Olink®

Olink® Reveal

Validation methods and results

Introduction

Olink® Reveal provides powerful, NGS-based proteomics to effortlessly uncover biological insights. It measures ~1,000 proteins while consuming only 4 µL of sample, with a new streamlined workflow to simplify implementation and reduce waste. Amidst these improvements quality, rigor, and transparency are important values for Olink to maintain. This document describes how each individual biomarker assay and the product as a whole was tested and validated. While the validation principles are the same, some of the procedure details differ between Olink Reveal and the previous products based on NGS readout, Olink Explore 3072 and HT. The validation data for each protein biomarker is available for download from our website.

Technology

Olink Reveal is based on Olink's innovative Proximity Extension Assay (PEA™) technology[1,2], where multiplexed oligonucleotide-labelled antibody probe pairs bind to their respective target protein in the sample. Following hybridization of the matched oligonucleotide sequences, a PCR reporter sequence is formed by a proximity-dependent DNA polymerization event. The DNA barcode is then amplified using PCR, which is subsequently detected and quantified using Next Generation Sequencing (NGS) readout, see Figure 1. Finally, the protein concentrations are calculated using a dedicated processing software and results are reported in the relative concentration unit NPX™ (Normalized Protein eXpression). Internal and external controls have been developed by Olink for data normalization and quality control. These have been designed to enable monitoring of the technical performance, as well as the quality of individual samples, providing information at each step of the Olink protocol. For detailed information regarding our controls and data normalization processes, please see the Olink White paper *Data normalization and standardization*[3] on our website.

Olink assay verification process (NGS-based products)

Every assay included in Olink Reveal undergoes a stringent 3-step, 15-factor analytical verification process.

Sensitivity, specificity, sample distribution for commonly used sample matrices (plasma, serum, cerebrospinal fluid [CSF], tissue, and cell lysate samples), as well as sample dilution linearity are all tested with defined acceptance criteria. In the first step the protein biomarkers are tested using a 96-plex format with qPCR readout. If the biomarker passes all test criteria in step one, it qualifies for the second step where a 192-plex format with NGS readout is used. The tests and acceptance criteria for steps 1 and 2 are very similar. The main differences are the plex grade and readout method, as described above.

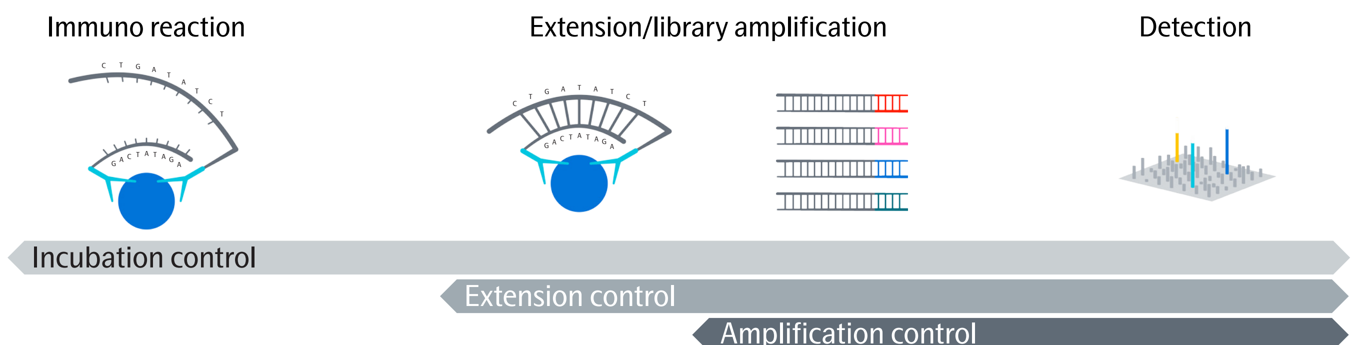

Figure 1. Olink® assay procedure (above) and built-in controls (below). The internal controls enable monitoring of the three core steps of the method and are used for quality control.

Figure 2. Example of NGS screening result. 1) Showing the calibrator curve. 2) Specificity test. 3) A range of disease and healthy samples tested in three different dilutions. Biomarker A failed in the test due to a non-approved dilution pattern for the marked samples and will hence not be part of further testing or included in the final product, while biomarker B passed and continues to the final test step.

Figure 2 shows the verification result from the second step. 1) A calibrator curve is defined using recombinant antigens, to evaluate the measurement range of the assay. 2) Specificity test pools are used to identify and exclude biomarkers with non-specific binding, where correct antigens should be detected in both set of pools. 3) To test dilution linearity, each assay is measured in a set of plasma, serum, and CSF samples from diseased and healthy individuals, as well as, control samples that are all run in 3 different dilutions. , To pass this test, the assay should show the same relative pattern of NPX levels across the samples at each dilution, and the plots should show good separation with NPX values proportional to the dilution factor used.

The biomarker assays passing all test criteria in the first two steps, were incorporated into Olink Reveal. In the final step, all assays are thoroughly tested together using the final panel design. The results and methods for step 3 are described in the following Chapter.

Olink® Reveal validation methods and results

Validation was performed at Olink R&D in Uppsala, Sweden. The results showed that the product could deliver robust and reliable proteomic data and provide a general idea of the predicted protein level in plasma samples.

Sample information

Both recombinant antigens and commercially available EDTA plasma samples were used during validation to evaluate the sensitivity, plasma protein level, precision, and scalability. The plasma samples included healthy subjects (n=21) and patients with a range of diseases including cardiovascular (n=20), autoimmune/inflammatory (n=20), neurological diseases (n=20) and cancer (n=20). Most of the disease samples had multiple diagnosed diseases. External control samples were placed on each plate in all tests.

Sensitivity

To determine the analytical measurement range for each assay, 32-point calibration curves were created using 2-fold dilutions of the antigens in 3 repeated runs starting with an initial antigen concentration of 0.0005 mg/mL. The measurement range is defined by the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ). Quantification limits are calculated using relative error <30% and CV <30% and are reported in pg/ml. The uppermost point of the calibration curve is referred to as Hook. The high dose Hook effect is seen when there is an antigen excess relative to the reagent antibodies, resulting in incorrectly low results. In such cases, a significantly lower value may lead to erroneous interpretation of results. If the antibodies are not

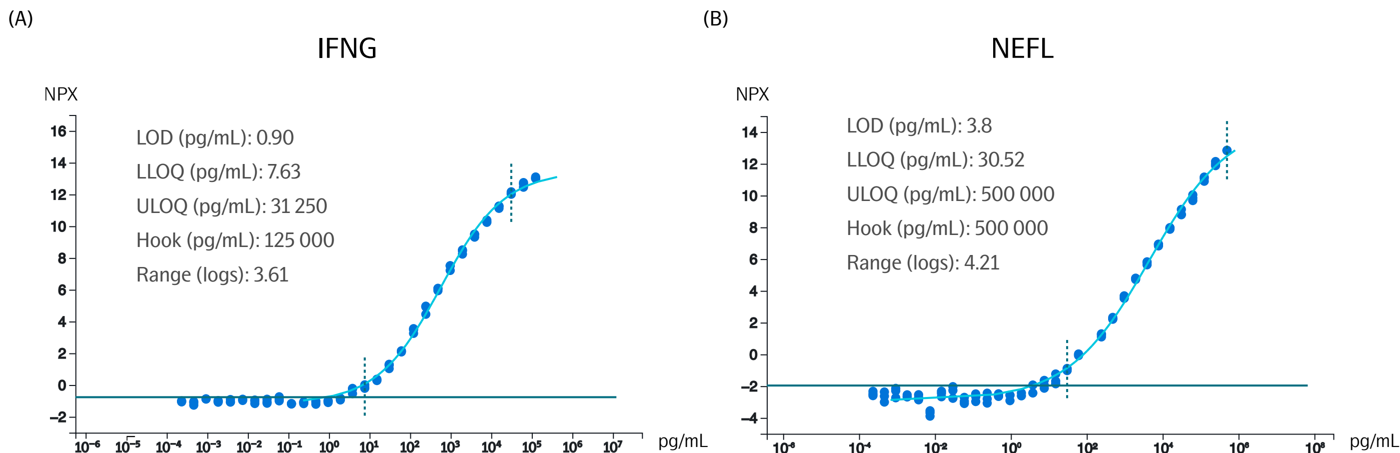

Figure 3. Calibrator curve for assay IFNG (UniProt: P01579) has reached hook (A), and NEFL (UniProt: P07196) has not reached hook (B).

saturated by the antigen (i.e., not reached hook), ULOQ will be set as the highest antigen concentration in the test. Additional information is reported in the Olink Reveal validation data, indicating whether the hook is reached.

The LOD was calculated based on 48 negative controls (NC) in the validation runs. LOD is defined as 3 standard deviations (SDs) above background as follows, and is reported in NPX:

LOD = Median (NCs) + 3SD(NCs) *or* Median (NCs) + 0.2, whichever is highest.

If a low sequencing signal is generated, i.e., the maximum number of counts in NCs ≤ 150 counts, the LOD is set to a counts level using a read count threshold. The read count threshold is equal to 2 times the maximum counts in NCs (or 150, whichever is highest).

Two examples of calibrator curves with their corresponding measuring ranges are shown in Figure 3. Note that for some assays, no suitable antigen was available, and calibrator curves could therefore not be established.

Expected protein levels in plasma

To assess likely plasma protein levels, EDTA plasma samples including healthy subjects and patients with a range of diseases were run three times in parallel. Examples of the plasma protein distribution in different groups are presented in Figure 4, where the mean NPX value from triplicate runs is presented for each sample. CXCL8 is a well-known chemokine that plays a crucial role in inflammatory responses. The distribution plot shows that CXCL8 is upregulated in most of the tested disease samples comparing to healthy controls.

Precision

Intra (within-run) variation was calculated as the mean coefficient of variance (CV) in a total of 36 individual control samples in 6 separate runs. Inter variation (between runs) was calculated as the mean CV, for the same control samples among 6 separate runs. The distributions of both intra- and inter- CVs are shown in Figure 5. The median intra-CVs and inter-CVs observed were 7.22% and 4.18%, respectively.

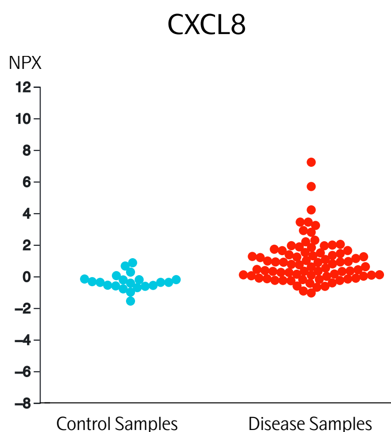

Figure 4. CXCL8 distribution in heathy control samples (blue) and disease samples (red).

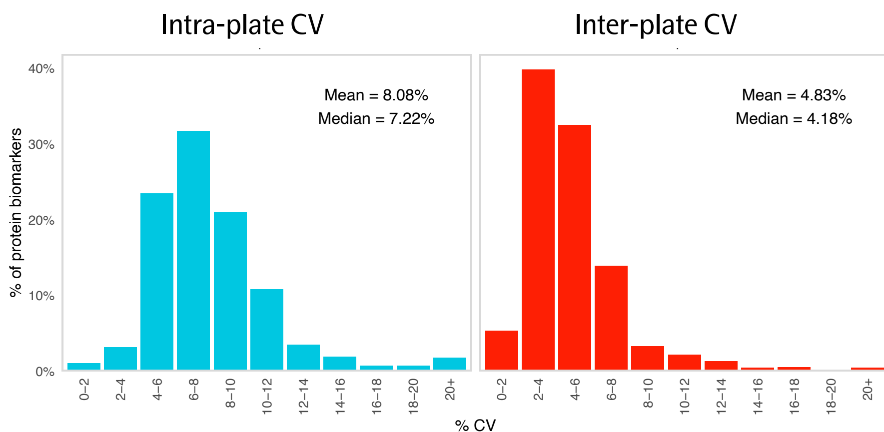

Figure 5. Distribution of intra-and inter- CVs with data above LOD.

Specificity

Specificity is of the highest importance for all immunoassays, especially for high-multiplex platforms where cross-reactivity could occur. For more information about how we test our products for specificity, please see the Olink White paper *PEA™: Exceptional specificity in a high multiplex format*[4], which is available on our website.

All assays included in Olink Reveal went through specificity testing during development of Olink® Explore HT. None of the assays exhibited cross-reactivity according to those tests and consequently, specificity testing was not repeated in the development of Olink Reveal.

Endogenous interference

Endogenous interference from heterophilic antibodies, e.g. human anti-mouse antibody (HAMA), and rheumaoid factor is known to cause problems in some immunoassays. Previous testing on Olink® Target panels showed no interference due to HAMA or RF, which indicates sufficient blocking of these agents. Testing was performed on the Olink Explore 384 CARDIO panel and confirmed absence of any inference due to HAMA and RF. Further testing on HAMA and RF was therefore not repeated for Olink Reveal.

Bilirubin, lipids and hemolysate are plasma and serum components known to interfere with some immunoassays. This has been evaluated for potential impact on other Olink panels at different added concentrations. In rare cases, altered signals are observed by the addition of hemolysate. The reason is most likely due to the specific analytes leaking out of the disrupted blood cells. Interference by bilirubin and lipids has previously been evaluated, and disturbance was only observed at extreme levels corresponding to 8 or 10 times normal values[5, 6].

Scalability

To compare the performance of Olink Reveal with the previous products, Olink Explore 3072, Olink Explore HT and Olink Target 96 Inflammation, 48 samples were run in multiple replicates on all four products. Pearson correlation coefficients were calculated for each of the overlapping protein biomarkers passing the following criteria: 1) run QC, 2) above LOD, 3) with more than 4 samples detected in both compared products and 4) IQR ≥ 0.6 on both compared products.

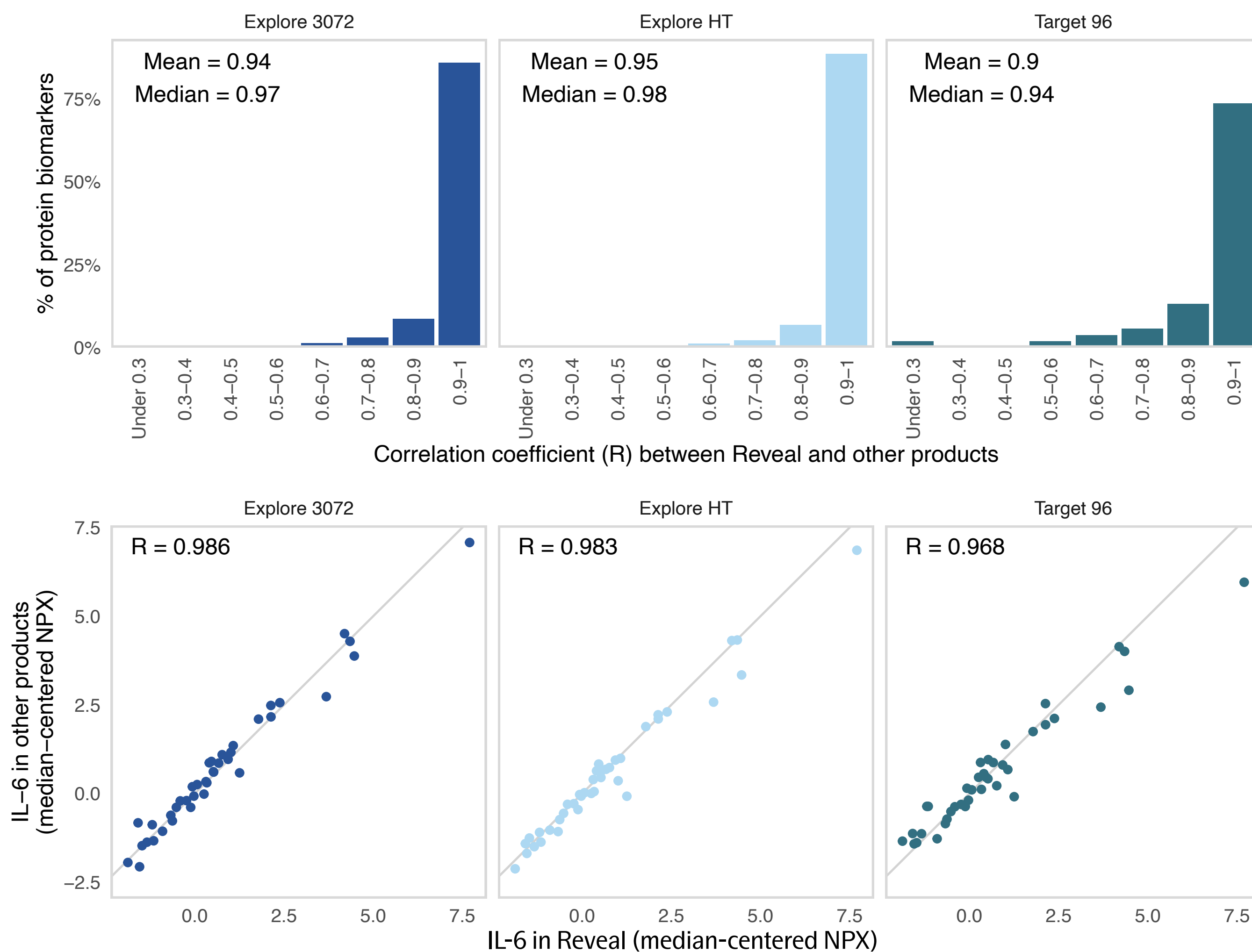

Figure 6. Distributions of correlation coefficients (top) and NPX correlation plots showing the results for the biomarker IL6 (bottom) for Reveal versus Explore 3072, Explore HT, and Target 96 Inflammation.

The distribution of correlation coefficients for all calculated overlapping assays, as well as the correlation plot of one protein (IL-6) are shown in Figure 6. This demonstrates a remarkable correlation among the different products. The median correlation coefficient (R) is 0.97, 0.98 and 0.94 when comparing Olink Reveal to Olink Explore 3072, Olink Explore HT and Olink Target 96 Inflammation, respectively.

Conclusion

The thorough, comprehensive series of QC, verification and validation processes described here illustrate Olink's commitment to quality and transparency to customers. The successful validation of Olink Reveal shows that this method for NGS-based proteomics is ready to deliver data with a more streamlined workflow while maintaining Olink's industry-leading specificity and data quality.

References

1. Lotta Wik, Niklas Nordberg, John Broberg, *et al*. Proximity Extension Assay in Combination with Next- Generation Sequencing for High-throughput Proteome-wide Analysis. *Mol Cell Proteomics 20* (2021).
2. Assarsson E, Lundberg M, Holmquist G, *et al*. Homogenous 96-Plex PEA Immunoassay Exhibiting High Sensitivity, Specificity, and Excellent Scalability. *PLoS One* April (2014).
3. Olink White paper Data normalization and standardization
4. Olink White paper PEA™: Exceptional specificity in a high multiplex format
5. http://emedicine.medscape.com/article/2074115-overview
6. http://www.nlm.nih.gov/medlineplus/ency/article/003479.htm

www.olink.com

© 2025 Olink Proteomics AB, part of Thermo Fisher Scientific.
Olink products and services are For Research Use Only. Not for use in diagnostic procedures.
All information in this document is subject to change without notice. This document is not intended to convey any warranties, representations and/or recommendations of any kind, unless such warranties, representations and/or recommendations are explicitly stated.
Olink assumes no liability arising from a prospective reader's actions based on this document.
OLINK, NPX, PEA, PROXIMITY EXTENSION, INSIGHT and the Olink logotype are trademarks registered, or pending registration, by Olink Proteomics AB. All third-party trademarks are the property of their respective owners.
Olink products and assay methods are covered by several patents and patent applications https://www.olink.com/patents/.

1588, v1.0, 2025-01-27