicine.

The growing reliance on protein biomarkers highlights the need for a robust development pipeline to ensure reliable protein measurements capturing true biological signal.

In this White Paper, we will review:

- The invaluable and increasing role of protein biomarkers in clinical research.
- Critical steps and considerations in the biomarker development pipeline.
- How Olink's Proximity Extension Assay overcomes common challenges associated with multiplex immunoassays.

Why do we need biomarkers?

Biomarkers are essential for capturing objective insights into the dynamics of human biology. They are measurable indicators of specific biological states or conditions and can thereby provide us with a better understanding of disease pathophysiology by:

- Enabling early detection of disease, as changes in biomarker levels often occur before clinical symptoms.
- Improving diagnostics through providing objective measurements.
- Guiding the selection of the right treatment and monitoring treatment response.
- Providing a basis for developing new therapeutics, both by enabling insights into affected pathways and identifying new druggable targets (1).

Why proteins?

The concept of biomarkers started gaining considerable attention with the mapping of the entire human genome in the late 20th century, enabling researchers to identify genetic variants as biomarkers for various diseases. Genomic biomarkers have been especially prominent in the oncology field, where specific mutations have been linked to certain cancers (e.g. BRCA1 and BRCA2 mutations are associated with a higher risk of breast and ovarian cancer). These discoveries paved the way for the development of targeted therapies and started the shift toward personalized medicine.

Although genomic biomarkers provide valuable pathological insights, they do not reflect the true dynamic nature of disease initiation and progression. RNA-based approaches offer dynamic insights into gene expression, however, this does not always directly correlate to functional relevance due to a lack of a strong correlation between mRNA and protein levels.

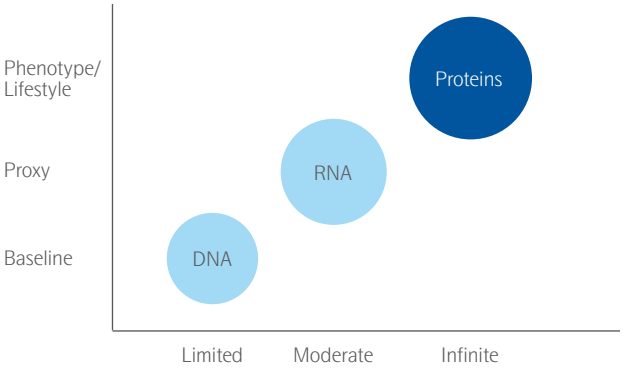

Figure 1. Correlation of analytes to real-time biology.

Protein biomarkers are vital for studying real-time human biology as they:

- Provide a direct measurement of biological activity and disease state, aiding diagnosis and patient monitoring.
- Reflect dynamic changes in the body, such as those caused by disease progression or treatment response, adding to their value in the drug development workflow.
- Undergo post-translational modifications affecting their function and activity, thereby serving as key indicators of disease states undetectable by genetic markers.
- Can be measured in easily accessible body fluids (plasma, urine, saliva), enabling less invasive and more practical clinical applications.

Today, there is a growing trend towards integrating genomics and proteomics to enhance biomarker discovery and obtain a holistic view of complex diseases.

In its early phases, protein biomarker research relied on the use of individual markers. However, these single biomarkers often lack the specificity needed for understanding complex diseases involving multiple pathological processes. For example, while neurofilament light polypeptide (NF-L) plays a key role in neurodegenerative disorders such as Alzheimer's disease (AD) (2), it is also implicated in other, unrelated conditions, such as cardiovascular disease, depression, anorexia, and schizophrenia (3). Hence, despite its relevance to neurodegeneration, NF-L cannot be used as a single biomarker for diagnosis and monitoring disease progression.

To address this, a strategy beyond the use of single biomarkers is needed for gaining a deeper understanding of complex pathologies. Protein signatures have been widely shown to generate a more nuanced and comprehensive understanding of the disease state and treatment response. An example of this is presented in a recent study by del Campo M. and Teunissen C.E. et al. (4) which identified two multiplex biomarker panels consisting of 8 and 9 proteins that accurately differentiated AD patients from controls and other dementia types.

Multiplex protein signatures therefore have the potential to enhance diagnostic accuracy, reveal disease subtypes, and enable tailored treatments, offering deeper insights into disease progression and treatment response. Altogether, this advances the field toward precision medicine.

What are the characteristics of a good biomarker?

The close connection between protein biomarkers and human biology highlights the need for a systematic approach to choosing the right biomarker.

Essentially, a physiologically relevant biomarker should:

- Have a measurable dynamic range of expression levels. Changes in these expression levels should be significantly different between the groups measured and the significance must remain consistent when moving from larger sample numbers to smaller groups.
- Be easily and safely sampled and measure (e.g. plasma is preferred over CFS due to a less invasive sampling).
- Be detectable robust, and less susceptible to pre-analytical variation (with characterized effects caused by factors such as endogenous interference, daily rhythm, and storage conditions that can inform the development pipeline).
- Be reproducible and specific for the measured target and ideally consistent across populations, genders and ethnic groups (or with established variation between these groups).

These factors are carefully assessed during assay development, which will be covered in the next section.

What do we want from an assay?

The growing use and reliance on protein biomarkers in understanding real-time human biology underscores the need for a robust and efficient protein biomarker development pipeline.

Key considerations during assay development include:

- Ensuring that levels in biological samples are within the measuring range of the assay.
- Maintaining consistent sample dilution within the measuring range.
- Achieving high specificity in target binding.
- Minimizing factors that interfere with assay performance.
- Preventing cross-reactivity with other assays in multiplex formats.
- Delivering reproducible results and robust assay performance.

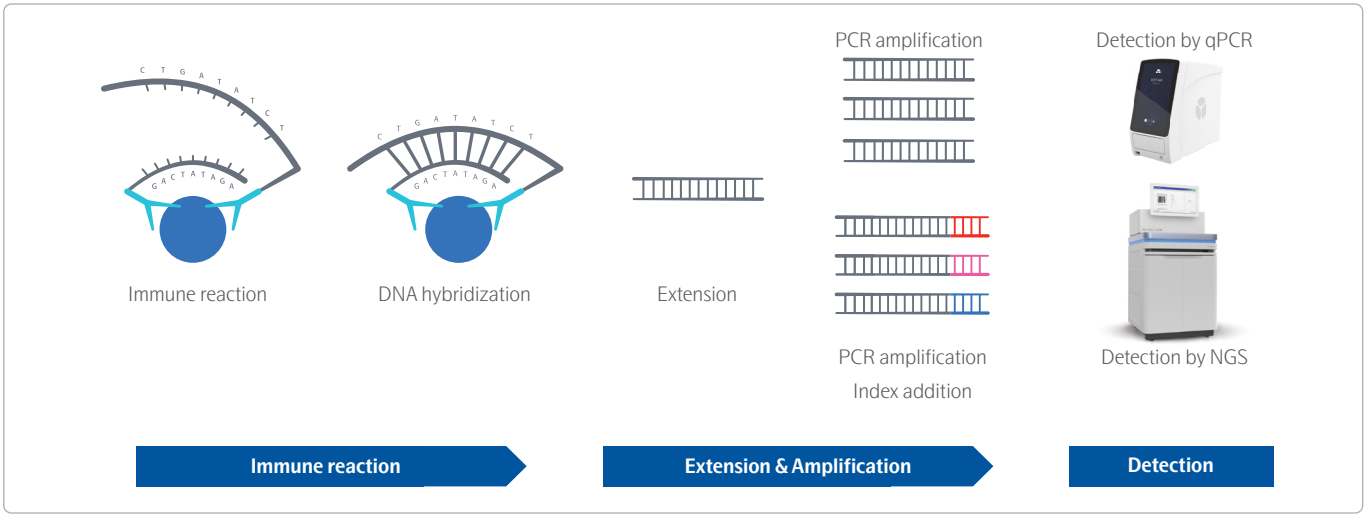

Figure 2. The main pre-read out steps in PEA™.

With the Proximity Extension Assay (PEA™) technology at its core, Olink's product portfolio is uniquely positioned to address these challenges and deliver a robust, scalable and highly sensitive multiplex analysis platform.

The dual-recognition, DNA-coupled readout, provided by PEA offers exceptional specificity even at high multiplexing levels. Two matched antibodies labelled with unique DNA oligonucleotides simultaneously bind to a target protein. In close proximity, their DNA oligos hybridize and are extended by DNA polymerase. This DNA "barcode" is unique for each protein and is quantitatively proportional to the initial concentration of the target protein. After PCR, the resulting DNA amplicon is quantified either by qPCR or NGS, depending on the specific Olink product used (Figure 2). The exponential amplification properties of PCR achieve a strong readout signal, providing exceptional assay sensitivity while requiring minimal sample volume (1-4 µL, depending on Olink panel used).

The protein biomarker development pipeline – key steps and considerations

The assay development process starts with the selection of the target (including identifying the part of the target) that the assay will be developed against. This selection is driven by the intended application, such as profiling the plasma proteome, detecting inflammation markers or drug targets, and other desired focus areas. Antigens are then designed according to the selected target, and antibodies are developed (Figure 3).

To maximize antibody-antigen affinity and reduce nonspecific binding, factors such as homologous proteins, cleavage products, and monomer or heteromer formations are considered throughout the development pipeline.

Following design and production, all Olink assays undergo a rigorous three step, 15-factor analytical verification process (Figure 4). Factors such as specificity, sensitivity, detectability and dynamic range are repeatedly assessed throughout the different stages of the assay screening and verification process.

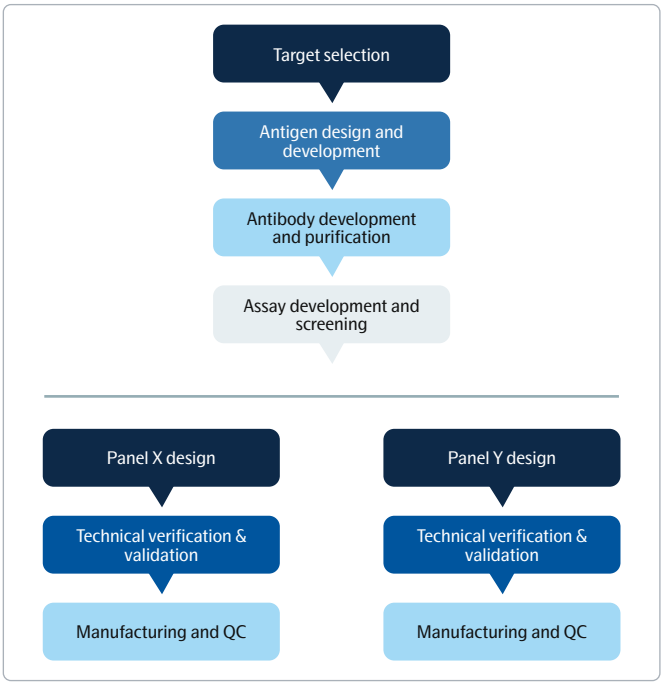

Figure 3. The critical steps of assay design, development and screening which precede downstream product development.

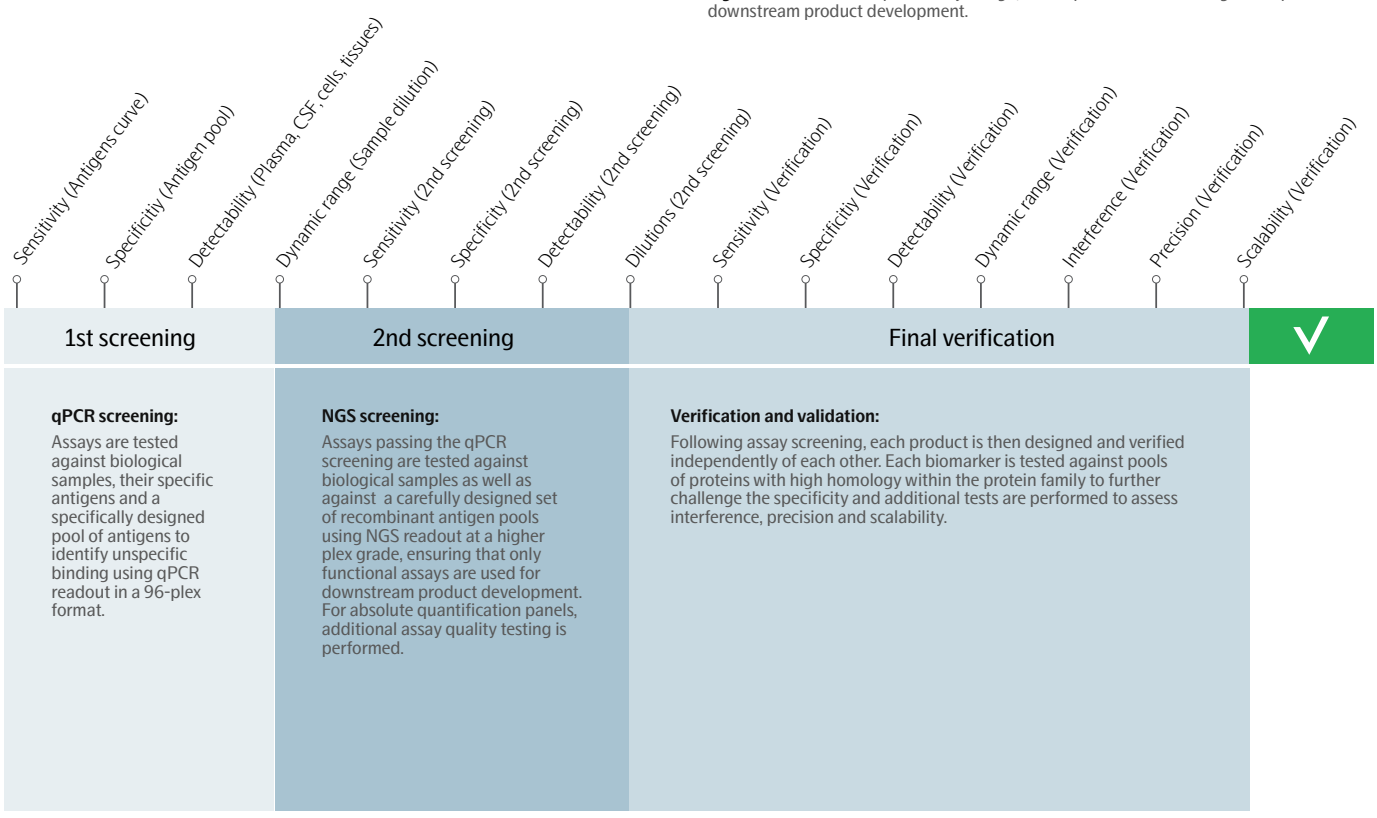

Figure 3. The critical steps of assay design, development and screening which precede downstream product development.

Figure 5 shows an example of a well-performing assay in the second phase of screening with NGS. The antigen curve shows the dynamic range of the assay, while the specificity pools show a lack of cross reactivity. To ensure that true biological signal is captured, the assay is tested against biological samples from various disease conditions and healthy controls. Plasma and serum are used, however, other sample types, such as CSF, cell and tissue lysates, may be tested as well. Samples are run at different dilutions to determine the optimal dilution of the assay and ensure that all samples dilute consistently in the measuring range. As some assays may be sensitive to contaminants, contamination controls (e.g. samples containing skin or saliva) are also included in the run. If the assay shows deviating sample dilution patterns or cross-reactivity with unrelated antigens, it fails the NGS screening.

After the assays have passed the screening and are included in the panel, additional factors are assessed in the final product verification, such as interference testing and assessment of precision (intra- and inter-CVs) and scalability. Validation data for all Olink panels is available for download on the Olink website.

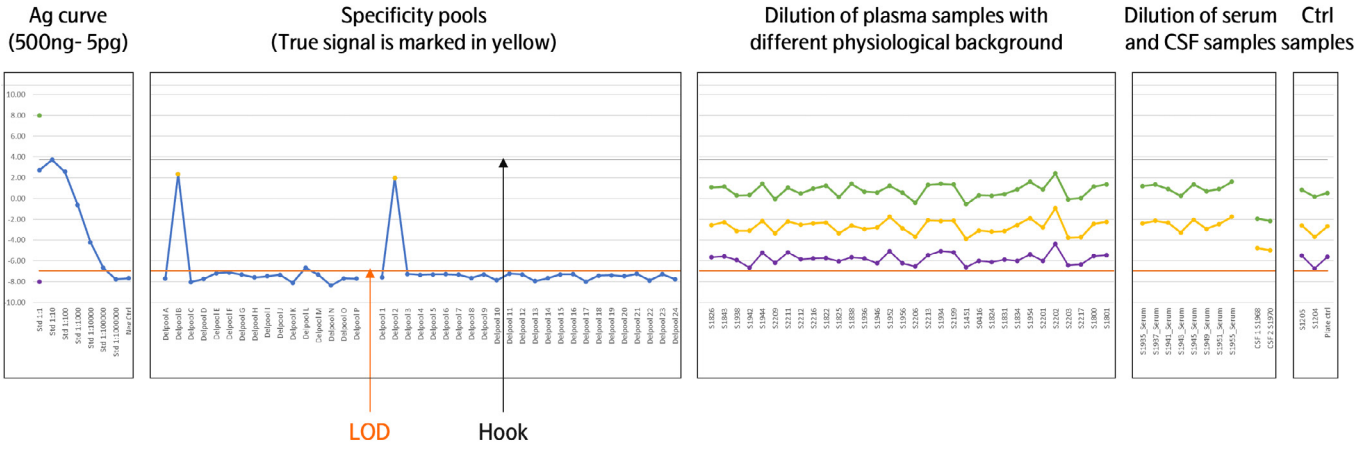

Figure 5. Assay screening: Example of a well performing assay.

How is assay specificity tested?

As mentioned, assays are developed with a strong emphasis on specificity and reducing non-specific binding, which is rigorously tested throughout the development pipeline. The cornerstone of Olink assay specificity is the dual antibody recognition and high-fidelity DNA-coupled measurement in the PEA protocol. As explained, for each protein target, two oligonucleotide-coupled antibodies (PEA probes) must bind in close enough proximity to enable the oligos to hybridize and form a unique DNA template for detection. This overcomes the problems normally associated with multiplexed immunoassays, since any potential antibody cross-reactivity will not contribute to a detection signal.

The addition of blocking reagents to the incubation solution in the immunoreaction step of the PEA protocol further minimizes unspecific binding. The blocking capacity is tested in a manufacturing QC step which includes 4 assays with mis-matched antibodies tagged with matched DNA probes. As a result, these antibodies can only get into proximity due to unspecific binding. In this reaction, high signals are detected if there is no blocking reagent in the solution. However, signals are eliminated when the blocking reagent is added to the incubation solution (Table 1).

Table 1. Incubation Solution QC. Testing for blocking capacity.

	Assay 1	Assay 2	Assay 3	Assay 4	
No blocking	8,1	11,7	10,4	12,6	
Lot 1	-0,3	-0,3	-0,1	-0,2	✅
Lot 2	-0,2	-0,5	0,0	0,2	✅

To assess potential crosstalk in the multiplex format and ensure that each assay in the panel is specific for its desired target, each assay is tested against pools that contain antigens for all assays of the panel. For example, during the product development of Olink Target 96 panels, the specificity was tested by creating a test sample consisting of a pool of antigens, which was then incubated with all 92 antibody probe pairs from the panel (5). Only if there was a correct match would a reporter sequence be created and serve as a template for subsequent real-time qPCR. Ten sub-pools of antigen were evaluated to cover the 92 assays in the Olink panel (Figure 6).None showed significant signal from the proteins tested.

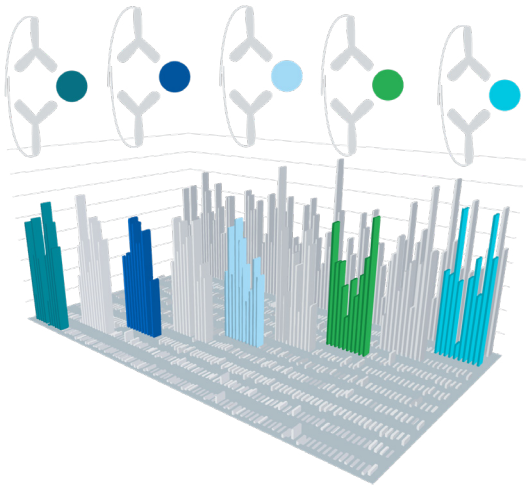

Figure 6. Each assay is exposed to samples containing either subsets or all of the selected antigens. A given assay should only generate a signal when the corresponding antigen is included in the sample.

Readout of cross-reactive events due to the presence of homologous proteins is another common problem for multiplex immunoassays. To address this during Olink product development, a set of highly related proteins were used to search for cross-reactive recognition and to further challenge specificity (6). Despite testing the most closely related proteins, cross-reactivity was not observed (Table 2).

Table 2. No readout of cross-reactivity.

Analyte	Related protein	Coverage (%)	Identity (%)	Cross-reactivity (%)
FAPB4	FABP9	99	64	0.0
FR-alpha	FR-beta	87	77	0.1
KLK11	KLK8	90	94	0.0
EN-RAGE (S100A12)	S100P	98	45	0.0
CDH3	CDH1	97	54	0.0
CDH3	CDH2	87	46	0.0
CDH3	CDH3	87	45	0.0
MK	PTN	71	51	0.0
Dkk-4	Dkk-3	90	27	0.0

Evaluating assays for scalability

Another factor that is evaluated during Olink product development is the effect of different plex grades on assay performance. In the example below, assays were run in a 1-plex and 96-plex format with qPCR readout and show high correlation (Figure 7). Similarly, assays run in a 21-plex format with qPCR were correlated with assays run in a 5400-plex format with NGS readout, with a correlation factor of 0.99 (Figure 8).

This unique scalability positions the Olink platform as an ideal solution for studies spanning discovery to clinical translation, with uncompromised data quality regardless of plex grade. This accelerates biomarker research by avoiding the complications of the "technology gap" usually encountered when using multiple methodologies at different stages.

Ensuring robust performance and reproducible results

Achieving robust assay performance and ensuring that reproducible data is generated is an essential part of the protein biomarker development pipeline. A number of technical factors can affect data acquisition and analysis in multiplex immunoassays. Hence, a selection of appropriate quality controls must also be addressed. Olink has developed a built-in quality control (QC) system using internal and external controls for its multiplex biomarker panels, allowing control over the technical performance of assays and samples.

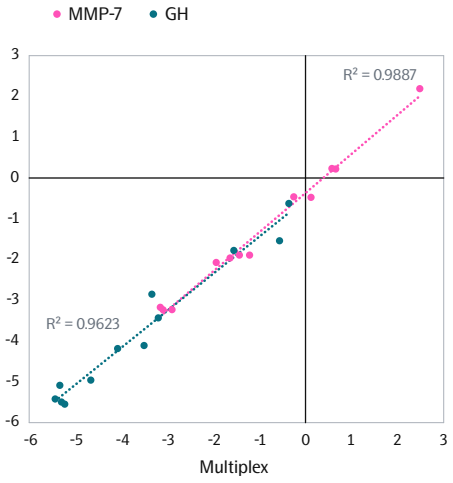

Figure 7. qPCR 1-plex vs 96-plex

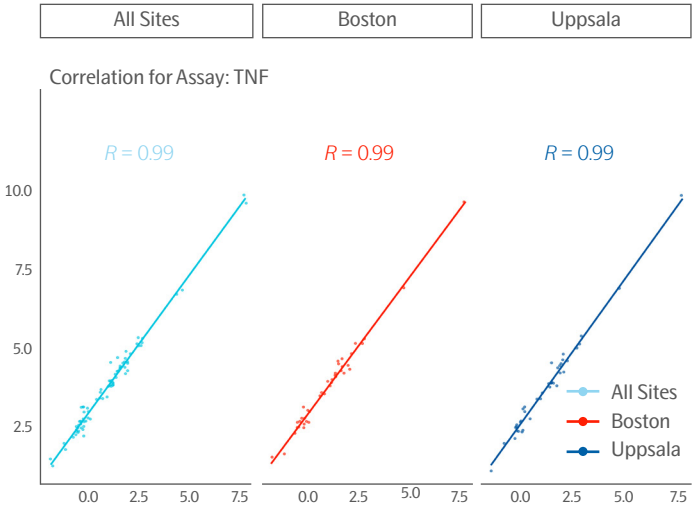

Figure 8. qPCR 21-plex vs NGS 5400-plex

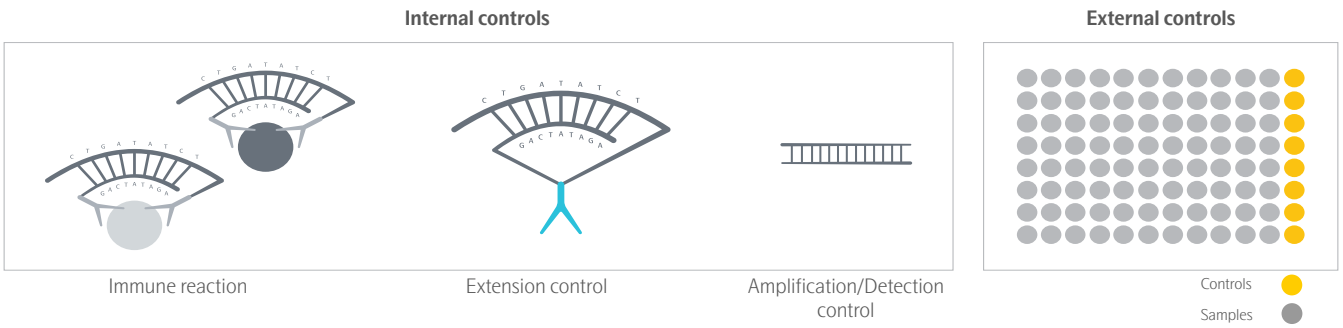

Figure 9. Olink's unique system of internal and external controls. The specific positioning of controls in the assay plate may vary according to the type of panel being run.

The internal controls are spiked into every sample and are designed to monitor the three steps of the Olink protocol (immuno, extension and detection controls). Additionally, each sample plate contains a designated row of external controls. In a 96-plex format, these include sample controls, negative controls and inter-plate controls. For panels with absolute quantification, a calibrator sample is added (Figure 9).

Assay development will not solve all research questions

Apart from resolving assay development challenges, several additional factors are essential to ensure a study yields meaningful biological insights and supports broader clinical applications. This includes clearly defining the research question and performing a thorough study design by addressing whether the study is exploratory or targeted, what the parameters of highest importance are, as well as what the long-term goal of the study is. A critical step in the study design is to consider the biological variability in the expression levels of the protein biomarker of interest, as this will impact the number of samples needed for the study to have sufficient statistical power (7). Finally, selecting appropriate statistical methods and conducting accurate, robust data analysis and interpretation are critical for drawing reliable and meaningful conclusions.

Olink's Scientific Support and Data Science teams can offer customized support with study planning and statistical analysis to help maximize the value and information output from your studies run using Olink panels. Get in touch with our Support teams at olink.com/support

PEA™ has overcome technological challenges in proteomics

By relying on the dual antibody recognition and hi-fidelity DNA-coupled measurement, PEA provides exceptional readout specificity. In addition, the signal amplification using PCR allows for high sensitivity and a broad dynamic range spanning fg/mL-mg/mL.

Olink's product portfolio offers an end-to-end solution for biomarker research, from exploratory studies to clinical translation by allowing for simultaneous measurement of thousands to a single digit number of analytes with a single, uniquely scalable platform. It includes a selection of pre-configured and customizable panels, while offering a simple, wash-free workflow and the lowest sample volume requirement among multiplex immunoassay platforms.

- Olink® Explore HT
- Olink® Target 96
- Olink® Target 48
- Olink® Target 48 Mouse Cytokine
- Olink® Flex
- Olink® Focus
- Olink® Signature Q100

In addition, Olink has developed tools and resources to support the entire biomarker journey, including:

- A set of tools that facilitate both study design and statistical analysis (Olink Analyze and Shiny apps), which are freely available to anyone using the PEA technology. Our Scientific Support and Data Science team can offer guidance and support with study design and customized statistical analysis.
- Olink® Insight, a knowledge platform that empowers users to understand and utilize the power of proteomics – from study design to data analysis.
- Olink ® Concordance Test, a product that enables you to demonstrate adherence to Olink Analysis Service standards, thereby ensuring the highest level of data accuracy and reliability.

Get access to PEA™

Start leveraging the PEA technology by selecting an option that works best for you:

- Run your samples at Olink Analysis Service
- Find an Olink Certified Service Provider
- Set up Olink in your own lab

Contact us at info@olink.com so we can guide you through your choice.

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