Olink®

Validation methods and results

Olink® Explore HT

Introduction

Olink® Explore HT is the next-generation solution for high-throughput protein biomarker discovery at any scale. It measures ~5,400 proteins while consuming only 2 µL of sample, with a new methodological design and streamlined workflow. Quality, rigor and transparency are important values for Olink. This document describes how each individual biomarker assay and the whole product was tested and validated. The validation data is available on our website www.olink.com.

Technology

The Explore HT platform is based on Olink's innovative Proximity Extension Assay (PEA™) technology[1,2], where multiplexed oligonucleotide-labelled antibody (Ab) probe pairs bind to their respective target protein in the sample. When the antibody pairs bind in proximity, the matched oligo sequences hybridize forming a PCR reporter sequence through a 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).

Built-in quality control (QC) system

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 data quality of individual samples, providing information at each step of the Olink protocol (see Figure 1).

Three internal controls are added to each sample: One Immuno Control, one Extension Control and one Amplification Control. The Immuno Control (an assay targeting a non-human antigen) monitors all three steps starting with the immuno reaction. The Extension Control is an antibody linked to two DNA tags in close proximity that provides a constant signal independent of the immuno reaction. This monitors the extension and readout steps and is used for data normalization across samples. Finally, the Amplification Control (a synthetic double-stranded DNA template) monitors the amplification and readout steps. Internal controls are mainly used to monitor the run quality at sample level, including both customer samples and external control samples.

Each 96-well sample plate contains 10 external control samples. Negative Control is included in duplicate on each plate and consists of buffer run as normal sample to assess potential contamination of assays. Plate Control is included in 5 replicates on each plate. The median of the Plate Control replicates is used to normalize each assay and compensate for potential variation between runs and plates. Sample Control is included in triplicate on each plate. These samples are used to assess potential variation between runs and plates, for example to calculate inter-assay and intra-assay CV, as well as for troubleshooting.

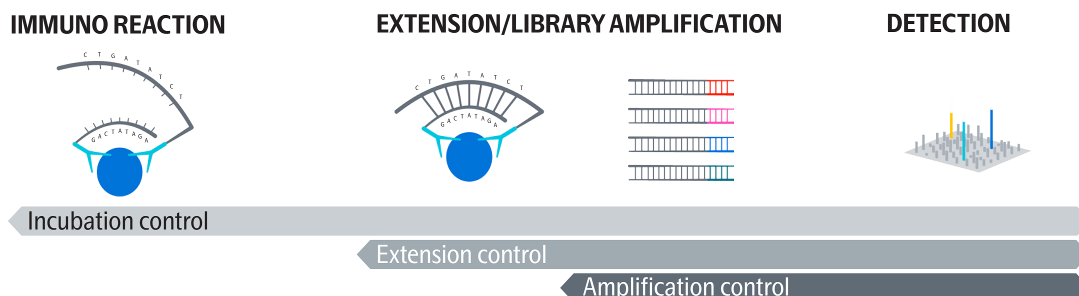


Figure 1. Olink® assay procedure (above) and controls (below). The internal controls enable monitoring of the three core steps of the method and are used for quality control.

Data normalization

Data that have passed QC undergo normalization and NPX values are generated. The NPX values are calculated in two main steps. The ~5,400 biomarkers in the library are arranged into 8 blocks depending on protein abundance and sample dilution factor as described in the Olink Explore HT User Manual. First, the counts of each biomarker from a sample are divided by the Extension Control count for that sample and block. A log2 transformation is then applied. In the last step the median of the Plate Controls is subtracted for each biomarker.

Olink Explore HT verification process

Every assay included in Olink Explore HT undergoes a stringent 3-step analytical verification process.

Sensitivity, specificity, sample distribution for commonly used sample matrices (plasma, serum, CSF and tissue and cell lysate samples) as well as sample dilution linearity are 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 is allowed to move on to 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 2 shows examples of results from step 2 in the verification testing where NGS readout is used.
1) A calibrator curve is defined using recombinant antigens, to evaluate the sensitivity and measurement range of the assay.
2) Specificity test pools are used to identify and exclude biomarkers with non-specific binding. Here the goal is to detect the correct antigen only (all other sub pools should have NPX levels below LOD). The test is repeated, and similarly only one sub-pools should be identified. If several peaks are identified the assay cross-reacts, i.e. picks up signal contribution from more than one antigen.
3) To verify the assay performance and expected NPX levels in real samples a range of plasma, serum and CSF (cerebral spinal fluid) samples from diseased and healthy individuals, as well as Explore HT Control Samples are tested. These samples are run in 3 different dilutions. Here the goal is to verify that all samples dilute well and that the NPX level pattern is repeated across dilutions.

The biomarker assays passing all test criteria in the first two steps, are incorporated into the Olink Explore HT panel. In the final step, each protein biomarker is thoroughly tested together using the final panel design. The results and methods for step 3 are described in the following section.

Methods and results

Explore HT validation was performed at Olink R&D in Uppsala, Sweden. The results showed that Explore HT could deliver robust and reliable proteomic data, and also 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 in Explore HT validation to evaluate

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

the sensitivity, plasma protein level, precision, specificity and scalability. The plasma sample included healthy subjects (n=21) and patients (n=80) with a range of diseases, including cardiovascular (n=20), autoimmune/inflammatory (n=20), neurological (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, 16-point calibration curves were created using 4-fold dilutions of the antigens, starting with an initial antigen concentration of 500 mg/mL. The uppermost point of the calibration curve is referred to as Hook. The measurement range for each protein is defined as from Limit Of Detection (LOD) to Hook-1NPX, or if Hook is not reached, as LOD to the uppermost point in the calibration curve.

The LOD is calculated based on the Negative Controls (NC) in the validation runs. LOD is defined as 3 standard deviations (SD) above background and reported in NPX. LOD can be calculated in two ways, and whichever value is highest is used.

LOD = Median (NCs) + 3SD (NCs) or Median (NCs) + 0.2

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).

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. Additional information is reported in the Olink Explore HT data sheet, indicating whether the hook is reached.

Two examples of calibrator curves with their corresponding measuring ranges are shown in Figure 3. Note that for some assays,

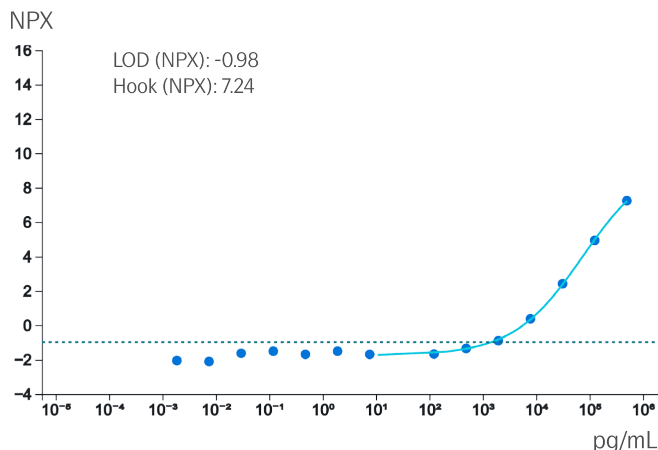


Figure 3b. Calibration curve for assay and SEPTIN3 (UniProt: Q9UH03) has not reached hook.

no suitable antigen was available and calibrator curves could therefore not be established.

Olink Explore HT offers a broad dynamic range spanning over 10 orders of magnitude, allowing the detection of high to very low abundance proteins (fg/mL to mg/mL).

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 on Explore HT three times in parallel. An example of the plasma protein distribution in different groups is presented in Figure 4, where the mean NPX value from triplicate runs is presented for each sample. NPPB is a well-known biomarker in cardiovascular studies. The distribution plot shows that NPPB has lower NPX levels and smaller variability within healthy control samples compared to the patient group.

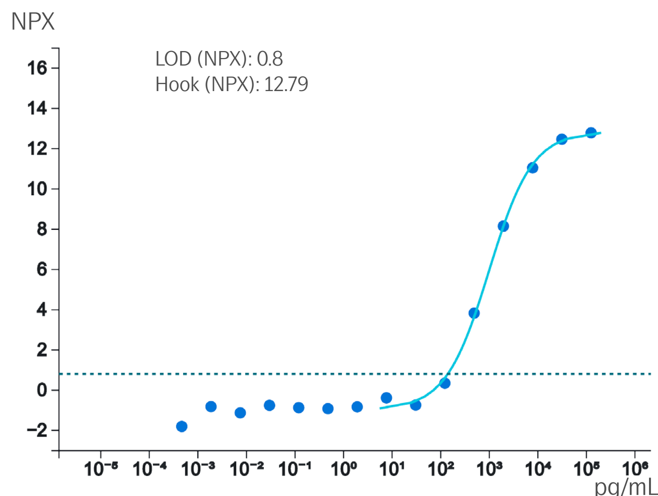


Figure 3a. Calibration curve for assay NPPB (UniProt: P16860) has reached hook.

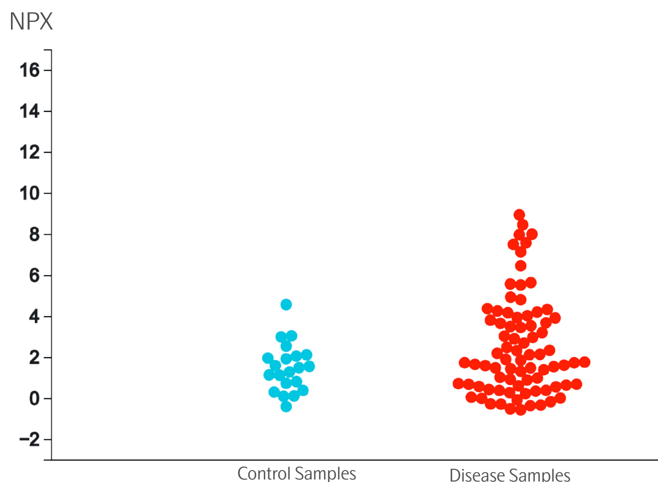


Figure 4. NPPB distribution in heathy control samples (blue) and disease samples (red).

Precision

Intra (within-run) variation was calculated as the mean coefficient of variance (CV) for 6 individual Sample Controls, within each of 3 separate runs. Inter variation (between-runs) was calculated as the mean CV, for the same 6 Sample Controls, among 3 separate runs. The distribution of both intra- and inter- CVs are shown in Figure 5. The median intra-CVs and inter-CVs observed are 10.2% and 8.8%, respectively.

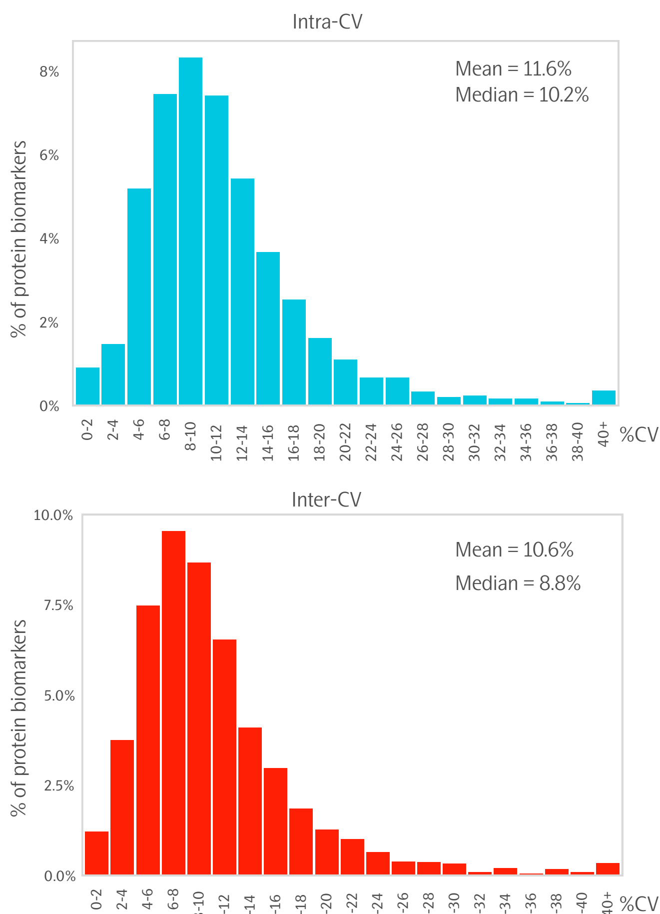


Figure 5. Distribution of intra- and inter CVs

Specificity

Specificity is of the highest importance for all immunoassays, especially for high-multiplex platforms such as Explore. For more information about how we test our products on specificity see the Olink White paper *PEA™: Exceptional specificity in a high multiplex format*[3] on the Olink website www.olink.com.

All Explore HT assays have gone through a predefined protocol with three levels of specificity testing. The third specificity test on the Explore HT product is described below.

In practice, it is not possible to test all Explore HT protein assays against each other to evaluate potential cross-reactivity. Here an alternative approach was used. First, all protein families in Explore HT were evaluated with regards to their amino acid homology with other proteins of the same protein family. The UniProt database was used to define the family of each protein, and the homology (% sequence identity) was determined with Python (Pairwise2) using full length protein sequences. Then, 179 antigens with high homology (over 80%) were selected to create the specificity test pool in order to investigate potential signal contribution from closely related proteins. The specificity test pool was divided into 64 sub-pools, each antigen was present in two different pools. All pools were diluted to 50 and 5 ng/mL and tested with Explore HT twice. If 5 ng/mL of antigen was detected in both pools and antigen with 50 ng/mL gains higher signal than 5 ng/mL, the detection was considered to be true and specific.

In total, 99.5% of assays (5389/5416) in Explore HT exhibited no cross-reactivity according to the tests described above. A total of 27 assays revealed a cross-reactive signal, meaning a signal contribution from a different protein. In all cases, this was to a closely related member within its protein family. The results for the 27 assays where cross-reactivity was observed are presented in the Explore HT validation data file. Note that the in vivo concentrations of the protein biomarkers may be much higher or lower than the concentration tested (5 ng/mL). Therefore, the signal contribution at endogenous level may differ between proteins and individuals.

Endogenous interference

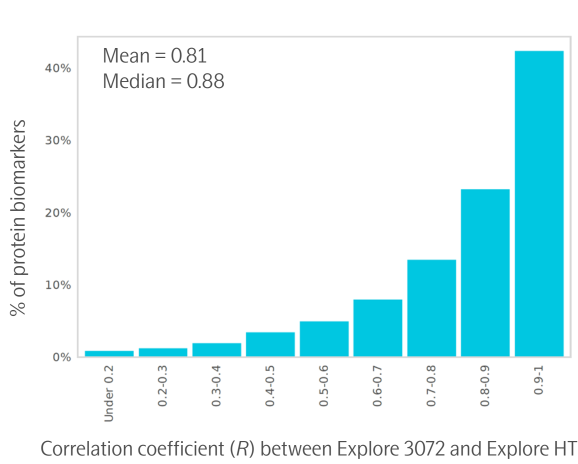
Endogenous interference from heterophilic antibodies, *e.g.* human anti-mouse antibody (HAMA), and rheumatoid factor is known to cause problems in some immunoassays. Previous testing on Olink Target panels showed no interference due to HAMA or RF, which indicates sufficient blocking of these agents. Testing was also performed on the 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 Explore HT.

Bilirubin, lipids and hemolysate, are plasma and serum components known to interfere with some immunoassays. These have been evaluated for potential impact on other Olink panels at different added concentrations. In rare cases altered signals were 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[4, 5].

Scalability

To compare the performance of Explore HT with Explore 3072 panels, 116 samples were run on both products in 3 repeated runs. Pearson correlation coefficient was calculated for each of the 2839 overlapping protein biomarkers using mean value of 3 replicates.

The correlation of assays for one protein (IL-6), as well as the distribution of correlation coefficients for all overlapping assays are shown in Figure 6. The median correlation coefficient (*R*) is 0.88. Proteins with lower correlations tended to have narrow dynamic spread among the samples tested and/or were close to the limit of detection.



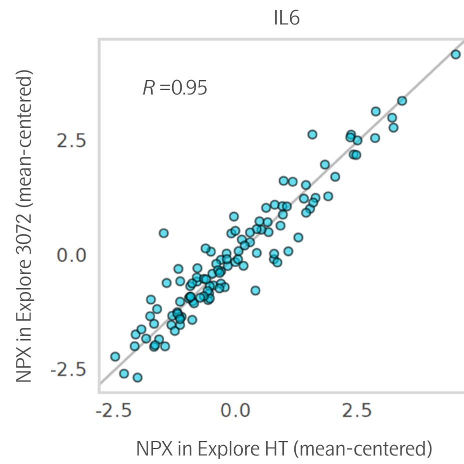


Figure 6. Distribution of correlation coefficient (left). NPX correlation plot showing the results for the biomarker IL6 on Explore 3072 versus Explore HT (right).

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 Explore HT shows that this next-generation solution for high-throughput biomarker discovery is ready to deliver robust, reliable proteomics data at any scale of project imaginable, while maintaining Olink's industry-leading specificity and data quality. This ensures that customers can gain true biological insights with great confidence.

References

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