Olink®

Setting New Quality Standards for Multiplex Immunoassays

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Chapter 1
Multiplexing: Challenges and Opportunities

Immunoassays in Clinical Research

Biomarkers are fundamental to accelerate clinical research and drug development. They broadly fall into two categories: those linked to disease (such as susceptibility, diagnostic, prognostic or disease activity biomarkers) and those associated with drug action (such as predictive, pharmacodynamic, safety or efficacy biomarkers). Among these, proteins are especially valuable due to their central role in all biological functions and direct involvement in disease mechanisms.

The importance of proteins as biomarkers highlights the need for methods that can accurately and precisely quantify them. Immunoassays are commonly used for their accessibility and versatility, ranging from traditional methods like enzyme-linked immunosorbent assay (ELISA) to more advanced technologies. They have known drawbacks, however, including shortcomings on dynamic range, analytical sensitivity, and target specificity. Despite these challenges, immunoassay methods are constantly evolving, with ongoing efforts to standardize and improve quality, so that a complete realization of the great potential of protein biomarkers in clinical research can be achieved.

Advantages of a multi-analyte approach

There are limitations associated with traditional immunoassays that hinder progression from single biomarkers to biomarker signatures consisting of multiple analytes. Several innovations in multiplex immunoassay formats are fortunately changing this landscape. Measuring multiple analytes from a single sample simplifies the identification, validation and implementation of protein signatures. A biomarker signature not only offers a more holistic approach to understanding diseases, but also potentially increases discrimination power of statistical models. Additionally, multiplex immunoassays offer practical benefits, such as a reduction in assay costs, time to results, and sample volume requirements.
Diseases are often the result of a complex network of biological changes, not just a single pathological process. Consequently, protein signatures provide a more nuanced and comprehensive understanding of the disease state and treatment response.

Technological advances that enable multiplexing

In recent years, we have witnessed a series of technological advancements that progressively unlocked the potential of multiplex protein analysis. Refer to the timeline below for key events. Among critical developments are the creation of planar (electro)chemiluminescence and bead-based immunocapture platforms. These technologies have been extensively applied and have successfully demonstrated the value of multiplexing to more efficiently identify protein signatures with biological and clinical relevance. However, with time it became evident that there are still technical challenges to be addressed to make multiplexing a tool that researchers can confidently rely on.

Innovations in immunoassay multiplexing

1980 – 1990's

Flourescent-based
Light-absorbing reactions to quantify protein concentrations. Small degree of multiplexing can be achieved by using distinct colors of flourescence for different proteins.

1990's

Chemiluminescent-based
Light-producing reactions to quantify protein concentrations. Some degree of multiplexing can be achieved by using distinct chemiluminescent tags or physical separation of reaction.

2000's

Bead-based
Color-coded microspheres to allow for quantification of hundreds of distinct proteins in a single sample.

Electrochemiluminescence-based
Electric current to stimulate a light-producing reaction that is proportional to the protein concentration. Multiplexing of up to ten proteins can be achieved by using patterned arrays where each spot on the array is coated with a different capture antibody.

2010's

Microfluidics cartridge-based
Microfluidic devices to separate and detect multiple proteins in a single sample. Degree of multiplexing is lower than bead-based assays.

Proximity Extension Assay (PEA)
Pairs of antibodies labeled with DNA oligonucleotides, which upon target binding and hybridization form a new DNA molecule by proximity extension. The resulting DNA barcode is then quantified using qPCR or NGS, allowing for highly multiplexed and sensitive protein measurements.

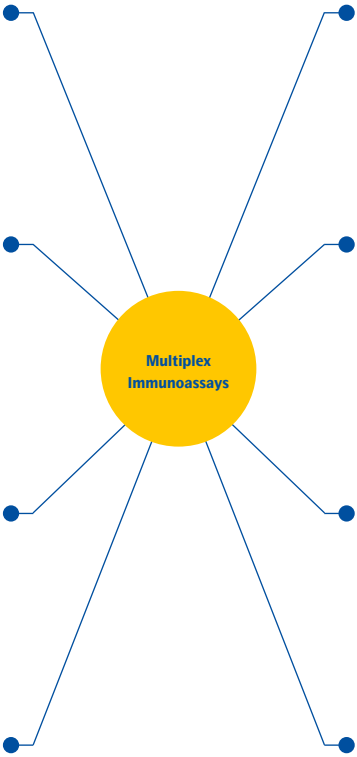
Common Challenges in Current Methods

Cross-reactivity
Non-specific binding of antibodies to the wrong antigen is more likely to happen with increasing antibody pairs present in the assay. Cross-reactivity impairs assay specificity and can give false-positive results.

Signal interference
Fluorescence-based readouts may have overlapping emission spectra that interfere with results. Light-based readouts may have light bleeding to adjacent spots and wells.

Low sensitivity
It is very difficult to maintain high sensitivity with increasing plex grade in an assay. Higher cross-reactivity and signal interference cause background noise, deteriorating ability to distinguish low abundance proteins from background.

High variability
When scaling up, issues like poor specificity, low sensitivity and signal interference increase. These may cause results to vary from run to run and deteriorate reproducibility of results.



Limited dynamic range
Immunoassay panels need to measure target analytes with compatible abundance. If not, dilution of the samples for high-concentration analytes lead to undetectability of low-level analytes. Poor sensitivity of multiplex assays with higher plex grade also limits the dynamic range.

Lack of quality controls
Quality control materials are traditionally not well developed for multiplex immunoassays. Ensuring the accuracy, reliability, and reproducibility of multiplex immunoassays requires stringent quality control measures at various stages of the assay process.

Complex data analysis
Multiplex immunoassays produce complex datasets that require advanced software capabilities for accurate interpretation of data and for close monitoring of data quality.

Laborious validation
More steps in the analytical validation are required to ensure that quantification of each analyte in the presence of all other analytes is accurate.

Challenges associated with multiplexing immunoassays

Although each multiplex immunoassay platform has its unique strengths and limitations, there are some common challenges that cause users to be unable or reluctant to implement panels with higher plex grade. Refer to the image above for details.

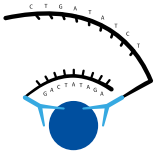
While the illustrated challenges in multiplex protein biomarker detection seem daunting, they can be overcome. In fact, the next generation of multiplex immunoassays address these very issues. The new solutions aim to bring high confidence to multiplex analysis by increasing specificity and sensitivity, expanding the dynamic range, and simplifying the processes of quality control, data normalization and analysis. In the following section, we will explore the last innovation mentioned in our timeline of multiplex advances: the proximity extension assay (PEA) technology.

Proximity extension assay (PEA) technology

PEA is an innovative method based on a dual-recognition immunoassay, where two matched antibodies labelled with unique DNA oligonucleotides simultaneously bind to the target analyte in solution. When the two antibodies are brought into proximity their complementary DNA oligonucleotide hybridize, serving as template for a DNA polymerase-dependent extension step. This creates a double-stranded DNA barcode which is unique for the specific antigen and quantitatively proportional to the initial concentration of target protein. PEA technology combines antibody- and DNA-based methodologies to provide a unique immunoassay that delivers unwavering performance regardless of the number of targets that are being multiplexed.

Pre-Readout steps in PEA

A

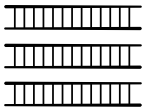


Binding of antibody pairs, which are labeled with complementary DNA oligonucleotides, to the target antigen in the solution.

B

Oligonucleotides that come into proximity are hybridized and extended with a DNA polymerase.

C



The newly created DNA barcode is then amplified through PCR, making it ready for readout through NGS or qPCR methods.

A differentiator of PEA is the ability to extensively control and monitor all steps of the reaction in each individual sample, bringing an unprecedented degree of confidence to multiplexing as depicted above. The simple yet sophisticated PEA innovation with integrated controls addresses the main challenges of multiplexing, as illustrated in the image "Addressing challenges" below

In addition, qPCR readout permits data to be extracted from extremely small sample volumes, down to 1 µL, which is ideal for precious samples that are limited or difficult to extract. It is also highly suitable for preclinical mouse model research, enabling efficient and ethical longitudinal studies with potentially reduced animal use.

White Paper

Read an in-depth exploration of the basis of PEA technology and its capabilities in the White Paper, *PEA – a high-multiplex immunoassay technology with qPCR or NGS readout.*

View article here

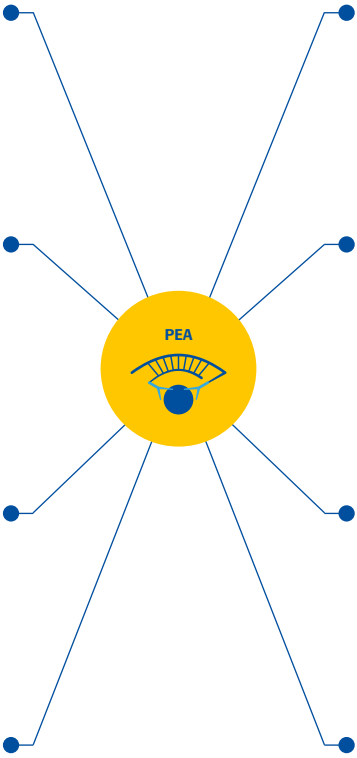
Addressing Challenges

Cross-reactivity
Signal readout only occurs if two antibodies have bound to the target analyte and their oligos are complementary to each other. This second layer of recognition, in addition to highly specific antibody pairs and highly optimized buffer components, eliminates readout of any potential cross-reactivity of individual antibodies to other targets.

Signal interference
DNA barcodes that form upon oligonucleotide extension are unique to each antibody pair and differ significantly from each other, excluding the possibility of erroneous cross-analyte reading.

Sensitivity
The exponential amplification properties of PCR achieve strong readout signal, providing high assay sensitivity no matter how many proteins are analyzed in parallel. Quantification of low abundance proteins using panels of various plex sizes becomes a reality.

Reproducibility
Triplicates of an external negative and interplate controls are included in each plate for normalization, allowing for optimal comparison of data derived from multiple runs. The use of automated microfluidics also contributes to exceptional repeatability and reproducibility.



Dynamic range
Broad dynamic range, covering up to 10 logs, depending on the panel used. Low abundance analytes can be measured in parallel to higher abundance analytes with no risk of signal saturation.

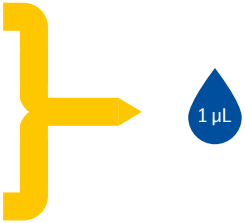
Quality controls
Specifically engineered internal controls are added to each sample and utilized to reduce intra-assay variability without the need for technical duplicates.

Data analysis
Dedicated data analysis software, designed to conduct data QC and convert qPCR data into either absolute protein concentration (pg/mL) or on relative quantification units. Tools to run statistical analyses such as creating sample distribution plots, PCA, and heatmaps, and running t-tests and ANOVA. Additional tools are available to increase study power and get insights into target pathways.

Assay validation
Transparent validation inspired by FDA guidelines is conducted. The analytical performace of the panels are rigourously vaildated for sensitivity, precision, and plex scalability.

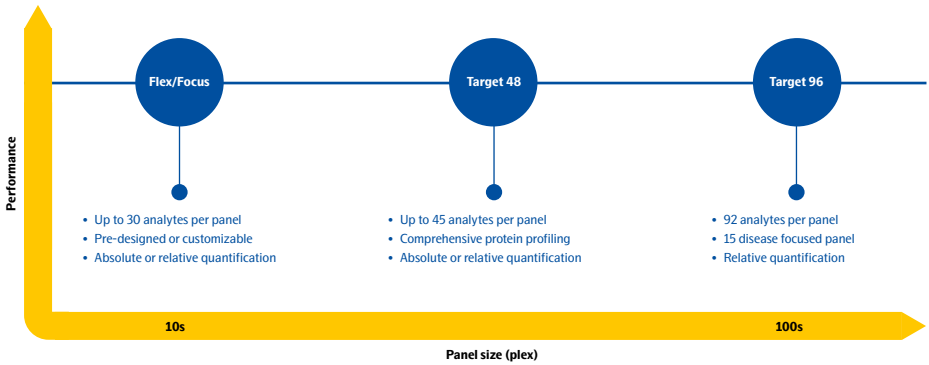
While PEA allows for large-scale high-plex studies with an NGS readout, the purpose of this eBook is to provide in-depth considerations on how to run routine PEA-based immunoassay panels in-house, independently of NGS capabilities. We will therefore focus on the PEA assays with qPCR readout that entails the use of an instrument called Olink® Signature Q100.

- Human plasma & serum
- Cerebrospinal fluid (CSF)
- Urine
- Tissue lysate
- Intestinal fluid / microdialysis
- Ocular fluids
- Saliva
- Synovial fluid
- Fine needle biopsies
- Mouse samples
- Cell lysates
- Conditioned media
- Extracellular vesicles



As depicted in the image below, with the same benchtop system, users are able to run panels ranging from tens to hundreds of proteins in parallel with unwavering performance. The identification of a promising biomarker signature typically begins with broad discovery studies. Validation and implementation of the signature will rely on more focused, targeted panels. Although high importance on data quality is generally placed on the latter, a high-performing screening panel is crucial for identifying robust signatures. This is why maintaining assay sensitivity and specificity when scaling up the plex is a game-changer in the field of precision medicine.

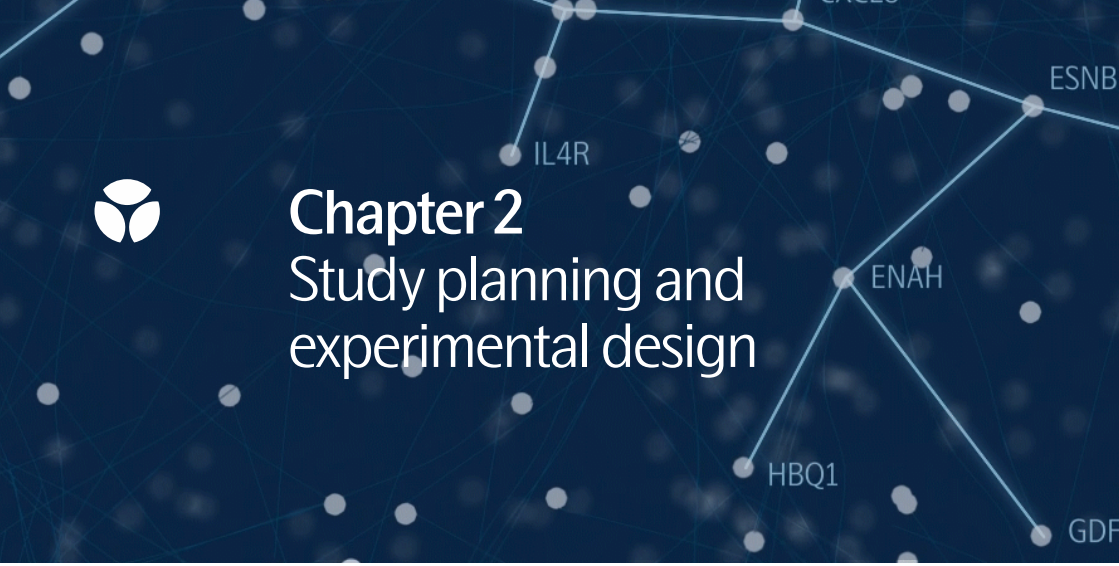
Signature Q100 benchtop and PEA panels with qPCR readout



In the next chapters this ebook will cover the essentials of planning and analyzing multiplex immunoassays, with an emphasis on the technical innovations of PEA. This includes all technical aspects of study planning and experimental design (power of the study, pre-analytical variability, data normalization), as well as an in-depth coverage of statistical analysis and data interpretation (QC, statistical pipeline and tools). In the last chapter, some prime examples of how biomarker signatures with potential clinical utility are identified and developed are detailed. ●

Key Take-aways

- Measuring several proteins in a multiplex panel is essential for a comprehensive disease understanding and for increasing statistical model discrimination while optimizing time, effort, and sample use.
- Innovations in multiplex immunoassays have enabled great advances in the development of biomarker signatures.
- Technical challenges are still present in most multiplex immunoassays. Issues like cross-reactivity and low sensitivity are particularly common in larger panels.
- The next generation PEA technology is designed to overcome major multiplexing challenges, ensuring high data quality across various panel sizes.



Chapter 2
Study planning and experimental design

2.1 Know your research question, select suitable controls, and power your study

A clearly defined research question and a thorough study planning process are imperative for generating reproducible data and gaining translatable biological insights for wider applications. This provides direction and focus throughout the study planning phase, particularly when assessing:

- experimental design
- sample collection and handling
- data collection and processing
- final statistical analysis
- limitations of the study

A critical part of the study design involves the selection of representative samples for the experimental group, as well as suitable controls. For a valid comparative analysis and meaningful results, subjects in both groups should be matched as closely as possible to reduce confounding factors, such as age, genetic diversity, sex, and underlying conditions. While the control group often consists of samples from healthy individuals, this is not always the best solution. For instance, in a study assessing potential biomarkers for secondary prevention of diseases, healthy controls are not a good comparison.

Another key factor to consider during this phase of planning is the selection of targets relevant to the research question. Biological variability in the expression levels of the protein of interest will impact the number of samples needed for the study to have sufficient statistical power.

UK Biobank

52,704 samples
1,461 biomarkers

China Kadoorie Biobank

2,024 samples
2,930 biomarkers

Arivale

300 samples
2,943 biomarkers

Streamlined biomarker selection

Olink® Insight is Olink's unique bioinformatics and biostatistical knowledge hub, intended to support every step of the biomarker research journey. One of its key features includes Normal Ranges for proteins, which incorporates data from three cohorts (55,000 people) across three continents and allows users to explore the biological variation of close to 3000 human protein assays.

This provides a tool for:

- **Guiding the selection of biomarkers,** by providing insights into the natural variation of protein expression levels and thus informing sample size/power calculations.
- **Validating findings,** by comparing the distribution of candidate biomarkers with the distribution observed in 'normal' cohorts.
- **Deeper biological insights**, by exploring how the expression of the proteins of interest varies depending on factors such as age, sex, genetic diversity, body mass index, and others.

For further assistance in target/analyte/biomarker selection, Olink Insight offers the **Pathway browser tool**, which enables researchers to use an open-source peer-reviewed database to explore the function, pathway and/or disease involvement of their proteins of interest. By using a list of genes, proteins, or even publications, this tool visualizes and illuminates the connections that matter, thereby informing biomarker selection and enhancing results interpretation.

Finally, Olink Insight's **Panel Builder** and **Panel Selection** tool allows users to either design their own panel or select one of our pre-existing products. To support translating a biological research question into a product recommendation, users can start their search from **Disease term** or **Biological process.**

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The power of a study is defined as the probability of correctly detecting a difference between the study groups, given that the effect is there. Along those lines, a power analysis will give an estimate on the number of samples that are needed, to ensure a high probability that a true difference will be observed.

A properly performed power analysis avoids:

- **An under-powered study,** which would lead to unreliable data and the inability to draw informative conclusions, thereby resulting in a waste of limited resources and ethical issues.
- **An overpowered study,** which aside from ethical concerns over using more subjects and resources than necessary, also raises statistical concerns. Power is substantially increased when numerous samples are analyzed, which will result in an exaggerated tendency to reject the null hypotheses, and to thereby observe differences that are not clinically relevant.

Ideally, a power analysis should be performed before collecting samples and running a study. However, power calculations can also be performed retrospectively, or when adding additional samples to the study is not an option. In this case, the calculation can tell us if we will have enough power to justify the study. Alternatively, retrospective calculations may be useful when designing a scaled-up study from a smaller pilot.

Need support with power analysis?

The Study Size Calculator on Olink Insight provides a web-based application to assist in power calculations for a balanced one-way ANOVA.

For more complex calculations and further statistical support, Olink's expert data science team can help you with customized statistical analysis to help ensure that you maximize the value and information output from your studies run using Olink panels.

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What is needed to perform a power analysis?

It is important to note that calculating power for a study is based on assumptions on the effect size – the magnitude of difference between the study groups. If there is no overlap between the data in the experimental group and the control group, there is a considerable difference. The effect size and the study power are therefore high, consequently requiring a smaller sample size. However, if the overlap is larger than the difference between the groups, the effect size is less significant, resulting in lower power, and a need for a larger sample size.

Effect size is calculated by dividing the difference of the means of the two groups with the estimated standard deviations for each group (Cohen's d). Researchers make educated guesses at these values, through literature review, by consulting Cohen's recommendations, or performing pilot studies. Due to the limitations of making such assumptions, studies are often designed to have 80% power.

Aside from effect size and standard deviation, power is directly related to sample size and the significance level. While it is possible to calculate or estimate one of these five variables if the others are kept constant, in reality, it is recommended to test several levels of the constant variables to see how the target variable (e.g. power or sample size) is affected. This provides a range of estimated numbers of subjects/samples needed to perform the study with enough power to draw reliable conclusions.

The significance level (often denoted α) is the probability of a false positive result. It is commonly set to 0.05 if one statistical test is carried out. However, in case of multiple testing, the significance level should be adjusted accordingly. This is commonly done by dividing the significance level with the number of tests which is called a Bonferroni correction.

White Paper

Read more about power analysis, including considerations for different types of clinical studies in the White Paper *Strategies for design of protein biomarker studies.*

View article here

2.2 Pre-analytical variability

Measuring proteins in biological samples can be affected by a number of pre-analytical variables, and thereby influence the data output and confidence with which conclusions are drawn. As mentioned, selecting appropriate references/controls through using strict matching criteria during sample collection is imperative to reduce the experimental variance and obtain meaningful data.

However, there is a set of factors related to sample collection, processing, and storage to be considered when measuring proteins in biological samples and interpreting results. While efforts to minimize the effects introduced by pre-analytical variability should aim to be consistent (through the development and implementation of standardized operating procedures), this is not always possible. This can be the case when multiple labs or platforms are involved or when biobank samples with uncertain history are used.

It is therefore important to document as much information as possible about the sample collection, handling, and storage process. Through this approach, it is possible to obtain meaningful results even with samples that have not been handled optimally.

Blood sample collection procedures

The rapid and non-invasive nature of blood sample collection, as well as the wealth of biological insights it can yield, makes serum and plasma sampling a common and attractive tool in protein biomarker research.

- Serum is the liquid part of blood after it has been allowed to coagulate fully for 30–60 minutes at room temperature. Blood is sampled in collection tubes with gels that separate the cells and the serum during centrifugation, when the clot is removed. Serum is therefore free of clotting proteins but contains the clotting metabolites that result from the coagulation process.
- Plasma is the liquid part of blood that has been treated with anti-coagulants (such as EDTA, heparin, or sodium citrate), after cells have been removed by centrifugation. While plasma is reflective of the blood as it circulates in the body as it has been prevented from clotting, anti-coagulants used for sampling can interfere with protein analysis.

Alternative sample types

Olink's panels are validated using EDTA plasma and serum samples. However, Olink assays have been demonstrated to perform well with a wide array of sample types (such as cerebrospinal fluid, dried blood spots, tumor biopsies, atherosclerotic plaques), requiring only minimal quantities of precious samples.

Our support team can provide detailed sample preparation guidelines for alternative matrice

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Temperature and duration of sample handling and storage

Proteins are sensitive to loss of structural integrity and activity due to proteolysis or aggregation; hence, the temperature and duration of sample handling and storage are major pre-analytical considerations.

Timely processing is critical as blood samples degrade rapidly – a pre-centrifugation delay in plasma sampling can have significant effects on the protein profile. However, Shein et al.[1] found that these effects are minimal within 8 hours of centrifugation delay if samples are handled at 4°C, but were seen already after just 1 hour if handled at room temperature (22°C). This type of sample mishandling can cause protein degradation, but also leakage of proteins from lysed blood cells (hemolysis), as longer delays have been shown to cause an increase in some plasma protein levels. The pre-centrifugation delay time should therefore be kept short and consistent between samples. As the effects of the protocol duration can also be highly protein-dependent, handling procedures should be pre-determined in an analyte-specific manner when possible.

Upon sample collection, storage in a single batch can control for variability in how the samples are handled. Long-term storage should be done at −80 °C, while freeze–thaw cycles should be minimized, as expression levels of certain proteins may be affected.

1. Shen et al., Strong impact on plasma protein profiles byprecentrifugation delay but not by repeated freeze-thaw cycles, as analyzed using multiplex proximity extension assays. Clinical Chemistry and Laboratory Medicine (2017).

Olink can help minimize pre-analytical variability

Olink's technical support can guide you on matters of pre-analytical variability. The more information you can provide regarding sample collection, preparation, and storage, the better the chances for our data scientists to identify potential problems to be able to normalize data between samples with different pre-analytical histories.

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2.3 Controlling for technical variation through experimental design considerations

Aside from pre-analytical variables concerning sample size, collection and processing, data acquisition and analysis in multiplex immunoassays can also be affected by a set of technical factors. Hence, a selection of appropriate quality controls must also be addressed. For many other multiplex immunoassays, validation steps are needed after the runs. To avoid this, Olink has developed a built-in quality control (QC) system using internal controls for its multiplex biomarker panels, allowing control over the technical performance of assays and samples. While this will be extensively covered in the next chapter on data normalization and statistical analysis, efforts to minimize technical variation need to be factored into the experimental design phase.

An important consideration for minimizing the risk of introducing technical bias in downstream analyses is an equal distribution of experimental variables (such as study

groups, treatment, sampling time points or demographics) across each sample plate prior to analysis. In other words, randomizing samples across each plate minimizes the possibility of missing true biological variation or misidentifying it as e.g. technical variations.

While the overall goal is to minimize the experimental variables across the study, different considerations will apply to different study types.

1. Small single plate study

For studies with one plate of samples or less, there are minimal experimental variables to account for. However, sample randomization will make the quality control process of the data more robust as technical variation can be more easily identified. By randomly assigning groups to wells, row and column group associations are removed. Thus, significant association with a row or column can be identified as technical variation, making QC process more robust.

2. Large/multi-plate study

This type of study typically means that analysis is performed at a single timepoint across multiple plates. The large number of samples allows for measuring small effect sizes and thus a higher statistical power of the study. However, an unequal distribution of the samples across plates can remove true biological variation following normalization. When samples are randomly assigned to plates, the plates can be considered equal and become comparable with normalization for technical variables.

How to randomize samples

The simplest way to randomize samples is to assign random numbers to them, then sort them and place them accordingly in your plate, or sort your tubes in the given order. Important to note is that randomization should be evaluated so that by chance some experimental variables were not placed on e.g. one sample plate.

The Olink Analyze R package available on Olink Insight includes a module for sample randomization. However, if you cannot randomize your samples yourself, Olink Analysis Service can help with this.

3. Continuous, multi-batch study

In a multi-batch study, variables relating to a study group are assessed with continuous or repeated monitoring. For instance, a study can start with single plate exploratory analysis, after which the acquired data can be used to drive a more extended analysis with more sample plates at a further timepoint. Key factors regarding the reproducibility of separate experimental runs within the same study should be considered, including:

- A change of reagent batch, which may introduce bias between samples run at different times.
- Biases or changed laboratory conditions, which may be introduced between timepoints in the longitudinal study.

It is therefore necessary to include bridging samples that are run at every batch of the study, if the data sets need to be merged. The protein levels measured for these bridging samples can be used as references between batches, to normalize and alleviate any potential bias.

4. Longitudinal study with multiple time-points

The primary objective of a longitudinal study is to compare and determine the changes in protein expression at different time points for the same subject. Additionally, single time points between different groups can be compared.

Therefore, all timepoints for one subject should preferably be plated on the same plate, to minimize technical variation, and samples should be randomized within and between plates. ●

How does Olink control for variation in multi-batch studies?

In the production of new reagent kits, Olink has QC processes to limit variation between batches, and to ensure a consistent performance. This is important since a new antibody batch could cause a shift in the data generated. To address this potential risk, Olink has introduced a thorough QC procedure with strict acceptance criteria. However, when kits are used in multi-batch studies, potential signal differences across multiple batches must be considered and adjusted for in the statistical analysis.

As Olink uses relative measurement in its assays (while also offering absolute quantification in lower-plex panels, to be discussed in the next chapter) the addition of bridging samples is an additional normalization step needed to allow the data to be comparable.

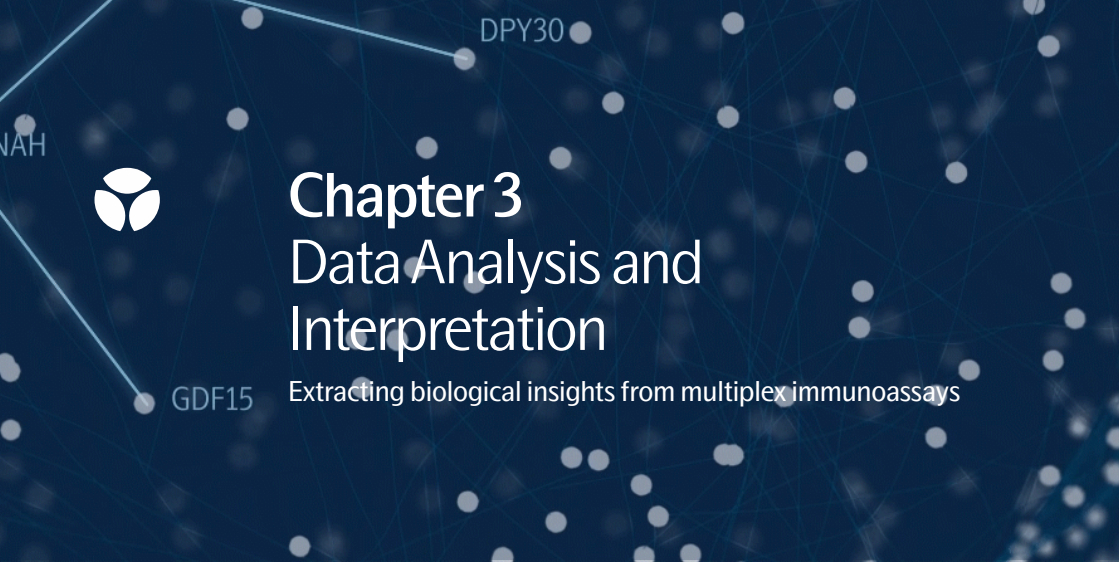
How to select bridging samples?

Olink recommends selecting a minimum of 8–16 bridging samples, while keeping in mind:

- the sufficient sample volume for bridging the entire study, and
- selected samples should represent the data set cases and controls.

The Olink Analyze R package can assist with selecting bridging samples based on the reference data, thereby identifying samples which passed QC and have high detectability, covering the dynamic range of the assays.

Discuss the full study design with an Olink representative to ensure that the appropriate numbers of kits and bridging samples are included.



Chapter 3
Data Analysis and Interpretation

Extracting biological insights from multiplex immunoassays

3.1. Data analysis in multiplex immunoassays

As the primary goal of running multiplex immunoassays is to identify and validate protein biomarkers for deeper biological insights and clinical applications, it is essential that the data analysis and interpretation step is executed with a high level of accuracy and robustness.

Both absolute and relative quantification are employed throughout the protein biomarker research pipeline, as protein levels are compared across different study groups, which are frequently based on health status (e.g., diseased and healthy) or treatment type (e.g., treated and untreated).

PEA technology enables absolute (Olink Focus, Olink Flex, Olink Target 48) and relative quantification (Olink Focus, Olink Flex, Olink Target 48, Olink Target 96) thereby providing an end-to-end solution for biomarker research.

Multiplex immunoassays can generate a large amount of data – the management, quality control, analysis and interpretation of the data requires an optimized software and set of tools that can ensure high data consistency and a robust data analysis process. To streamline data processing when running panels with a qPCR read-out, Olink has developed the Olink NPX Signature software. Furthermore, to support data analysis and interpretation, Olink offers a range of free online resources available on Olink Insight, as well as fee-for-service biostatistical support.

As mentioned in the previous chapter, a set of technical factors in a multiplex immunoassay run can also impact data analysis. To account for this, Olink uses a built-in QC system that monitors the performance of assays and samples, followed by appropriate normalization that alleviates systematic noise caused by sample processing or technical variation

3.2. Olink's built-in QC system

3.2.1. Internal controls

The QC system consists of four internal controls that are spiked into every sample and are designed to monitor the three steps of the Olink protocol (Figure 1):

- **Incubation control:** The Incubation control (a non-human antigen) monitors all three laboratory steps starting with the immuno reaction.
- **Extension control:** The Extension Control (an antibody linked to two matched oligonucleotides for immediate proximity that is independent of antigen binding) monitors the extension and readout steps and is used for data normalization across samples.
- **Detection control:** The Detection control (a synthetic double-stranded template that does not require any proximity binding or extension to generate a signal) monitors the readout step.

Samples that deviate from a pre-determined range for one or more of the internal control values will result in a QC warning in the Data analysis software and results file.

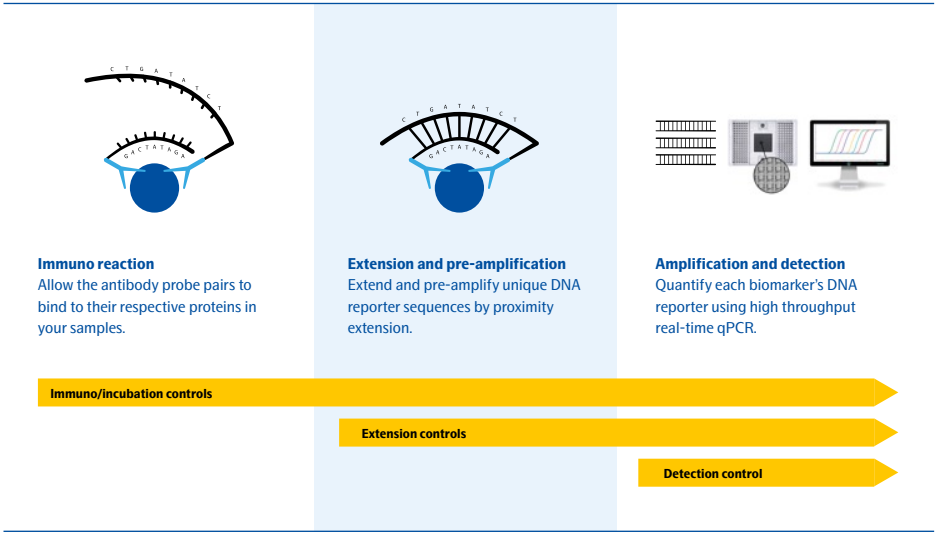


Figure 1. The internal controls enable monitoring of the three core steps in the Olink assay and are used for quality control and data normalization. Readout is performed by using Olink® Signature Q100.

3.2.2. External controls

Each sample plate contains eight control samples (Figure 2). For panels with absolute quantification, this includes:

- **Sample control** – added in triplicates and used to monitor and control the quality of reported output data by evaluating both accuracy and intra assay precision for all assays.
- **Negative control** – added in duplicates and consists of buffer run as a normal sample. These are used to monitor any background noise generated when DNA-tags come in close proximity without prior binding to the appropriate protein.
- **Calibrator** – added in triplicates, allowing for calculation of standard concentration units and is used in a second normalization step. It is designed to improve inter assay precision, enabling optimal comparison of data derived from multiple runs.

Both the Sample Control and the Calibrator are composed of pooled plasma from healthy donors spiked with recombinant proteins known to have low endogenous levels in normal plasma.

For panels with relative quantification only, an inter-plate control (IPC) is included in triplicate on each plate, and these are run as normal samples. The IPC is a pool of 92 antibodies, each with one pair of unique DNA-tags positioned in fixed proximity and can be seen as a synthetic sample, expected to give a high signal for all assays. The median of the IPC triplicates is used to normalize each assay, to compensate for potential variation between runs and plates.

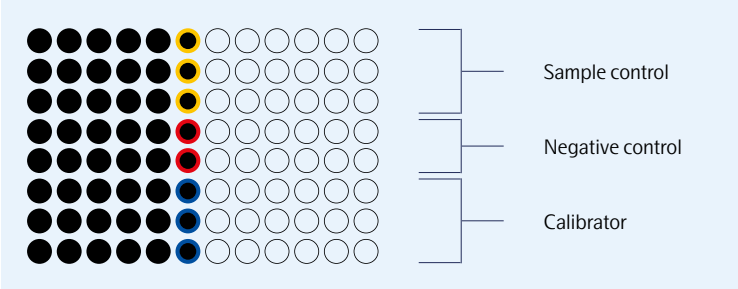


Figure 2. Sample plate layout with external controls. The specific positioning of controls in the assay plate may vary according to the type of panel being run.

3.3. Data normalization

Olink's PEA technique uses real-time quantitative PCR (qPCR) in the readout step to measure relative changes in protein expression. The qPCR detects the unique DNA sequence formed when complementary oligonucleotide-tags attached to pairs of analyte-specific antibodies hybridize and extend in the presence of DNA polymerase.

Olink translates the Ct values from the qPCR into an arbitrary relative quantification unit (NPX) using a series of computations. These operations are designed to minimize technical variation and improve interpretability of the results.

In qPCR, the x-axis value of the point where the reaction curve intersects the threshold line is called the Ct, or "threshold cycle." This indicates the number of cycles needed for the signal to surpass the fluorescent signal threshold line. NPX is derived from the Ct values obtained from the qPCR using the following equations:

Extension Control:

$$Ct_{Analyte} - Ct_{Extension\ Control} = dCt_{Analyte}$$

Inter-plate Control:

$$Ct_{Analyte} - dCt_{Inter\text{-}plate\ Control} = ddCt_{Analyte}$$

Adjustment against a correction factor:

$$Correction\ factor - ddCt_{Analyte} = NPX_{Analyte}$$

- ✔ If you know how to perform other expression data analysis, you can apply the same method to NPX data.
- ✔ NPX is relative, but on a concrete quantitative scale. For a given assay, 1 NPX increase is approx. 2x increase in protein concentration.
- ✔ Even if two different proteins have the same NPX values, their absolute concentrations may differ. NPX should be compared for each assay separately between samples within a run.

Study design-dependent normalization

Olink recommends one of two normalization methods depending on the study design.

1. For randomized studies, IPC normalization should be replaced with intensity normalization, which will increase statistical power and reduce technical variation between plates and projects.
2. For non-randomized studies, Olink recommends reference sample normalization. Running reference samples on all plates is a good strategy to minimize technical variation. When applied correctly, both intensity normalization and reference sample normalization can increase the power in a given study by reducing technical variation, since they are based on real samples in contrast to the IPC samples.

Read more about Olink's built in QC system and data normalization methods in the White Paper *Data normalization and standardization.*

View article here

3.4. Absolute quantification

In absolute quantification using the standard curve method, unknown protein concentrations are quantified based on known quantity of proteins in the calibrators. Firstly, a standard curve is created, after which unknowns are compared to the standard curve and a value is extrapolated. Common practice is to produce a standard curve to run on each plate, using a standard provided by the vendor, which is diluted into the correct concentration by the operator.

Olink has developed a different way of performing absolute quantification using a calibrator system and pre-defined calibration curves. This allows users to skip time-consuming dilutions series, thereby minimizing the source of technical errors, increasing the calibration curve and data accuracy, as well as leaving more wells for samples.

- ✔ A 24-point standard curve is established for each assay during product development (Figure 3).
- ✔ A single Calibrator point is measured in triplicates on each plate, and its median value is used to re-adjust the pre-defined calibration curve (Figure 4).
- ✔ Measured sample values are translated to pg/mL by relating back to the adjusted curve model.
- ✔ Accuracy is validated by the known concentrations in the triplicate Sample Controls.

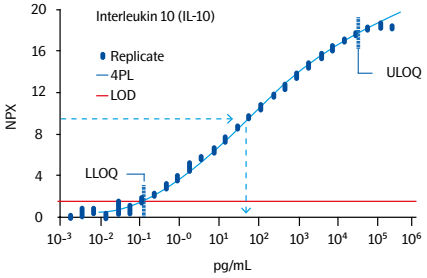


Figure 3: An example of a 24-point standard curve defined for each assay during development.

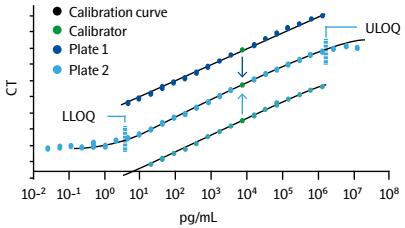


Figure 4: The Calibrator is run on each sample plate in triplicate and is used to adjust the predefined standard curves.

White Paper

Learn more about how Olink performs absolute quantification, how it ensures validation, verification, and lot-to-lot monitoring, as well as the high correlation between panels with relative and absolute quantification in the White Paper *Ensuring quality with flexibility: Olink® Flex validation & verification.*

View article here

3.5 What a basic statistical pipeline should look like

1. Data acquisition and clean up: Getting the data together

Firstly, the data is combined with its clinical/experimental annotations (e.g. cases vs controls, severe vs mild covid, etc.). In the case of multiple datasets, a normalization step is required for bridging the projects together, in which medians from overlapping/bridging samples are adjusted, thus making the datasets comparable. Finally, the data clean up involves addressing potential missing titles, mis-formatted datasets, QC warnings, and removal of samples that were found to be non-representative in later steps of analysis.

While this step would also include removing technical replicates, Olink's PEA platform enables singlicate measurements due to its integrated QC system (as explained above), thereby removing the need for technical replicates.

To facilitate an efficient way to review the data quality and results, Olink has developed the NPX Signature software for data processing and calculating the protein concentration (either in NPX units or in pg/ml).

2. QC and exploratory data analysis: Assessing the overall shape of the data.

Once the data has been cleaned up, the next step in the pipeline involves looking at the overall shape of the data and specific data points, with the aim of assessing whether the data should be used as is, or whether editing is needed. Some of the most common ways to do this include using principal component analysis plots and looking for outliers/odd, non-normally distributed data, other unusual configurations, or samples that are not within standard ranges. With Olink data, individual NPX sample distributions are checked for outliers.

3. Statistical analysis: Using the data to answer your biological questions

After the acquisition, clean up and QC steps, the data should correspond to the researcher's basic assumptions. A statistical test that matches the study and that is most fit to answer the biological question should then be selected.

Furthermore, annotation and visualization are implemented to present the data in a form that will conveys the key results in an understandable and authentic way (e.g. box plots, volcano plots, statistically significant proteins – adjusting for multiple testing, adjusting annotations to existing knowledge of key proteins or pathways of interest, etc.).

For instance, group comparisons presented in a volcano plot provide fast and easy testing of a single hypothesis for all proteins analyzed in the study. The test can establish associations between protein levels and the variable of interest. This can include, but is not limited to T-tests, ANOVA, and linear regressions.

Linear mixed models can be used to study patient protein level changes over time that are different between groups, while hierarchical clustering can be used to get an overview of the data, and to identify subgroups of similar samples or proteins based on protein profiles.

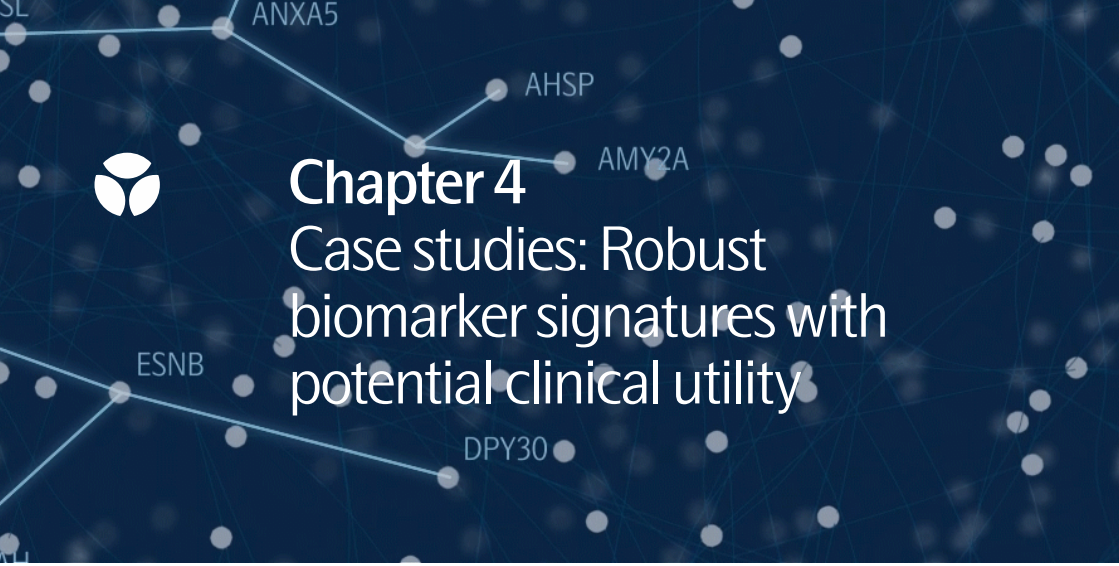
The statistical analyzer in Olink Insight enables a seamless, user-friendly experience for statistical analyses of Olink data. The additional layers of curated data in Olink Insight provide for accelerated insights into the obtained results.

4. Biological interpretation: How the proteins identified may relate to specific biological questions

Once the data analysis is finalized, further research insights can be obtained by applying additional biological context, for instance, by assessing how the differentially expressed proteins are related to the disease or pathway of interest. This process can be guided by using a comprehensive database of annotations, which provides data- and literature-derived information on pathway coverage and disease-related biomarkers, with scores and rankings of connections.

As an alternative approach, pathway enrichments can be looked at in a more quantitative way, when researchers are attempting to identify new pathways of interests and generate new hypotheses. Two common ways of achieving this are Over Representation Analysis and Gene Set Enrichment Analysis. Pathways can only be interpreted based on the background/context the data comes from, e.g., if only an Inflammation-focused panel was run, the available pathways would be given based on a background of proteins related to inflammation.

Olink Insight's numerous layers of annotations (such as tissue specificity, drug target information, biological processes, pathway information, publications, etc) provide additional biological context to data analysis and interpretation. ●



Chapter 4
Case studies: Robust biomarker signatures with potential clinical utility

As discussed in chapter 1, protein signatures comprised of multiple markers are more likely to capture disease complexity across various disease areas. Furthermore, an increase in the discrimination power of statistical models can be expected when more analytes are incorporated. The following examples illustrate the contribution of PEA-derived protein signatures to several real-world studies, such as in understanding disease mechanisms for identification of therapeutic targets, elucidating mode of action of drugs, monitoring disease progression and prognosis, achieving differential diagnosis and patient stratification, establishing biomarkers of drug efficacy, and predicting treatment response and risk of adverse events.

Understanding disease mechanisms for identification of therapeutic targets

Inflammatory Bowel Disease

Ulcerative colitis is one of the two major forms of inflammatory bowel diseases. There is a poor understanding of the early drivers of immune dysregulation that lead to it. With the aim of better characterizing systemic inflammation that occurs with this disease, researchers utilized the Olink® Target 96 Inflammation panel to measure 92 proteins from the plasma of patients up to 15 years before diagnosis, along with a treatment-naïve cohort. Up-regulation of six pro-inflammatory and tissue-repairing pathways was found several years before ulcerative colitis diagnosis. These differentially expressed proteins served as a protein signature with an Area Under the Curve (AUC) of 0.92 in a validation set receiver-operator curve. Moreover, the effect of shared genetic and environmental risk factors on the preclinical protein markers was assessed by comparing healthy twin siblings of patients with ulcerative colitis and healthy blood donors. This study highlights

how a comprehensive and robust screening of proteins allowed the identification of a signature with high performance to uncover key inflammatory proteins leading to a chronic disease, which can guide development of new therapies.

> *"A set of inflammatory proteins are up-regulated several years before a diagnosis of ulcerative colitis. These proteins were highly predictive of an ulcerative colitis diagnosis, and some seemed to be up-regulated already at exposure to genetic and environmental risk factors."*

Bergemalm D and Halfvarson J et al. Systemic Inflammation in Preclinical Ulcerative Colitis. Gastroenterology. 2021 161(5):1526-1539.e9.

Heart Failure and Diabetes

Diabetes is common in patients with heart failure. It can negatively impact clinical outcomes and treatment in patients. In this study, researchers brought more clarity into the pathophysiological processes associated with type 2 diabetes in heart failure. They utilized the Olink® Target 96 Cardiovascular Disease III panel to measure 92 proteins in 1572 plasma samples from patients with heart failure with reduced ejection fraction (HFrEF) with or without Diabetes. Results revealed that 9 proteins were differentially regulated in those patients with Diabetes in both index and validation cohorts. Biomarkers remained differentially expressed after adjusting for age, sex, BMI, hypertension, ischemic etiology of HF, and glomerular filtration rate. After network analysis, EGFR and pathways related to inflammation were identified as important hubs in patients with heart failure and diabetes and can potentially be explored as treatment targets.

> *"A better understanding of the pathophysiology of diabetes in heart failure with reduced ejection fraction is a prerequisite for identifying novel treatment targets and effectively treating diabetes in these patients."*

Tromp J and Sama IE et al. Distinct Pathological Pathways in Patients With Heart Failure and Diabetes. JACC Heart Fail. 2020 8(3):234-242. doi:10.1016/j.jchf.2019.11.005

Elucidating the mode of action of drugs

Psoriasis

Apremilast (Otezla® by Celgene) has been approved for the treatment of psoriasis and psoriatic arthritis (PsA), but its mode of action is not fully understood. Researchers applied Olink® Target 96 to measure 92 proteins involved in inflammation using synovial fluid cells from patients with active Rheumatoid Arthritis or Psoriatic Arthritis. IL-12/IL-23p40 was revealed as a major downstream target of Otezla® in the inflamed joints of immune-mediated inflammatory arthritis. These findings could be used to expand indications of existing drugs for other IL-12/IL-23-driven inflammatory diseases.

"Our findings could explain the efficacy of apremilast seen in psoriasis and PsA and holds promise for testing apremilast in other IL-12/IL-23-driven immune-mediated inflammatory diseases."

Kragstrup TW et al. IL-12/IL-23p40 identified as a downstream target of apremilast in ex vivo models of arthritis. Ther Adv Musculoskelet Dis. 2019;11:1759720X19828669. doi: 10.1177/1759720X19828669.

Immune-mediated diseases

Two patients with gastrointestinal symptoms had a SOCS1 haploinsufficiency identified through next-generation sequencing. One of them was treated with the JAK1 inhibitor ruxolitinib and went in remission. To gain further understanding into the mechanism of action of this drug for this specific condition, peripheral blood was analyzed for several immune-related markers, including the circulating cytokine profile. For this purpose, they used Olink® Target 48 and showed an increase in IFN-γ, CCL-3/4/5, CXCL-8/9, IL-6, IL-17, TNF-α, TGF-α, and Oncostatin-M before ruxolitinib therapy that normalized after 1 year of treatment, except for IFN-γ, which was only slightly reduced by this treatment. This study highlights how comprehensive cytokine profiling can provide insights into the pathophysiology of many human immune–mediated diseases and validate the use of tailored therapies.

"Hyper activation of the JAK-STAT signaling underlies the pathophysiology of many human immune–mediated diseases. Herein, the study of 2 adult patients with SOCS1 haploinsufficiency illustrates the severe and pleomorphic consequences of its impaired regulation in the intestinal tract."

Rodari MM et al. Insights into the expanding intestinal phenotypic spectrum of SOCS1 haploinsufficiency and therapeutic options. J Clin Immunol. 2023 Aug;43(6):1403-1413. doi: 10.1007/s10875-023-01495-7.

Multiple Sclerosis

Multiple sclerosis (MS) is a progressive life-long debilitating disease characterized by inflammatory demyelination and neuronal damage. The clinical course greatly varies, but a large portion of patients experience disease activity as relapses following initial symptoms presentation. Treatment at this stage is essential to prevent irreversible neuronal damage. Intravenous methylprednisolone is the standard treatment for a multiple sclerosis relapse, even though it fails to alleviate symptoms in a quarter of patients. Alternatives such as immunoadsorption have gained interest, but its mechanisms are still elusive. In this prospective study, patients with steroid-refractory acute multiple sclerosis relapses receiving either immunoadsorption or higher dose of methylprednisolone therapy were compared. The former treatment was shown to have higher efficacy to treat relapses. In order to uncover the underlying mechanisms of each treatment, extensive flow cytometry data was conducted along with comprehensive serum chemo- and cytokine profiling with Olink® Target 48. Cytokine networks were affected by both treatments, with immunoadsorption reducing cytokines necessary for B cell maturation and B cell-derived cytokines. This was in line with the observed reduction of B cell subsets in the periphery, and led the authors to conclude

that B cell modulation is a central mechanism of immunoadsorption. This study underscores how Olink Target 48 is an excellent complement to flow cytometry data, so that underlying mechanism of action can be elucidated.

> *"To complement the extensive flow cytometry analysis of our cohort, we decided to run larger-scale serum chemo-and cytokine profiling"*

Pfeuffer S et al. Immunoadsorption versus double-dose methylprednisolone in refractory multiple sclerosis relapses. J Neuroinflammation. 2022;19:220. doi: 10.1186/s12974-022-02583-y.

Monitoring disease activity and prognosis

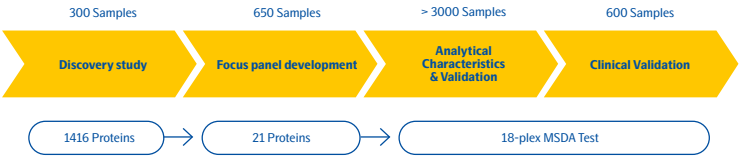
Multiple Sclerosis

Octave Bioscience screened over 1400 proteins with Olink® Target 96 and other methods to find a robust signature to quantitatively assess disease activity in serum. Subsequently, based on statistical, analytical, and biological factors, 21 proteins were selected for inclusion in a custom panel with Olink® Focus. This 21-plex panel was then developed and thoroughly analytically validated. A narrowed down 18-plex panel went through clinical validation in more than 600 samples from independent cohorts from 4 different sites, to develop a laboratory developed test (LDT) for MS disease assessment referred to as MSDA (MS Disease Activity) test. The analytical validation of MSDA demonstrated this panel is accurate, precise, sensitive and stable. Moreover, the inclusion of analytes involved in several aspects of MS pathophysiology, including immuno-modulation, neuroinflammation, myelin biology and neuronal integrity increases its value. Perhaps unsurprisingly, this comprehensive panel outperformed the single analyte test of neurofilament light for monitoring disease activity.

> *"Analytical validation of this multi-protein, serum-based assay is the first step in establishing its potential utility as a quantitative, minimally invasive, and scalable biomarker panel to enhance the standard of care for patients with MS."*

Qureshi F, Hu W, Loh L, et al. Analytical Validation of a Serum-Based Assay for Disease Activity Assessments in Multiple Sclerosis. (2023) Proteomics Clinical Applications, DOI: 10.1002/prca.202200018

Octave Bioscience MSDA Test



White Paper

This White Paper called *Development and validation of customized PEA biomarker panels with clinical utility* describes the development and validation of customized protein biomarker panels. The technical performance of a custom 19-plex panel is verified, with the aim of guiding a decision on whether the technology is fit-for purpose for future clinical utility, such as in early-phase clinical trials.

View article here

The white paper above details proposed steps for a technical verification of custom lower-plex PEA panels with qPCR readout, demonstrating the feasibility of this platform for both screening and verification studies.

The Octave Bio study showcases the readiness of PEA with qPCR readout to be used from discovery to clinical utility. Another great example is a study that screened proteins that allow early detection of ovarian cancer utilizing Olink® Target 96 panels. A biomarker signature with 11 proteins was identified in a discovery cohort and validated in two replication cohorts. Based on that, a custom panel was developed and validated in a process followed an FDA inspired "fit-for-purpose" analytical validation protocol. You can learn more about this study here.

Achieving differential diagnosis and patient stratification

Kawasaki Disease

Kawasaki disease (KD) is a life-threatening pediatric disease characterized by vasculitis that can lead to coronary artery aneurysms if appropriate treatment is not administered early enough. As symptoms are unspecific, there is urgency in developing diagnostic tools to distinguish KD from similar conditions. In light of this, researchers have employed Olink® Target 48 to perform a comprehensive cytokine profiling on minimal sample volumes extracted from pediatric patients. Of note, elevated IL-17A, IL-17C, and IL-17F distinguished KD from other conditions and correlated with CAA development. IL-17 cytokine levels could be used as a diagnostic tool for KD and are predictive of CAA development. Given the low sample volume requirement and the low abundance of IL-17, PEA is an ideal tool for sensitive immunophenotyping of pediatric inflammatory diseases with the aim of identifying diagnostic biomarkers.

> *"Our findings demonstrate the utility of PEA technology in aiding the diagnostic evaluation of pediatric inflammation diseases."*

Brodeur, K.E. et al, Elevation of IL-17 cytokines distinguishes Kawasaki disease from other pediatric inflammatory disorders. Arthritis Rheumatol. 2023. https://doi.org/10.1002/art.42680

COVID-19 and Sepsis

The pathophysiology of severe COVID-19 and sepsis presents as dysregulated inflammatory responses that can result in Acute Respiratory Distress Syndrome, kidney injury, multiorgan failure and septic shock. For effective management of patients with COVID-19 and sepsis it is essential to be able to distinguish the two diseases. Researchers set out to identify protein signatures that have high power of discrimination using Olink® Target 96 Organ Damage, Immune Response and Inflammation panels among healthy, COVID-19, community-acquired pneumonia (CAP), non-pneumonia sepsis, and septic shock patients. COVID-19 and sepsis shared 42 differentially altered proteins, but a higher systemic inflammatory response was observed in sepsis. Protein biomarkers (TRIM21, PTN, CASP8, CSF1) accurately differentiated COVID-19 from CAP-sepsis with higher accuracy than standard clinical markers. The study provides a deeper understanding of the immune responses to COVID-19 vs. CAP-sepsis, while also revealing diagnostic and severity signatures that could be used to personalize care for COVID-19 and sepsis patients.

> *"We set out to compare systemic host responses during the acute stages of COVID-19 and sepsis, to capture potential disease-, pathogen-, and organ-specific proteomic profiles."*

Palma Medina et al. Respiratory Research (2023) 24:62. https://doi.org/10.1186/s12931-023-02364-y

Alzheimer's Disease

Alzheimer's Disease accounts for 60% to 80% of patients with dementia, and misdiagnosis is estimated to be 30%. This high proportion of misdiagnosis risks efficiency of clinical trials. Due to the complexity and multifactorial causes of AD, a signature of protein biomarkers is more likely to capture varying disease pathophysiology or discriminate between non-AD dementias. To identify a robust signature, authors utilized 11 Olink® Target 96 panels to broadly screen proteins in CSF samples from patients with mild cognitive impairment, AD and non-AD dementia (797 participants). Based on this screening, a specific Olink® Focus panel was able to successfully distinguish AD from controls (AUC=0.96) and another Focus panel distinguished AD from non-AD dementia (AUC=0.87). This study highlights the ability of running discovery screening studies to identify signatures that can be further developed for disease insights and patient stratification in clinical trials.

> *"This study also highlights the effectiveness of our methodological workflow to discover and validate new biofluid-based biomarkers, leveraging the combination of large well-characterized cohorts with robust and translatable technologies."*

del Campo M. and Teunissen C.E. et al. Nat Aging (2022) 1-14 CSF proteome profiling across the Alzheimer's disease spectrum reflects the multifactorial nature of the disease and identifies specific biomarker panels.

Establishing biomarkers of drug efficacy

Atopic Dermatitis

A new dual inhibitor of Janus kinase and spleen tyrosine kinase called ASN002 (Asana Biosciences) was developed to treat patients with Atopic Dermatitis (AD). There is a need for biomarkers that help determine optimal dosage. Researchers used four Olink® Target 96 panels to screen for biomarkers in participants receiving ASN002 or placebo (3 different doses of 20, 40 and 80mg) over a 28-day period. ASN002 significantly downregulated several serum biomarkers involved in Th1, Th2 and Th17/Th22. They also observed a decrease of the atherosclerosis-associated biomarker E selectin/SELE in ASN002-treated patients. This type of study demonstrates the possibility of relating disease activity scores to biomarker changes and identifying responders vs non-responders.

> *"Based on serum biomarker analyses, our study showed that ASN002 provided greater and more significant modulation of many key AD circulatory biomarkers compared with placebo, particularly at high dosages."*

Bissonnette R and Guttman-Yassky E et al. The oral Janus kinase/spleen tyrosine kinase inhibitor ASN002 demonstrates efficacy and improves associated systemic inflammation in patients with moderate-to-severe atopic dermatitis: results from a randomized double-blind placebo controlled study. Br J Dermatol. 2019; 181(4):733-742. doi:10.1111/bjd.17932

Charcot-Marie-Tooth

Charcot-Marie-Tooth (CMT) is one of the most common inherited neurological disorders and a specific efficacy biomarker for clinical trials is much needed. Using five Olink® Target 96 panels to screen proteins in subjects with CMT subtype 1A (CMT1A) and controls, researchers were able to identify a 2-fold increase of transmembrane protease serine 5 (TMPRSS5) in subjects with CMT1A from two independent cohorts. TMPRSS5 is most highly expressed in Schwann cells of peripheral nerve, which is consistent with the fact that Schwann cells are the most affected cells in CMT1A. These findings indicate that TMPRSS5 could serve as a biomarker of efficacy to increase efficiency of clinical trials for CMT1A treatment.

> *"These data identify the first Schwann cell-specific protein that is elevated in plasma of CMT1A patients, and may provide a disease marker and a potentially treatment-responsive biomarker with good disease specificity for clinical trials."*

Wang H and Shy ME et al. Ann Clin Transl Neurol. 2020 7(1):69-82. Transmembrane protease serine 5: a novel Schwann cell plasma marker for CMT1A. doi:10.1002/acn3.50965

Predicting treatment response and risk of adverse events

Gastric cancer

Gastric adenocarcinoma is a common and lethal cancer that is often detected too late. Among the few treatment options is preoperative chemotherapy, but patient responses are highly heterogeneous. Biomarkers to identify patients who respond to preoperative chemotherapy were identified using the Olink® Target 96 Inflammation panel. Serum biomarkers correlated with tumor microenvironment features and could predict tumor regression, overall survival, and progression-free survival of patients receiving preoperative chemotherapy. A pretreatment serum protein signature including PDL-1 and CCL20 that accurately identify non-responders was revealed. A serum response predictive score was also developed to stratify patients based on treatment response. These predictive tools could transform the management of gastric adenocarcinoma by enabling more personalized treatment strategies.

> *"A pretreatment serum protein scoring system is established for response prediction. Together, these findings highlight the fundamental but largely underestimated role of systemic immunity in the treatment of gastric cancer, suggesting a patient stratification strategy based on pretreatment serum immune proteomics."*

Tang Z, Gu Y, Shi Z, et al. Multiplex immune profiling reveals the role of serum immune proteomics in predicting response to preoperative chemotherapy of gastric cancer. (2023) Cell Reports Medicine, DOI: 10.1016/j.xcrm.2023.10093

Immune checkpoint inhibitor toxicity

Immune checkpoint inhibitors (ICIs) are promising treatment options for melanoma and non-small lung cancer. In spite of this, it is known that they can be associated with immune-related adverse events (irAEs) in a significant number of patients. With the aim of identifying predictive biomarkers of irAEs, researchers have screened 235 patients treated with ICIs using the Olink® Target 96 Inflammation panel. Combining those data with immune cell population data by flow cytometry allowed them to identify an early serum immune signature predictive of development of irAEs in ICI-treated patients. Early identification of those at increased risk for irAEs would be a personalized approach to increase patient safety.

> *"We propose that the analysis of serum proteins combined with the detection of proliferating T cell subsets 1 to 2 weeks after the start of therapy can help identify those patients at higher risk of later ICI toxicity"*

Nuñez NG, Berner F, Friebel E, et al. Immune signatures predict development of autoimmune toxicity in patients with cancer treated with immune checkpoint inhibitors. (2023) Med, 10.1016/j.medj.2022.12.007

Rheumatoid Arthtitis

Treatment of Rheumatoid Arthritis (RA) with anti-TNF biologics is extremely effective for a proportion of patients. However, around one third of RA patients fail to respond. Taking this into account, this study sought to identify biomarkers predictive of treatment response by screening plasma from 144 RA patients on anti-TNF therapy with 4 Olink® Target 96 panels. Machine learning identified a 17-protein signature to identify responders (AUC=0.88). The implementation of such a signature could significantly improve therapeutic outcomes by enabling the early identification of individuals most likely to benefit from anti-TNF therapy.

> *"We have developed a ML based classifier ATRPred (anti-TNF treatment response predictor), which can predict anti-TNF treatment response in RA patients with 81% accuracy, 75% sensitivity and 86% specificity. ATRPred may aid clinicians to direct anti-TNF therapy to patients most likely to receive benefit, thus save cost as well as prevent non-responsive patients from refractory consequences."*
>
> Prasad BP and Shukla P et al. PLoS Comp Biol 2022 18(7):e1010204 ATRPred: A machine learning based tool for clinical decision making of anti-TNF treatment in rheumatoid arthritis patients DOI: 10.1371/journal.pcbi.1010204

Conclusion

PEA represents a revolutionary advance in the field of multiplexing immunoassays, setting a new quality standard for identifying robust protein signatures. The inherent complexities of multi-protein assays are finally addressed to allow for accurate quantification of all analytes in a panel with virtually no cross-reactivity. The growing number of publications is a testament to the full confidence researchers can now place in their multiplex data. By overcoming the challenges that have long plagued multiplex immunoassays, PEA offers a reliable way to explore and validate complex protein interactions and biomarkers. ●

Learn more at www.olink.com

Olink Proteomics, Salagatan 16F , SE-753 30 Uppsala, Sweden 1482, v1.4, 2025-09-29