Olink®

White paper

PEA: Exceptional specificity in a high multiplex format

Introduction

Specificity and its importance in the context of multiplex testing

There are a growing number of methodologies available to measure proteins in biological samples that depend on the use of an affinity reagent to detect each target molecule. Specificity describes the ability of an assay to accurately identify and measure the defined protein of interest, while minimizing the detection of off-target proteins. This is typically achieved by using specific antibodies or other binding agents that are highly selective for the target protein. Problems may arise if:

- The affinity reagent does not recognize the intended target

  or

- there is cross-reactivity between the affinity reagent and additional targets or other components in the reaction.

There are two types of cross-reactivity. The first is true biological cross-reactivity, in which case the proteins are very homologous and binding to an epitope on both proteins could occur both in vivo and in vitro. The second type is technical cross-reactivity from non-specific binding due to the method used. This white paper focuses on the latter.

Consequences of poor specificity

Without sufficient specificity, there is likely to be inaccurate quantification and misidentification of the target proteins, which can have serious implications. In studies aimed at identifying protein biomarkers that can provide actionable biological insights, improve the diagnosis, treatment, and monitoring of diseases and help develop and optimize new therapies, the consequences of poor specificity in the assay method used could be disastrous.

- Valuable time, money and precious samples wasted on studies that provide incorrect and misleading information
- Misidentification of proteins leading to erroneous conclusions and misdirection of subsequent research
- Misplacement of future resources focused on irrelevant proteins and incorrectly implicated biological pathways
- Even if stable signatures are identified for a biological state, they may not be attributed to the correct proteins, and incorrect identification of potential new therapeutic targets can lead to costly failures in drug development projects
- Unnecessary delays in gaining key biological insights that could expediate better understanding of disease, development of more effective therapies and ultimately, better outcomes for patients

Method validation

This white paper shows how the accuracy of the Olink® Proximity Extension™ Assay (PEA™) technology has been validated regarding recognizing the intended target protein. Since there is no gold standard to compare with, a proxy must be used. The key for an accurate multiplex method is to have good specificity, and this is something both Olink and Olink's customers have validated using orthogonal methods.

You will learn how the unique features of the PEA technology ensure the fidelity of each individual assay for its target protein and have overcome the problem of cross-reactive readout in high-multiplex analyses.

PEA technology features

The dual-recognition, DNA-coupled readout, provided by PEA provides exceptional specificity even at high multiplexing levels. 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 by qPCR or NGS. This dual antibody recognition and hi-fidelity DNA-coupled measurement mean that PEA is able to provide truly exceptional readout specificity. This overcomes the problems normally associated with multiplexed immunoassays, since any potential antibody cross-reactivity will not contribute to a detection signal. This degree of specificity is a hallmark of PEA.

How Olink® validates specificity

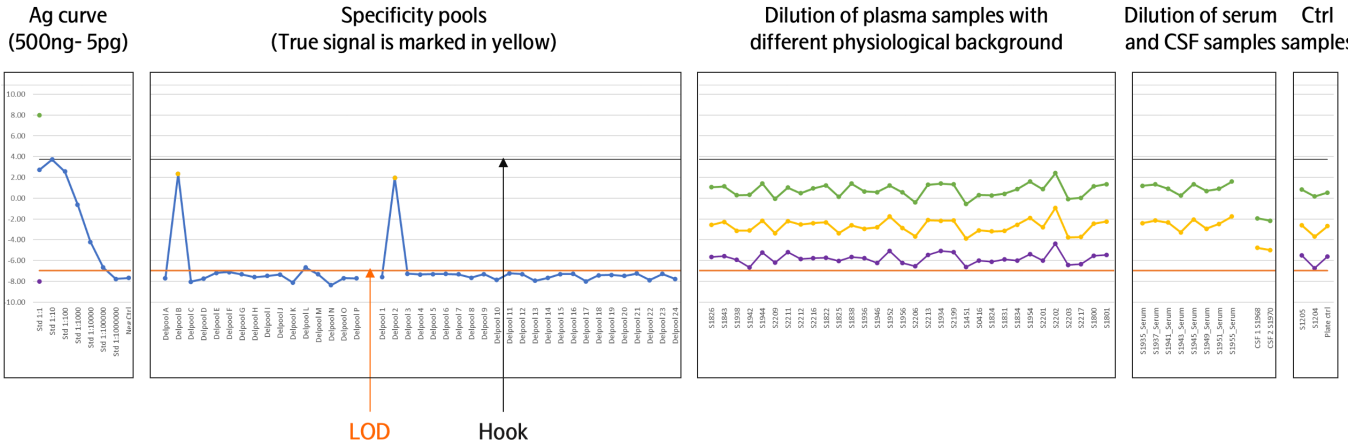

Figure 1 Illustration of an assay that passed NGS screening. This assay was deteced in relevant plasma and serum matched samples. If the assay had shown a deviating sample dilution pattern, it would have failed the NGS screening.

Specificity testing for Olink® Explore panels

Rigorous specificity testing was used during the development of Olink Explore, both as part of the assay selection process, and afterward as part of the formal product validation procedure. All biomarkers undergo a three-step analytical verification process with three levels of specificity testing.

- qPCR screening: A first screening against a specially designed pool of antigens to identify unspecific binding using qPCR readout
- NGS screening: A second screening against a carefully designed set of recombinant antigen pools using NGS readout
- Verification and validation: Finally each biomarker is tested against pools of 96 selected Olink Explore proteins with high homology within the protein family to further challenge the specificity

The graph in Figure 1 shows an assay that has passed the NGS screening.

Specificity testing for Olink® Target, Flex and Focus panels

Validation of the readout specificity for all Olink panels with qPCR readout is carried out using a simple, sequential approach in which pools of protein analytes are tested with all antibody probe pairs in the panel (see Figure 2).

The analytical performance of the panels has been carefully validated for sensitivity, dynamic range, specificity, precision, and scalability, and the results are summarized in the Data Validation documents for each panel. These documents can all be found on the Olink website.

Assay readout specificity study

In a study by Assarsson et al. (1), assay readout specificity of the Olink platform was tested for one Olink panel. To ensure that the antibodies in each assay were specific for their desired targets, each assay's response was measured against all of the 92 proteins in the panel, as well as against an additional 107 proteins. In principle, 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. 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 as illustrated in Figure 2. None showed significant signal from the proteins tested.

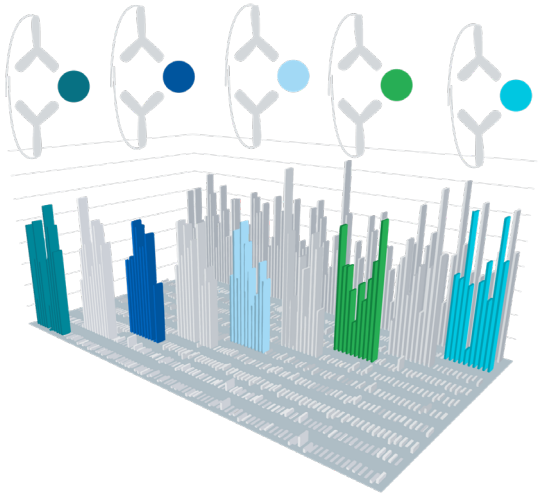

Figure 2 Each assay is exposed to samples containing either subsets or all of the selected antigens. A given assay should only generate a signal when the corresponding antigen is included in the sample.

Readout of cross-reactivity

Readout of cross-reactive events are a common problem for multiplex immunoassays, such as sandwich ELISA. The dual recognition of PEA and multiple blocking reagents included in the immunoreaction step, prevent the readout of non-specific binding using Olink panels.

In one experiment, a set of highly related proteins were used to search for cross-reactive recognition and to further challenge specificity (2). The analytes and their related proteins are listed in Table 1.

Table 1. 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	49	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

Despite testing the most closely related proteins, cross-reactivity was not observed. FR-alpha showed some recognition of its highly homologous relative FR-beta (77% identity and 87% coverage), although at a non-significant level in plasma (0.1%). This systematic approach demonstrates that the Olink assays can distinguish between very similar human proteins, and yet again highlights the high specificity of PEA.

Assay performance independent of plex-grade

Olink has assessed how PEA protein measurement and quantification is affected by different multiplex grades. The assay level and performance of ~1000 proteins have been compared between all of the proteins in the same 1000-plex probe pool versus being in eleven 100-plex pools.

Analysis of the data showed very high assay-level NPX™ correlation between the 100- and the 1000-plex environments, indicating that performance of PEA and subsequent signal amplification by PCR is comparable regardless of the assay plex-grade of the reaction.

TERMINOLOGY

NPX is an arbitrary, relative quantification unit. Olink normalizes the raw values into the relative quantification unit NPX, using a series of computations. These operations are designed to minimize technical variation and improve interpretability of the results.

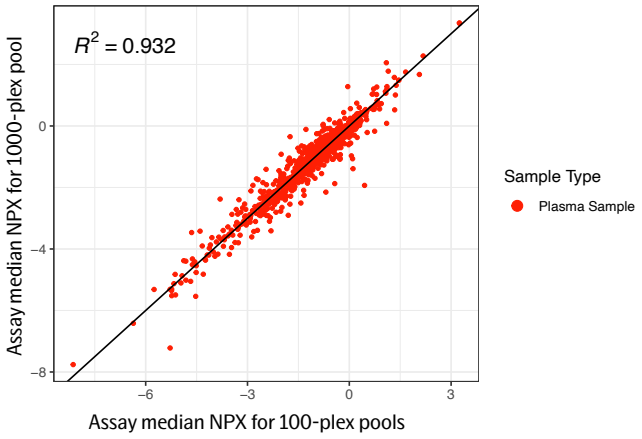

Figure 3. Protein-level NPX correlation between assays run in 1000 multiplex grade (Y-axis) and the same assays run in eleven smaller 100 multiplex grade reactions (X-axis). Each dot indicates the median NPX levels for three replicates in the plasma sample.

How Olink users validate specificity

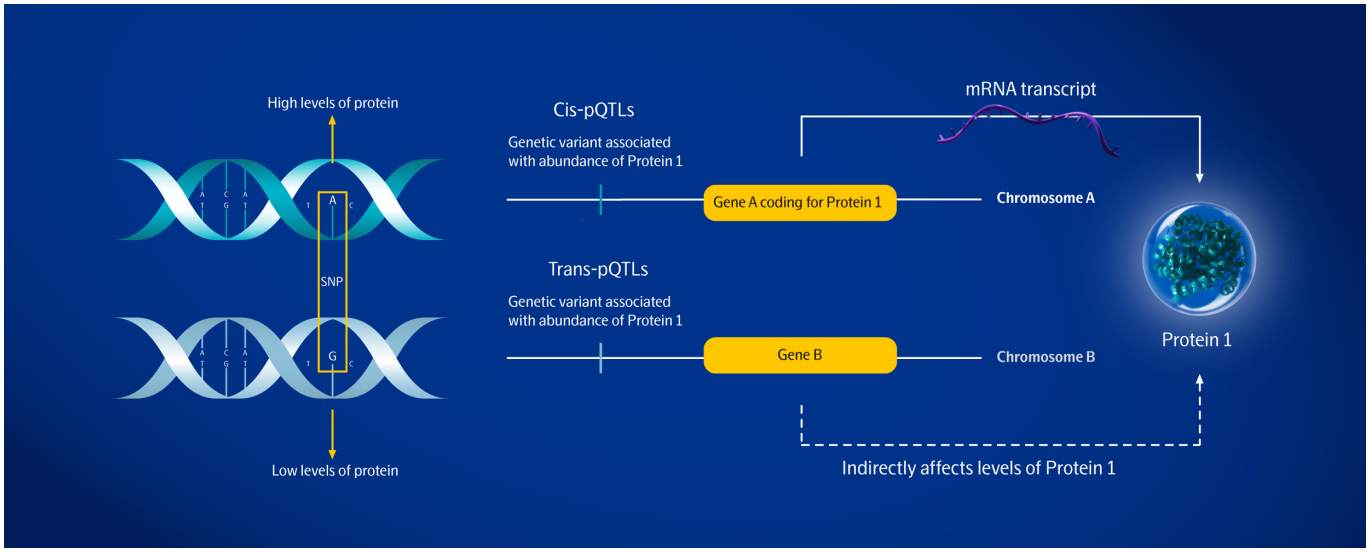

Figure 4 Schematic representation of a cis- and trans-pQTL, and how they affect protein levels. A cis-pQTLs is a genetic variant (typically a SNP) that lies very close to (<1 Mb) or within the gene that codes for the protein (Protein 1) being measured. Trans-pQTLs are located more than 1 Mb away from the gene encoding the affected protein and may be on a different chromosome altogether (chromosome B). Trans-pQTLs are assumed to regulate the protein being studied indirectly and can reveal new mechanisms of how Gene B could be related functionally to Gene A.

Genetic validation of Olink® protein biomarker assays

Compelling evidence that Olink's PEA assays robustly measure their intended protein targets comes from numerous proteogenomic studies, where Olink data is combined with genetic data, such as from genome wide association studies (GWAS). This enables the identification of gene variants associated with regulation of plasma protein levels, known as protein quantitative trait loci (pQTLs). If these genetic variants are located near or in the gene encoding the affected protein, they are called cis-pQTLs. See Figure 4. As well as being invaluable tools for ascribing causality to a protein in a disease or other biological process, cis-pQTLs also provide excellent validation that the protein being measured is the one targeted by the assay, since there is direct genetic evidence linking the genetic and protein data in the same genomic location.

A growing number of proteogenomic studies have shown that Olink-based protein measurements consistently identify a high frequency of cis-pQTLs compared to alternative highplex proteomics methods, leading to many important, novel discoveries that may otherwise have been missed, or mis-attributed, as exemplified below.

Proteogenomic links to human metabolic diseases

Koprulu et al.(3) describes a cis-pQTLs-focused proteogenomics analysis of 2,923 plasma proteins measured in 1,180 individuals using the Olink Explore 3072 platform. This identified a total of 1,553 independent genetic associations for 914 unique proteins, representing the discovery of 256 previously unreported cis-pQTLs, many of which were linked to metabolic diseases.

Importantly, 125 of the 256 newly reported cis-pQTLs were associated with 101 proteins that were previously investigated in aptamer-based proteogenomic studies. The earlier investigations failed to identify cis-pQTLs in these proteins, despite using sample sets up to 30 times larger than the Olink study.

> *"Here, we identified more than 200 unreported cis-pQTLs by capitalizing on recent assay developments."*
>
> Koprulu et al., 2023, *Nature Metabolism*

The genetic regulation of protein expression in cerebrospinal fluid

A study from Hansson et al. (4) used Olink Target 96 panels (CVD II, Inflammation, Neurology, Neuro Exploratory) to perform proteogenomic analysis of plasma and cerebrospinal fluid (CSF) samples from more than 1,500 individuals. This resulted in the identification of 117 cis-pQTLs for 145 CSF proteins of the 398 that were measured. Meta-analysis suggested that almost half of the cis-pQTLs identified had previous evidence of association with gene expression in brain tissues. Mendelian Randomization (MR) also suggested causal roles for several proteins in neurological diseases such as Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease.

> *"The main novelties included the use of highly specific proximity extension assays in a large cohort. New possible treatment targets for several neurological diseases were nominated."*
>
> Hansson et al., 2022, *EMBO Molecular Medicine*

Proteomic profiling platforms head to head

In a study by Katz et al. (5) Olink Explore 1536 and an aptamer-based platform were compared head-to-head in a proteogenomic analysis of several hundred individuals. In their assessment of accuracy and biological insights provided, Olink was superior in terms of frequency of cis-pQTLs and phenotypic associations. For several proteins where the Olink assay showed a clear genetic/phenotypic association that wasn't seen with the aptamer platform, correlation of protein levels with gold-standard ELISAs for those proteins also correlated better with the Olink data

> *"To draw strong biological conclusions from proteomic analysis, accuracy is paramount... Olink held an advantage, as a higher percentage of proteins on that platform had cis pQTLs."*
>
> Katz et al., 2022, *Science Advances*

Genomic and drug target evaluation

In a study from the SCALLOP consortium by Folkersen et al. (6), Olink Target 96 CVD I was used for a proteogenomic analysis of 90 proteins in over 30,000 individuals with the data compiled from 15 cohorts. This resulted in identification of 451 pQTLs mapped to 85 proteins. Among these, there were 170 cis-pQTLs, and MR revealed 11 proteins with previously unknown causal links to human disease, suggesting potential new drug target candidates or repositioning opportunities.

> *"We identified and replicated 315 primary and 136 secondary pQTLs for 85 circulating proteins to yield new insights for translational studies and drug development."*
>
> Folkersen et al., 2020, *Nature Metabolism*

Correlation with other methods

Comparing proteomics platforms is complex and difficult, and the degree of correlation between different platforms will vary depending on reagents and samples used.

A comparison of PEA™ with established immunoassays

Olink's multiplex assays correlate well with standard, single ELISAs according to a study by Siegbahn et al. (7).

In the study, clinically approved single-plex assays were run in a total of 10 000 samples from three large cohorts. The correlation between the single-plex assay and Olink Target 96 CVD III multiplex assay is shown in Figure 5 for GDF-15. Note that the Olink panel only needed 1 µL of sample.

> *"PEA results provided the same associations to outcome—cardiovascular death—as conventional assays and can, we believe, be used as a substitute."*
>
> Siegbahn et al., 2017 *Science/AAAS*

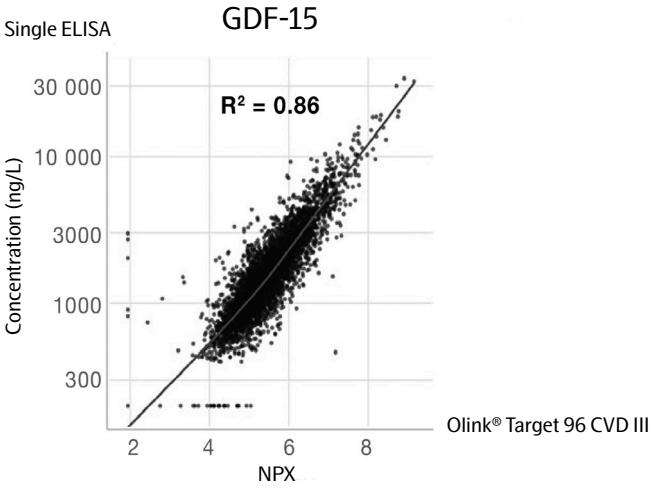

Figure 5 Scatter plot of comparison between a single ELISA and corresponding PEA™ results for GDF-15.

NFL compared between several different methods

Neurofilament light chain (NFL) concentration in blood is a biomarker of neuro-axonal injury in the nervous system and there now exist several assays with high enough sensitivity to measure NFL in serum and plasma. In a study by Andreasson et al. (8) they aimed at setting a standard for clinical measurements of NFL, where the first step was to establish a matrix in which they would give consistent results .

Four analytical methods for measuring NFL were used (Olink, Simoa, Ella and Atellica).

The correlation among the different analytical methods was exceptionally high (Spearman correlation coefficient $\rho \geq 0.96$) and the differential spiking showed that samples spiked with CSF showed higher commutability compared to spiking with recombinant human NFL protein. Serum was also shown to be a better matrix than plasma.

Conclusion

As the data presented in this white paper show, PEA ensures high-quality measurement of proteins with exceptional specificity, whether measuring a single protein or several thousand simultaneously.

Crucially, this is supported by a growing literature showing cis-genetic associations with protein levels measured using PEA, providing the highest level of confidence in the specificity of the technology.

References

1. Assarsson et al., Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. PLOS ONE 9 (2014).
2. Olink White paper Development and validation of customized PEA biomarker panels with clinical utility (2017).
3. Koprulu et al., Proteogenomic links to human metabolic diseases. *Nature Metabolism* (2023).
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7. Siegbahn et al., A comparison of the proximity extension assay with established immunoassays, in Advancing precision medicine: Current and future proteogenomic strategies for biomarker discovery and development, p. 22-25. *Science/AAAS* (2017).
8. Andreasson et al., Assessing the commutability of candidate reference materials for the harmonization of neurofilament light measurements in blood *Clinical Chemistry and Laboratory Medicine (2023)*

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