White paper

Measuring protein biomarkers with Olink — technical comparisons and orthogonal validation

Introduction

Olink panels use the proprietary Proximity Extension Assay (PEA) technology, a unique method where each biomarker is addressed by a matched pair of antibodies, coupled to unique, partially complementary oligonucleotides, and measured by quantitative real-time PCR. This enables a high level of multiplexing while maintaining exceptional data quality.

To date more than 1 100 000 samples have been analyzed using Olink panels and more than 250 publications presenting Olink data have been published, illustrating how the high-quality data the PEA technology produces is having a great impact in the field of protein biomarkers.

In this paper we would like to address the question: "How does the data from an Olink study compare to that from other technologies?" We present examples of comparisons ranging from single-plex ELISAs to high multiplex assays and also provide useful tips for comparisons between Olink data and data from other technologies.

Single-plex antibody-based assays

ELISA

Olink's multiplex assays correlate well with single-plex fully automated ELISA used in clinical labs for routine analysis according to a study by Siegbahn *et al* (1). In the study, single-plex assays for *in vitro* diagnostic use were run in a total of 10 000 samples from three large cohorts (ARISTOTLE, PLATO and STABILITY). The correlation between the single-plex Elecsys® assay (Roche Diagnostics) run on cobas® analyzer that uses an electrochemiluminescence (ECL) readout, with the corresponding Olink assay in multiplex, is shown in Figure 1 for Growth-differentiation factor 15 (GDF-15) run on the Olink CVD III panel. Note that the Olink panel only needed 1 µl of plasma to measure 92 proteins simultaneously compared to the 35 µl required for the Elecsys® GDF-15. This is an important feature of PEA technology, since clinical sample volumes are frequently volume limited.

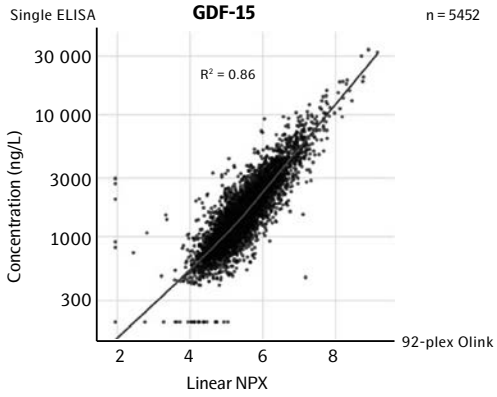


Fig 1. GDF-15. Correlation between Elecsys® assay with the corresponding Olink assay in multiplex, run on the Olink CVD III panel.

The following example (Fig 2) is a direct comparison, using the same antibodies, between a PEA assay included in several of Olink's multiplex panels with a commercially available ELISA. In this example, a comparison of the Interleukin-6 (IL-6) assay showed a strong correlation between the Olink 92-plex PEA assay and a single-plex ELISA from R&D Systems™. This ELISA required 100 µl and the Olink panel 1 µl of sample.

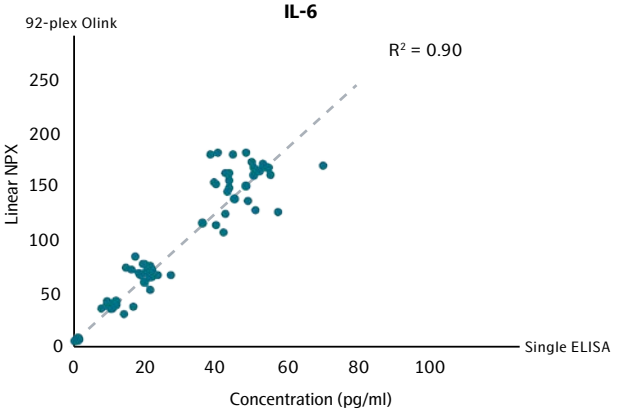


Fig 2. IL-6. Correlation between single-plex ELISA from R&D systems with the corresponding Olink assay in multiplex.

CMIA

Arrigo *et al*. performed an investigation of the accuracy of quantification of brain natriuretic peptide (BNP) using the PEA technique compared to the FDA approved chemiluminescent microparticle immunoassay (CMIA) using the Architect i2000 platform (Abbott Diagnostics) (2). A total of 120 plasma samples from 30 stable chronic hemodialysis patients were compared.

BNP values obtained by CMIA and PEA showed high overall correlation using Spearman's rank test (rho=0.865, P<0.0001), see Figure 3.

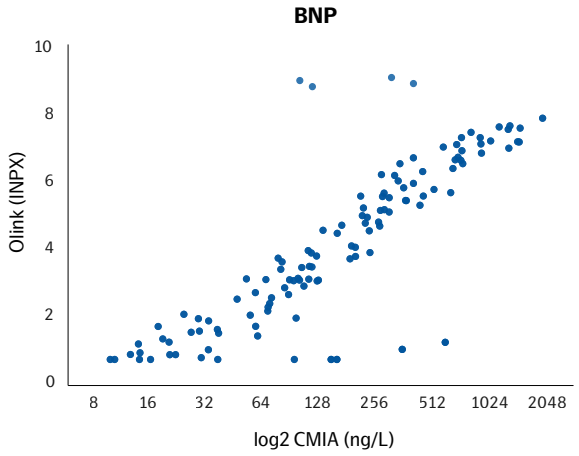


Fig 3. BNP. Correlation of Olink and CMIA assays for BNP.

This close correlation was affected by two outlier samples, probably reflecting pre-analytical sample variation that differentially affected the two assay methods. When these samples were excluded from the analysis, a Bland-Altman plot showed a very high correlation across the entire concentration range tested between the two technologies (rho=0.966, P<0.0001).

The authors conclude that "this pilot study showed that multiplex assays based on PEA technology allow linear and accurate quantification of plasma BNP over a large range of concentrations, while only requiring one microliter of plasma." They also suggest this warrants larger studies to confirm results for BNP.

High-sensitivity single-plex assays

Neurofilament light pepetide (NFL) is a highly sensitive biomarker for monitoring active injury of the central nervous system (CNS), including degenerative diseases such as multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS) and dementia, as well as traumatic brain injury (3). Levels of NFL in both CSF and plasma are utilized for monitoring injury in the CNS, although the less invasive plasma samples are better suited for monitoring disease progression. The levels of NFL are 50-fold lower in plasma compared to CSF and available ELISA assays are not recommended for measurements in blood. A fully optimized single-plex assay for NFL, covering the range for both matrices, is the high-sensitivity Simoa NF-light® assay from Quanterix.

Olink also developed an NFL-assay (for its Neuro Exploratory panel), using the same antibodies as the Simoa assay (NF-light® from UmanDiagnostics, Umeå, Sweden). The Olink NFL-assay covers the full range of NFL levels in CSF and plasma and shows strong correlation to the Simoa NFL-assay in plasma samples from MS patients and controls (Fig 4).

This shows that the PEA technology enables measurement with high sensitivity in a 92-plex format, matching the performance of the high-sensitivity single-plex "gold standard" assay, while simultaneously providing data on 91 additional proteins using just 1 µl of plasma or CSF.

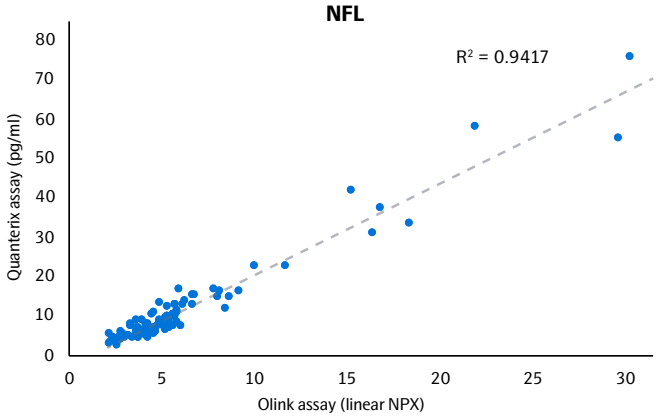


Fig 4. NFL. Comparison of Olink and Simoa NFL assays. Samples kindly provided by Prof. Tomas Olsson (Karolinska Institute, Stockholm).

Multiplex antibody-based assays

In a study by Mahboob *et al*, the Olink Oncology I panel was used to analyze plasma samples from patients with Duke's staged colorectal cancer (CRC) to identify biomarkers for early CRC detection (4). To validate the Olink results against a technology used in FDA approved assays, an identical sample set was analyzed using the established Bio Plex Pro™ Human cytokine 27-plex panel based on the Luminex™ technology (note that this specific Luminex™ cytokine assay is not FDA approved).

The expression profiles of 13 common oncoproteins were compared between the platforms. Nine of these showed reasonable correlation between platforms, and for the PDGF subunit B the Spearman correlation was particularly strong (rho=0.87, p and q values = 0). Interestingly, significant correlation was seen between Olink-measured levels of CEA, IL-8, prolactin and the different CRC stages.

The authors of the article suggest that the Olink-identified proteins are prospective novel markers of CRC progression that hold potential to be utilized in clinical oncology (4).

Platform evaluation and comparison of Olink, MSD and Myriad RBM

One of Olink's customers performed a head-to-head platform evaluation of Olink, MSD and Myriad RBM to determine which protein biomarker technology was most suitable for utility in their clinical trials.

Phase 1: Precision

In the first phase, assay performance was evaluated. Plasma samples from 10 normal subjects and 10 patients with chronic lymphocytic leukemia were analyzed in triplicate on the Olink Immuno-Oncology panel. The calculated intra-CV was 4% and the inter-CV was 6%. Detectability was 87%.

Phase 2: Linearity

In the second phase, eight samples showing high levels of proteins were analyzed in duplicate at four dilutions. The selected analytes of interest were: C-C Motif Chemokine Ligand 2 (CCL2), C-C Motif Chemokine Ligand 3 (CCL3), C-C Motif Chemokine Ligand 4 (CCL4), Interferon gamma (IFN-gamma), Interleukin 10 (IL-10), Interleukin 18 (IL-18), Interleukin 2 (IL-2), Interleukin 4 (IL-4), Interleukin 5 (IL-5), Interleukin 6 (IL-6), Interleukin 7 (IL-7), Interleukin 8 (IL-8) and Tumor necrosis factor alpha (TNF-alpha). In addition, calibrator curves for these analytes were produced.

Summary of results

In the comparison between the three technologies for the 13 proteins of interest, some individual proteins were more readily detectable on one platform compared to another, as might be expected (Fig 8).

The number of proteins detected in all eight samples was nine for MSD, seven for Olink and two for RBM. The number of proteins not detected in any of the samples was six for RBM, two for Olink and three for MSD (including IL-18 which was not assayed using MSD). In summary, RBM Myriad had worse detectability in this comparison, which is likely due to the requirement of 1:5 dilution for their assays while Olink and MSD can analyze undiluted samples. Olink and MSD had similar detectability. For TNF-alpha and IFN-gamma the MSD assays showed better detectability than Olink and were able to measure these proteins in all samples.

The customer found the precision and linearity data from the Olink assays to be excellent and despite the low detectability of TNF-alpha and IFN-gamma, the customer chose Olink from among the tested technologies and stated the following: "From our standpoint it makes a lot of sense to move forward with Olink for large scale biomarker discovery work for our programs."

The TNF-alpha and IFN-gamma Olink assays have now been improved and have a significantly increased detectability (see the Driving assay improvement information box on page 4). We have re-analyzed the samples used in the previously described comparison (undiluted), using these improved assays and the proteins are detected in all samples (Fig 5 and 6).

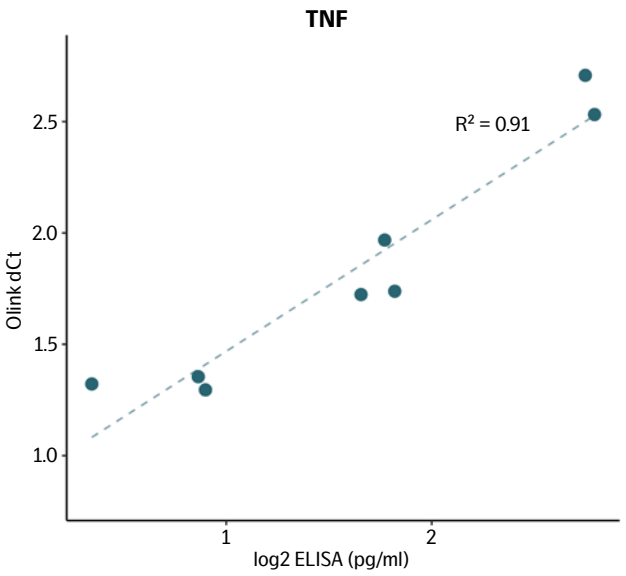


Fig 5. TNF. Correlation between high-sensitivity ELISA from R&D systems with the improved single-plex Olink TNF-alpha assay.

For TNF-alpha we have compared the PEA data to R&D systems high-sensitivity ELISA, which is the golden standard, and these data show very good correlation (R^2=0.91).

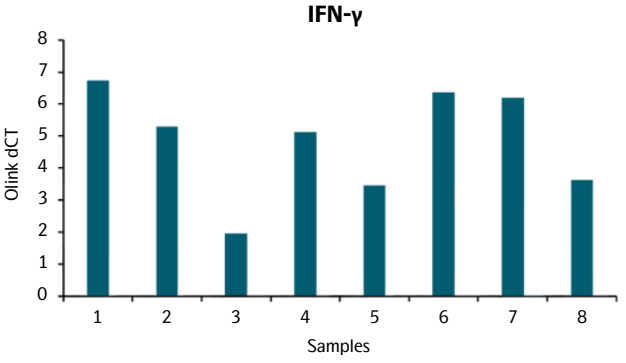


Fig 6. IFN-gamma. Higher sensitivity for IFN-gamma with the improved single-plex Olink assay.

With the improvement of these two assays, Olink now also matches MSD with 9 proteins detected in all samples for the selected 13 markers of interest. In summary, Olink is performing better than RBM Myriad and at least as well as MSD in all parts of this comparison.

Driving assay improvement

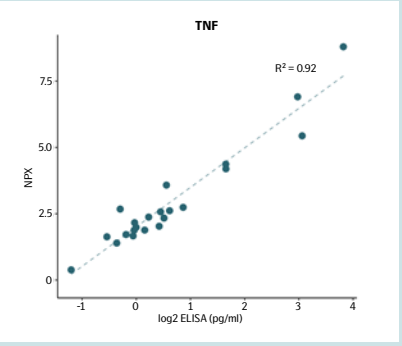
In some cases, Olink uses benchmarking against other technologies to further improve existing assays. Recent examples are the assays for TNF-alpha and IFN-gamma in the Olink Inflammation or Immuno-Oncology panels.

Background

The previous Olink TNF-alpha assay was specific for the biologically active, native trimeric form of TNF-alpha. The detectability of these trimers was relatively low in many samples. Since our customers expressed a strong wish to be able to also measure the soluble dimer and monomer versions of TNF-alpha present in serum and plasma, Olink decided to develop a new assay that can do this. Customers have also asked for a higher sensitivity for IFN-gamma, since the detectability of this marker has been relatively low, leading Olink to optimize and improve this assay as well.

Assay improvements

To develop these new assays, 23 antibodies for TNF-alpha were screened in 75 antibody combinations, and 16 antibodies for IFN-gamma were screened in 73 antibody combinations, using samples from healthy subjects and/or patients with different diseases where TNF-alpha or IFN-gamma might be elevated. This extensive work resulted in the identification of the optimal antibody combination for each assay. Both of the improved assays showed significantly higher detectability in plasma taken from healthy controls and a range of different disease types (Fig 7), and were able to measure signal above LOD/LLOQ in 100% of all tested plasma samples in the validation experiments. Comparison to gold standard ELISA from R&D Systems™ for the new TNF assay shows very good correlation (R^2=0.92). Calibrator curves and validation data for the new assays can be found on the Olink website.



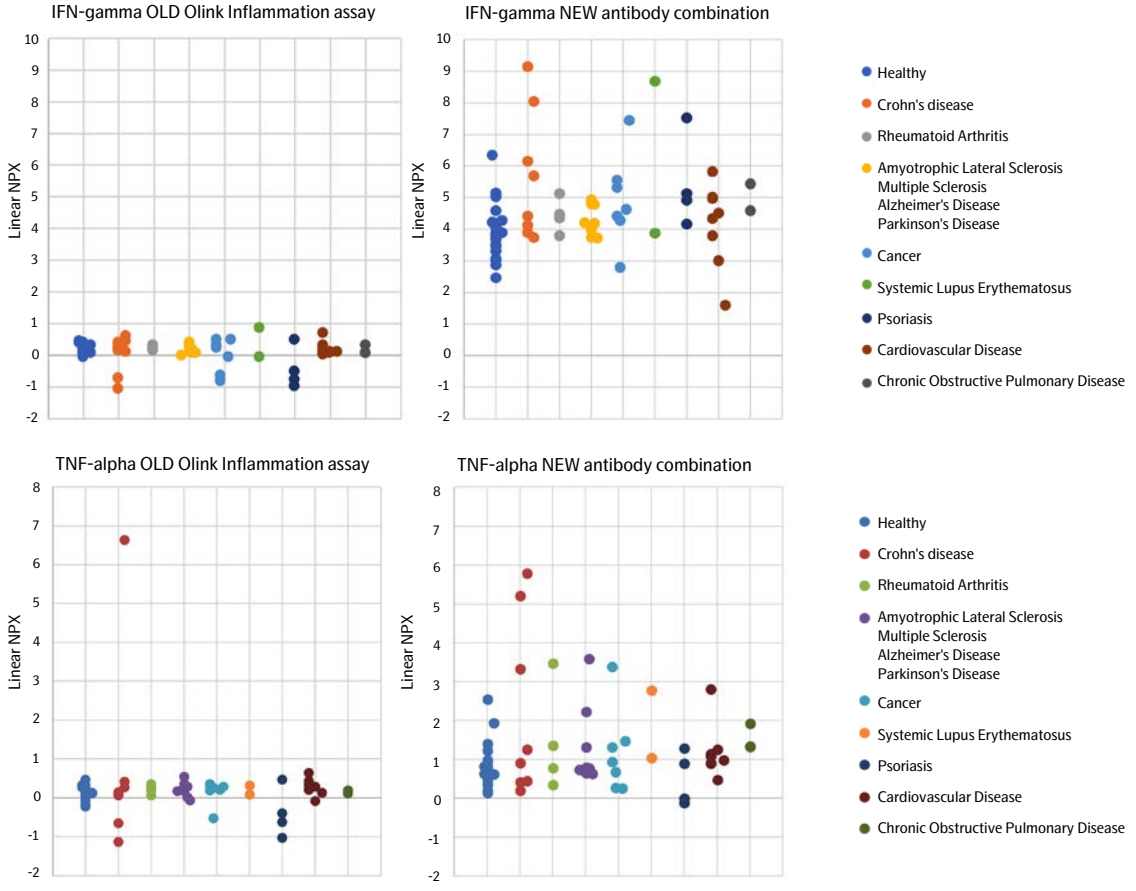


Fig 7. Illustrated assay improvement. The increased detectability with the final optimized Inflammation panel assays for INF-gamma and TNF-alpha in different diseases is shown in the plots to the right compared to the detectibility of the corresponding assay in the old panel, as shown to the left.

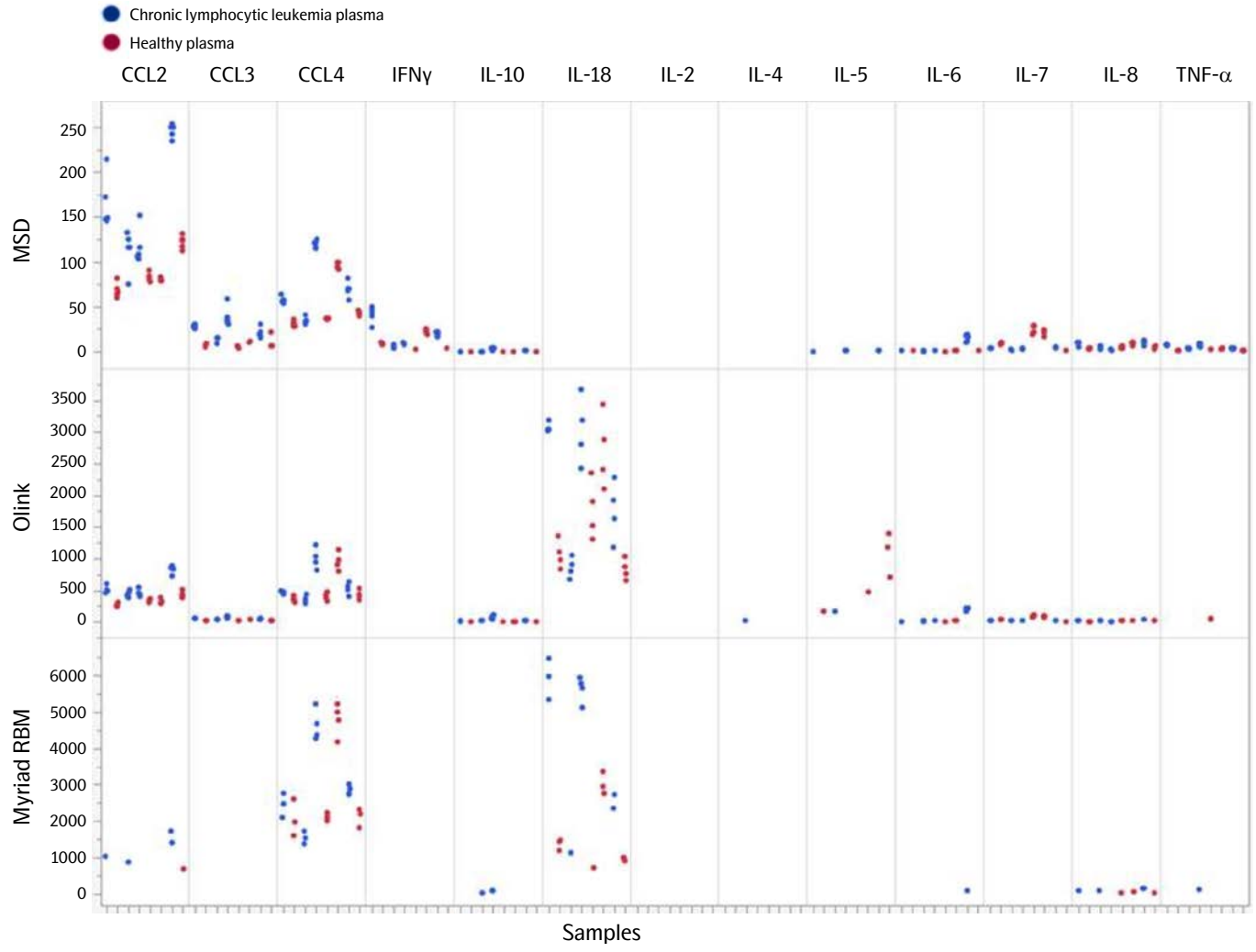


Fig 8. Illustrated comparison of MSD, Olink and Myriad RBM for utility in clinical trials. On the MSD and Olink platforms, the samples were run undiluted and with 1:2, 1:4 and 1:8 dilution, while Myriad RBM had four dilutions: 1:5, 1:10, 1:20 and 1:40 dilution. IL-18 was not tested on the MSD platform. The y-axis shows dilution corrected concentrations across platforms and the x-axis indicates the eight samples tested for each assay.

High multiplex non-antibody-based assay

In a study by Sun *et al*., protein data from Somalogic™ needed to be validated with another method. A subset of the samples were analyzed using the Olink Inflammation, CVD II and CVD III panels (5) and effect-size estimates were strongly correlated between the two platforms for a selected subset of protein quantitative trait loci (pQTLs) identified by the Somalogic analysis ($r = 0.83$).

The correlation was examined unidirectionally (Somalogic to Olink), however, so there was no data regarding any additional pQTLs that may have been identified using the Olink protein data alone. This may be significant given that the frequency of cis-pQTLs identified by Somalogic analysis among the large number of proteins examined was significantly lower than has been reported using Olink panels for protein measurements (6).

Genomics

SCALLOP (www.olink.com/scallop) is a collaborative framework for discovery of protein quantitative trait loci (pQTLs) and novel biomarkers using Olink panels for the protein analysis. In one landmark publication (6) they reported a substantial frequency of cis-pQTLs (41 for the 83 proteins examined). This provides very strong orthogonal validation of specificity via genomics, since the polymorphisms identified are co-located with the gene encoding the protein of interest.

In an unpublished study by Mälarstig *et al*. a significant number of CVD-linked proteins were measured in blood using Olink and evaluated for cis-pQTLs, using classic epidemiology and correlation studies. If individuals with higher risk of disease have higher levels of a protein, the protein may be associated with disease. However, this does not necessarily mean that the

protein causes disease; it may merely be a consequence of the disease process. To establish whether a protein causes disease, a genetic approach, insensitive to reverse causation, can be used. Instead of correlating the levels of the protein itself, gene variants that regulate the protein levels are used in the analysis, in combination with mendelian randomization. This approach requires prior knowledge of which genetic variants are linked to individual proteins.

"We performed a genome-wide association study of plasma protein levels measured using the Olink PEA CVD I-panel by combining data from 13 study cohorts, in total encompassing almost 22 000 subjects. A total of 90 proteins passed our quality control, and of those, 80 proteins had a cis-pQTL surpassing genome-wide significance ($p < 5\times10^{-8}$). This means that for 90% of the proteins tested we had orthogonal evidence that the assay detects the expected protein." (Anders Mälarstig, Pfizer and Karolinska Institute, personal communication.)

Similar associations between Olink-determined protein levels and GWAS data for a specific protein have been presented in a multi-omics study by Mahdessian *et al.* (7) The study strongly implicates MMP-12 in large-artery atherosclerotic stroke, with plasma MMP-12 levels associated both to genetic SNPs and clinical phenotypes, e.g. incident cardiovascular and cerebrovascular events and carotid intima-media thickness progression. In addition, the functionality of MMP-12 in atherosclerotic plaques by enhancing elastin degradation and macrophage invasion was supported by transcriptomics, proteomics and immunohistochemistry.

Lack of correlation — Who is wrong, who is right?

There are occasionally cases where the data for an Olink assay does not correlate with a standard ELISA – so in that situation, is it possible to figure out which assay is correct?

Background

A problem was discovered in a study where aliquots of cell lysates and cell supernatants were analyzed using the Olink Inflammation panel at Olink Analysis Service and significant differences were observed between different cell groups for Leukemia inhibitory factor (LIF) and Leukemia inhibitory factor receptor (LIF-R). When the same cell lysates and supernatants were analyzed using ELISAs for LIF and LIF-R by a customer, however, the significant results obtained by Olink could not be repeated using the ELISA.

Experiments and results

Olink carried out a series of experiments to investigate the matter further. The recombinant LIF and LIF-R proteins in the experiments came from four different vendors. The Olink LIF-R assay did not recognize the LIF-R antigen from the ELISA kit from the same vendor as the customer had used for their ELISA run in any of the experiments (Fig 9).

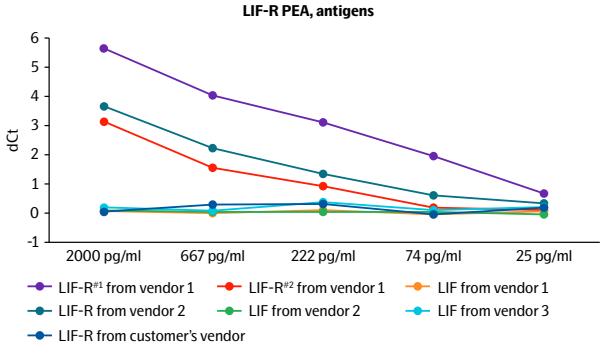


Fig 9. LIF-R PEA. The customer vendor's LIF-R antigen is undetected using the Olink LIF-R assay.

The Olink LIF assay did, however, recognize the LIF-R antigen from the vendor that the customer used (Fig 10).

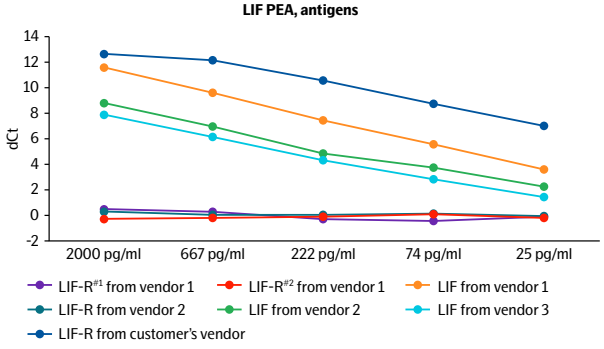


Fig 10. LIF PEA. The customer vendor's LIF-R antigen is detected using the Olink LIF assay.

The ELISA kit from the vendor that the customer used, gave significant reactivity only with LIF antigen from one of the other vendors, not with the other LIF or LIF-R antigens tested.

Conclusions

The results from Olink's investigations indicated that the LIF-R ELISA kit the customer used, actually measures a LIF-like protein sequence and that the Olink Inflammation panel measures LIF-R correctly. Contact support@olink.com for more information.

Comparing different technologies — important points to consider

There are several reasons why data might not correlate. Here is a summary of key points for what to consider before making comparisons with Olink data:

- Make sure to have a sufficient number of data points to perform a correlation and that the measuring range between these is sufficient to show correlation, not only the technical variability for the methods.
- To verify which exact protein is measured with the different methods, antigen spike-ins in the samples can be used.
- When making a direct correlation between the results of two different methods, e.g Olink and ELISA, make sure that the same samples have been used and that they are annotated correctly. Preferably the samples should be taken from the exact same vial and analyzed at the same point in time.
- NPX, Normalized Protein eXpression, is Olink's arbitrary unit which is in a Log2-format. When NPX data is to be correlated and/or plotted against the data of another method, remember to either linearize the NPX-data (2^{NPX}) or Log2-transform the linear data obtained from the other technology being tested. As an alternative to correlation plots, Bland-Altman can also be used.
- When looking to validate results by analyzing another cohort, the results will be most accurate if the same sample matrix has been used in both cohorts and the samples have been treated in a similar manner.
    - Please note that sodium-heparin plasma and EDTA plasma should not be considered to be the same sample matrix as the two anticoagulants will have their effect on different steps of the coagulation cascade, causing the sample types to have different compositions.
- If you do not find any clear correlation:
    - Investigate if the methods are measuring the same part of the protein, as the different domains of the proteins might be present in varying concentrations due to cleavage etc.
    - Check if the methods have similar measuring ranges, and that the results are within those ranges.
    - A Bland-Altman plot could tell you if you have problems at some specific range of the measurement, e.g. at the low concentration end.

Summary

Comparison of data using different technologies is a good way to validate results and this paper shows that Olink data can be validated using all the different technologies presented here. As described in the previous section, there are many important points to consider when performing comparisons between different technologies. To assist our customers with analysis of Olink data, we have a dedicated Data Science team and offer fee-for-service statistical services which can also include comparisons to other technologies.

References

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