Validation methods and results

Olink® Explore 3072

Introduction

Olink® Explore 3072 is a reagent kit for concentration measurements of human protein biomarkers. Explore comprise nearly 3000 validated protein assays arranged over eight Explore 384-plex panels, utilizing a miniaturized and automated library preparation protocol. The analytical performance of the product was carefully validated before market introduction and the results are presented in this document.

Technology

The Explore platform is based on the Proximity Extension Assay (PEA™) technology[1,2], where multiplexed oligonucleotide labeled antibody (Ab) probe pairs are allowed to bind to their respective target protein present in the sample. Following hybridization of the matched oligo sequences, a PCR reporter sequence is formed by a proximity-dependent DNA polymerization event. The DNA barcode is then amplified using PCR amplification, which subsequently is detected and quantified using Next Generation Sequencing readout. Finally, the protein concentrations are calculated using a dedicated processing software and results are reported in the relative concentration unit NPX (Normalized Protein eXpression). The assays on the Explore platform are performed in 384-plex format (see Figure 1).

Quality controls

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 assay performance, as well as the quality of individual samples, providing information at each step of the Olink protocol (see Figure 1). The internal controls are added to each sample and include 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 immune reaction. The Extension Control (an antibody linked to two matched oligonucleotides for immediate proximity that is independent of antigen binding) monitors the extension and read out steps and is used for data normalization across samples. Finally, the Amplification control (a synthetic double-stranded template) monitors the amplification and readout steps. 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 NPX processing software.

Each 96-sample plate contains eight control samples. Triplicates of an external negative control sample and a plate control sample are included in each sample plate and are used to improve inter-assay precision, allowing for optimal comparison of data derived from multiple runs. Two external sample controls are included in the control strip to estimate the precision (CV).

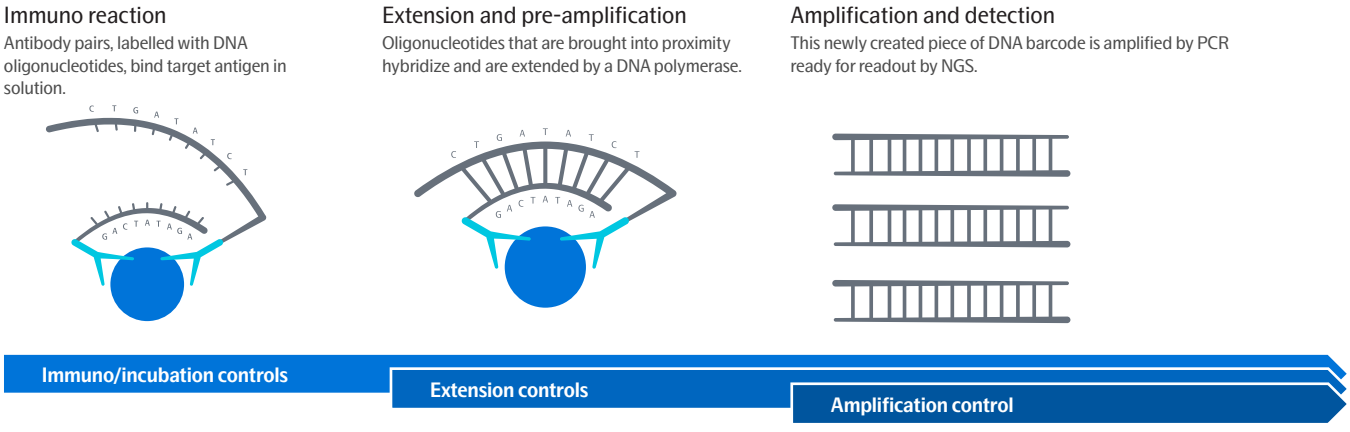

Figure 1. Olink® assay procedure (above) and controls (below). The internal controls enable monitoring of the three core steps of the method and are used for quality control and data normalization. Readout is performed using Illumina® NovaSeq™ and NextSeq™ instruments.

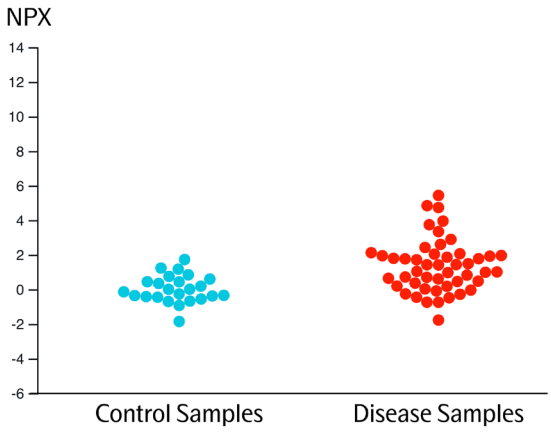

Figure 2. Example of a sample distribution plot for 24 healthy control samples and 48 disease samples.

Data analysis and protein concentration calculation

The NPX values are calculated in two main steps, followed by an optional between-plate-normalization step. First, the assay counts of a sample are divided with the Extension Control for that sample and a subpool of assays, here referred to as a block. A log2 transformation is then applied. In the last step the median of the three Plate Controls is subtracted for each assay. The optional additional step is intensity normalization which sets the median level of all assays to the same value for all plates.

Results

Explore validation data results are found on the Olink Explore website in a downloadable excel file. Validation data for each biomarker can also be found via the biomarker search function on the Olink website.

Performance characteristics

Sample information

Olink Explore 3072 was validated using commercially available serum and EDTA plasma samples from healthy subjects (n=24) and patients (n=48) with a range of diseases including cardiovascular (n=12) , autoimmune (n=12) and neurological diseases (n=12), as well as cancer (n=12). Most of the disease samples had multiple diagnosed diseases. These data provide a general idea of the NPX range to expect but cannot cover all potential levels in clinical samples.

The protein levels of the plasma samples are presented in sample distribution plots (see Figure 2). The y-axis shows NPX above background, which is defined as the median of Negative Control measurements.

Analytical measurement

Note that for some assays, no recombinant protein antigen is available and some of the data below can therefore not be presented.

Detection limit

Calibrator curves using recombinant antigen were determined in multiplex format. Limit of detection (LOD) is defined as 3 standard deviations above background and reported in pg/mL.

High dose hook effect

The high dose hook effect is seen when there is an antigen excess relative to the reagent antibodies, resulting in falsely low results. In such cases, a significantly lower value may lead to erroneous interpretation of results. Therefore, the hook threshold is determined for each analyte and reported in pg/mL.

Measuring ranges

The analytical measuring range is defined by the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ). Quantification limits of LLOQ and ULOQ are calculated using relative error <30% and CV <30% and are reported in pg/ml.

The calibrator curves are defined during the validation procedure and is found on each biomarker web page on the Olink Explore website. Two examples of calibrator curves are shown in Figure 3. Note that for some assays, no suitable antigen was available and calibrator curves can therefore not be presented.

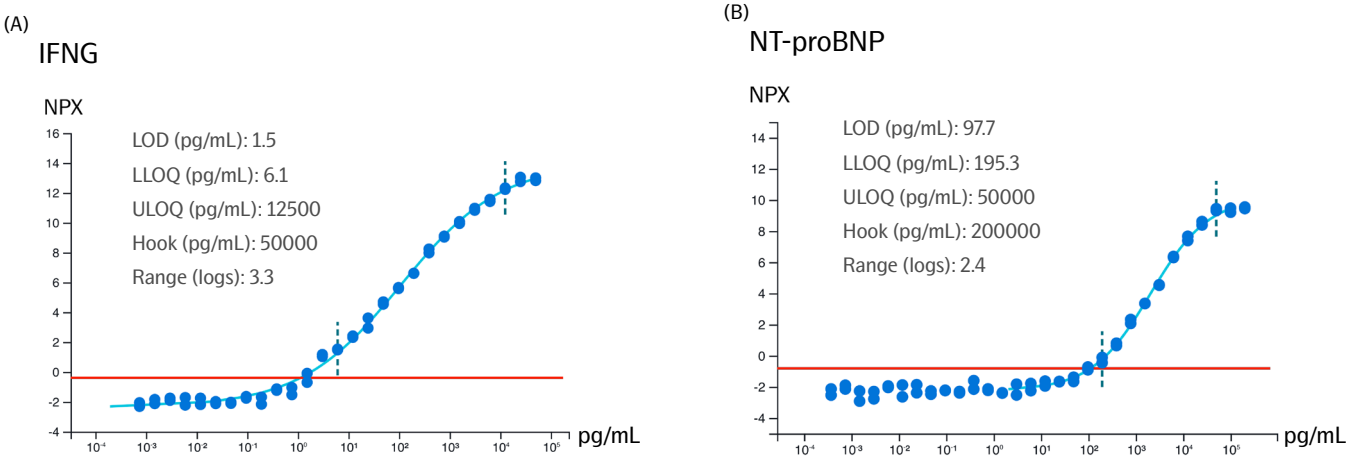

Figure 3. Calibrator curves from two assays and their corresponding analytical measurement data.

Precision

Repeatability

Intra-assay variation (within-run) was calculated as the mean coefficient of variance (CV) for 6 individual samples, within each of 7 separate runs during the validation studies for analytes where signal levels could be measured in serum and plasma. Inter-assay variation (between-runs) was calculated as the mean CV, for the same 6 individual samples, among 7 separate runs during the validation studies. The distribution of both intra-assay and inter-assay variations are shown in Figure 4.

The first four Explore 384 panels (INF, CARDIO, ONC, and NEURO) were introduced to the market in June 2020 and are here referred to as Explore 1536. Across its 1472 protein assays, the mean intra-assay and inter-assay variations observed were 8% and 11%, respectively (Fig.4 A)

The next four Explore 384 panels (INF II, CARDIO II, ONC II, and NEURO II), here referred to as Explore Expansion, were introduced to the market in Dec 2021. Across its 1472 assays the mean intra-assay and inter-assay variations were 9% and 17%, respectively (Figure 4B).

Reproducibility

Inter-site variations (between-site) were investigated during the validation at Olink's Analysis Service labs in Uppsala, Sweden and Boston, USA and at Olink's R&D lab. In addition, external Olink-certified laboratories around the world are running the Explore platform (see www.olink.com/service for details). With properly trained operators, our experience is that inter-site reproducibility meets required standards. For more information please contact support@olink.com.

Analytical Specificity

Assay specificity

Specificity is of utter importance for all immunoassays, and especially for highplex platforms such as Explore. Rigorous specificity testing was therefore performed during the development and validation. All Explore assays have gone through a predefined protocol with at least three levels of specificity testing:

1. Screening against an Olink designed antigen (Ag) pool developed over many years to detect unspecific binding.
2. After removal of poorly performing antibodies, a second screen was performed using a second Ag pool (n=92 Ags)
3. Validation of final product design was then performed against a pool of carefully selected proteins with documented high homology within their protein families (n=96 Ags).

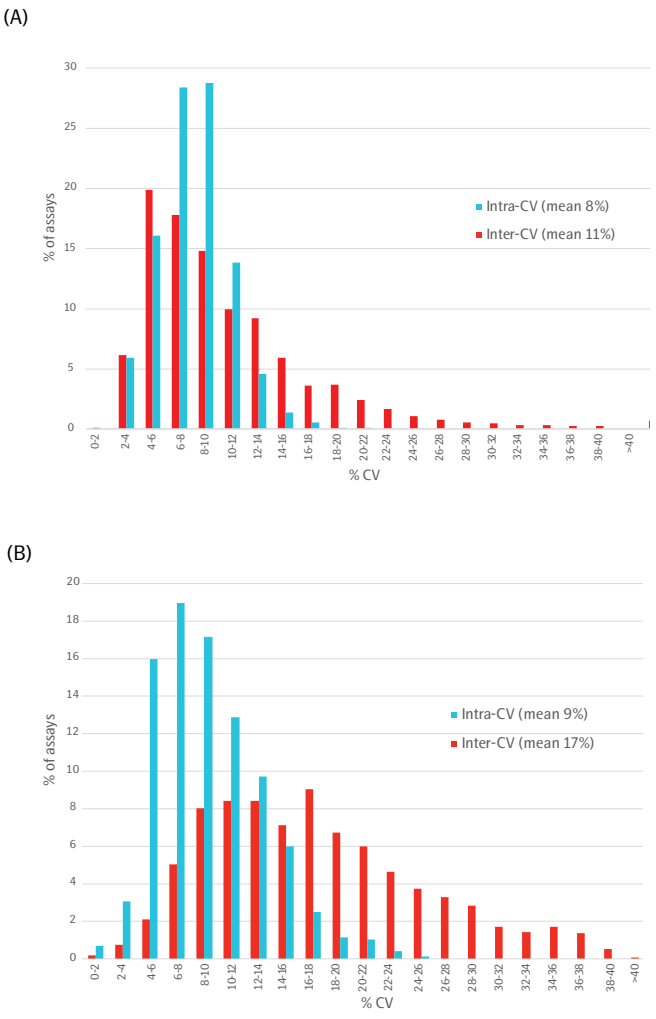

Figure 4. Distribution of intra-assay and inter-assay variations of (A) Olink® Explore 1536 and (B) Olink® Explore Expansion.

In the last step, specificity was tested by creating specificity test samples, consisting of pools of antigens, which were incubated with 96 antibody probe pairs. Ten sub-pools of antigens were created using 96 carefully selected antigens to stress test the specificity according to the following: All protein families in Explore were evaluated with regards to their sequence homology with other proteins of the same protein family. Proteins with the highest homology were selected to the specificity test sample pools. The Uniprot database was used to define the family of each protein, and the sequence homology (% sequence identity) was determined with Python (Pairwise2) using full length protein sequences.

Specificity test samples were created and the assay response to the samples were measured. In theory a signal from the pool where its corresponding antigen is included should be detected

(in the diagonal). No other signals should be observed except for the positive control pool with all 96 antigens that should detect all assays (last row in Figure 5). All Explore 3072 assays were tested against the specificity test sample pools.

For the first four Explore 384 panels (Explore 1536), 36 proteins from 15 protein families with high homology within their protein family (uniprot.org) plus 60 well-known key biomarkers from Target 96 panels were selected to the specificity test sample. For the second four Explore 384 panels (Explore Expansion) all 96 selected proteins belonged to the category with high sequence homology.

In total, 99.7% of assays (2936/2943) in Explore 3072 exhibited no cross-reactivity according to the tests described above. Nine assays revealed a cross-reactivity signal, and in all cases it was to a known closely related protein as shown in Table 1.

One example of low-level elevated response outside the diagonal represents CCL3. CCL3 shares ~58% amino acid sequence identity with its homolog protein, CCL4. The high homology is confirmed by PEA and displayed as a cross-reactive signal where the CCL3 assay recognizes CCL4. However, the signal contribution is less than 1.5% at endogenous levels. The assay CCL4 on the other hand does not recognize CCL3. For the 9 assays where cross-reactivity between homologous proteins was observed, it is noted and explained like above for CCL3 at its biomarker web pages.

Scalability

To compare the performance on assay level between an Olink panel with qPCR and NGS readouts, 308 samples were run on both the Target 96 and Explore platforms. Correlations were calculated for each of the 608 overlapping proteins and the median Spearman correlation coefficient (r_s) ranged between 0.69 and 0.83 (average 0.75). Proteins with lower correlations tended to have narrow dynamic spread among the samples tested and/or were close to the limit of detection.

The correlation between Olink Target 48 Cytokine and Olink Explore assays was also examined. The two panels have 44 overlapping protein assays. 200 samples were analyzed and the

Table 1. The nine Olink® Explore 3072 assays listed here showed cross-reactive signal contribution to a closely related protein.

Protein X		Protein Y		% Amino acid
Gene	UniProt	Gene	UniProt	Sequence homology
FOLR3	P41439	FOLR2	P14207	~83
CCL3	P10147	CCL4	P13236	~58
LHB	P01229	CGB3	P0DN86	~85
MYL3	P08590	MYL4	P12829	~82
MYL4	P12829	MYL3	P08590	~82
MYLGB	P14649	MYL3	P08590	~69
KIR2DL2	P43627	KIR2DS4	P43632	~88
KIR2DL4	P43632	KIR2DL2	P43627	~88
CACNB3	P54284	CACNB1	Q026041	~75

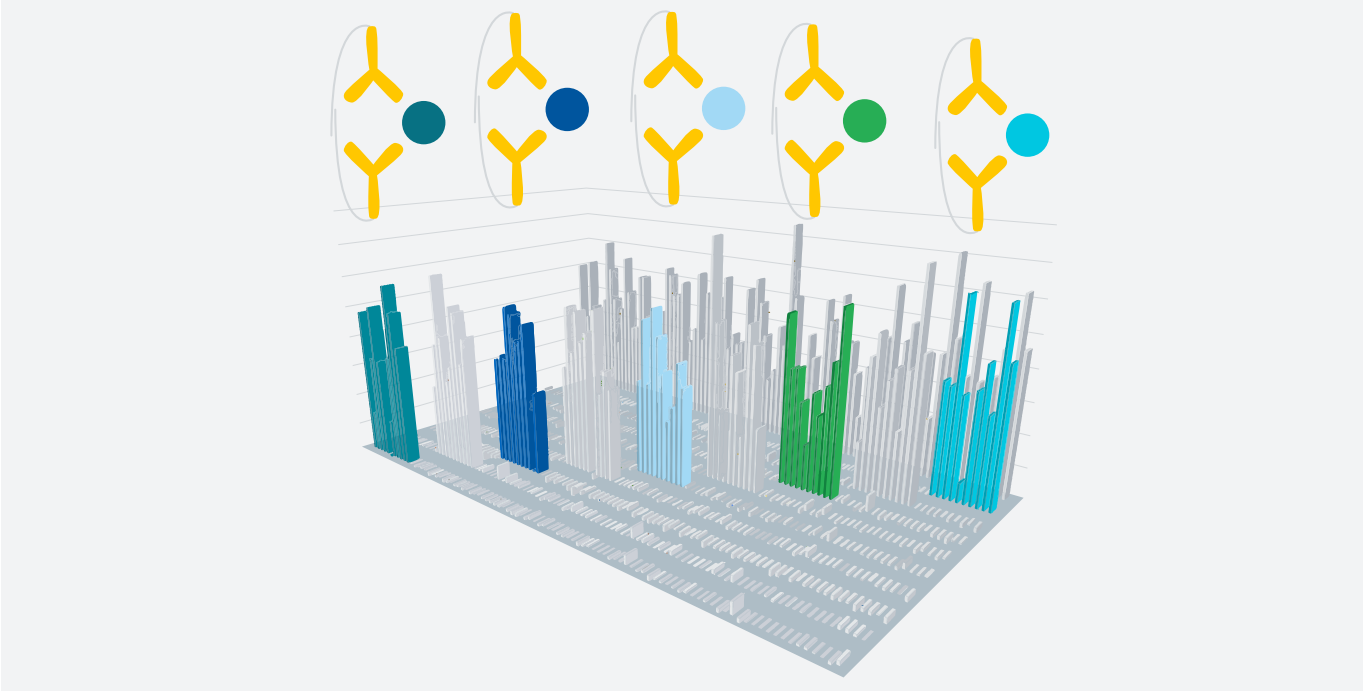

Figure 5. Assay readout specificity of the Olink® platform. For each assay, specificity is confirmed by testing antigens against the specificity test sample pools created. 99.7% of the assays exhibit no readout of cross-reactivity. Proteins with observed cross-reactivity readout are noted on the biomarkers' web pages.

median Spearman correlation coefficient (r_s) ranged between 0.50-0.99 (median 0.95). See Figure 6. For more information see the Olink White paper *Multiplex analysis of inflammatory proteins: A comparative study across multiple platforms*[3].

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 show no interference due to HAMA or RF, which indicates sufficient blocking of these agents. Testing was performed on the Explore 384 CARDIO panel and confirmed absence of inference due to HAMA and RF.

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[4, 5]. This test was not repeated for Explore.

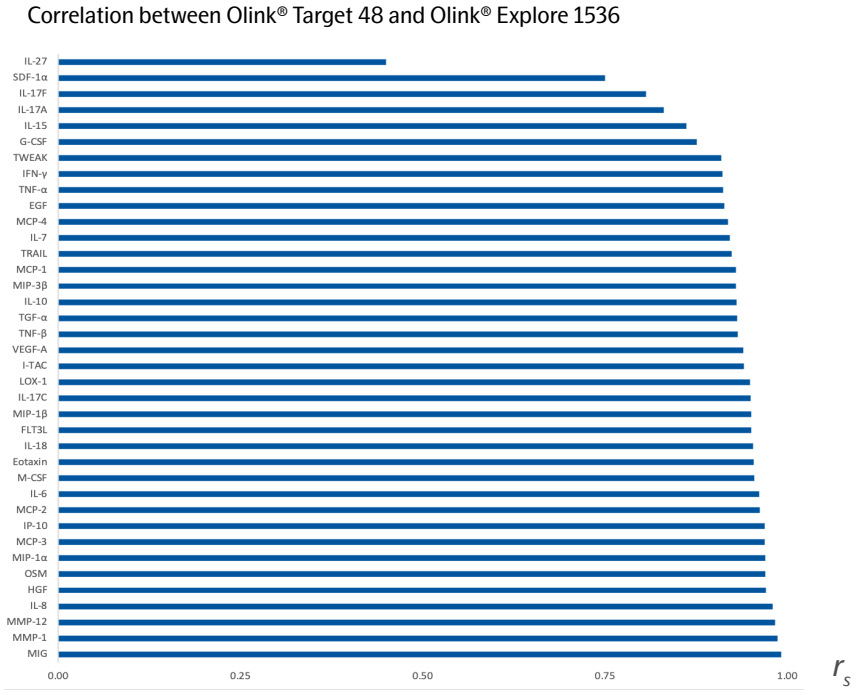

Figure 6. Spearman correlation coefficient for 39 overlapping assays with data above LOD on Explore and Target 48 Cytokine.

References

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1265, v1.1, 2023-06-19