Target 96 Oncology III

Introduction

Olink® Target 96 Oncology III is a reagent kit measuring 92 established and exploratory oncology related human protein biomarkers simultaneously. The analytical performance of the product has been carefully validated and the results are presented below. Please note that when a new panel is developed, both the individual assays and 92-plex panel as a whole are subject to our thorough validation procedure. If individual assays are subsequently improved or one or more assays are replaced in later versions of the panel, focus is placed on thoroughly validating the individual assays in question.

Technology

The Olink reagents are based on the Proximity Extension Assay (PEA) technology\(^1\), where 92 oligonucleotide labeled antibody probe pairs are allowed to bind to their respective target protein present in the sample. A PCR reporter sequence is formed by a proximity dependent DNA hybridization and polymerization event, amplified, and subsequently detected and quantified using real-time PCR. The assay is performed in a homogeneous 96-well format without any need for washing steps, see Figure 1.

Quality controls

Internal and external controls have been developed by Olink for data normalization and quality control purposes. These controls 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).

Data analysis

Data analysis was performed by employing a pre-processing normalization procedure. For each sample and data point, the corresponding Cq-value for the Extension control was subtracted, thus normalizing for technical variation within one run. Normalization between runs were then performed for each assay by subtracting the corresponding dCq-value for the Interplate Control (IPC) from the dCq-values generated. In the final step of the pre-processing procedure the values were set relative to a

Fig 1. Olink assay procedure (above) and controls (below). The internal controls enable monitoring of the three core steps in the Olink assay and are used for quality control and data normalization. Read out is performed by using the Fluidigm® Biomark™ or the Fluidigm Biomark HD system.
correction factor determined by Olink. The generated Normalized Protein eXpression (NPX) unit is on a log2 scale where a larger number represents a higher protein level in the sample, typically with the background level at around zero. Linearization of data is performed by the mathematical operation $2^{\text{NPX}}$. Coefficient of variation (CV) calculations were performed on linearized values.

**Performance characteristics**

**Sample types**
Performance with different sample types was evaluated for Olink Target 96 Oncology III by collecting matched EDTA-, acid citrate dextrose (ACD)- and sodium heparin-plasma, as well as serum samples from 4 healthy individuals. Table 1 summarizes response values for 32 normal EDTA plasma samples expressed in NPX, as well as relative differences compared to EDTA plasma. Variations observed between responses in heparin, citrate plasma and serum, as compared to EDTA plasma, were generally small, and all assays will therefore function without limitation in these sample types. In addition, cell lysates, tissue lysates and CSF were also evaluated.

**Analytical measurement**

**Detection limit**
Calibrator curves were determined for 91 out of 92 biomarkers simultaneously in a multiplex format. One protein biomarker (Q93096) lacked accessible recombinant antigen. Limit of detection (LOD) was defined as 3 standard deviations above background and reported in pg/mL for all assays where recombinant protein antigen was available, see Table 1 and Figure 2.

**High dose hook effect**
The high dose hook effect is a state of antigen excess relative to the reagent antibodies, resulting in falsely lower values. In such cases, a significantly lower value can be reported which leads to misinterpretation of results. Therefore, the hook effect was determined for each analyte, here reported in pg/mL for 91 out of 92 assays, see Table 1.

**Measuring range**
The analytical measuring range was defined by the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) and reported in order of log10, see Table 1. The upper and lower limits of quantification, ULOQ and LLOQ, respectively were calculated with the following trueness and precision criteria; relative error $\leq$ 30% and CV $\leq$ 30%, of back-calculated values, and reported in pg/mL, see Table 1.

Three assays with their analytical data are shown in Figure 2 and the distribution of measuring ranges of 90 assays and endogenous plasma levels are shown in Figure 3. Separate calibrator curves established for each assay may be viewed at www.olink.com/onc3.

**Fig 2.** Calibrator curves from 3 assays and their corresponding analytical measurement data.
Fig 3. Distribution of analytical measuring range, defined by the lower and upper limits of quantification (LLOQ-ULOQ), and normal plasma levels (highlighted bars) for 91 out of 92 analytes.
Table 1. Sample Types: Normalized Protein eXpression (NPX), Endogenous Interference, Analytical Measurement; Limit of Detection (LOD), Lower Limit of Quantification (LLOQ), Upper Limit of Quantification (UULOQ), High Dose Effect (Hook), Range and Precision indicative of assay performance are shown for 92 analytes. Not available (NA).

<table>
<thead>
<tr>
<th>Target</th>
<th>Olink Target</th>
<th>Normal plasma levels (NPX)</th>
<th>Relative to EDTA plasma (%)</th>
<th>Analytical measurement</th>
<th>Precision</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>10th %tile</td>
<td>Median</td>
<td>90th %tile</td>
<td>LOD (pg/mL)</td>
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<tr>
<td></td>
<td></td>
<td>186</td>
<td>526</td>
<td>1120</td>
<td>2244.14</td>
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</tbody>
</table>

**Interleukin-1 beta (IL1B)**

- Normal plasma levels (NPX): 46, 127
- Relative to EDTA plasma (%): 1.1, 2.1
- Analytical measurement: 2244.14, 2444.14, 500000, 100000
- Precision: 3.1, 7, 21

**Endogenous Interference**

- LOD: 2244.14, 2444.14, 500000, 100000
- LLOQ: 500000
- UULOQ: 1000000
- Hook: 102, 108, 15

**Analytical measurement**

- Range: 61.04, 61.04, 31250, 62500
- Intra: 2.7, 24
- Inter: 3.7, 24

**Precision**

- % CV: 3.7, 24
<table>
<thead>
<tr>
<th>Target</th>
<th>UniProt ID</th>
<th>Normal plasma levels (NPL)</th>
<th>Relative to EDTA plasma (%)</th>
<th>Analytical measurement</th>
<th>Precision</th>
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<tbody>
<tr>
<td>T-complex protein 1 subunit epsilon (CTES)</td>
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<td>Thiolipin 5-methyltransferase (TPMT)</td>
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<td>Thioredoxin domain-containing protein 15 (TXNDC15)</td>
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<td>Translationally-controlled tumor protein (TPT1)</td>
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**Notes:**
- **LOD:** Lower limit of detection
- **LLDQ:** Limit of linear detection
- **ULOQ:** Upper limit of quantitation
- **CV:** Coefficient of variation
Precision

Repeatability

Intra-assay variation (within-run) was calculated as the mean %CV for 6 individual samples run in triplicates within each of 9 separate runs during the validation studies. Inter-assay variation (between runs) was calculated between experiments with the same operator. The reported inter-assay %CV is the average of three operators’ %CV. Variation calculations were performed on linearized values for 92 analytes for which response levels could be measured in serum and normal plasma, see Table 1.

Across all 92 assays, the mean intra-assay and inter-assay variations were observed to be 6.4% and 20.1%, respectively. The distribution of both intra-assay and inter-assay variations are shown in Figure 4.

Replicability

Inter-site variations (between-site) were investigated during the validation of previous panels in betasite studies. Olink has Analysis Service labs in Sweden and the USA, and in addition there are many Olink-certified core laboratories around the world running the Olink platform (see www.olink.com/service for details). Our experience over several years is that inter-site reproducibility is very good providing that operators are properly trained. For more information please contact support@olink.com.

Analytical Specificity

Assay specificity

To test that the antibodies selected for use in our Target 96 Oncology III assays are specific for their desired targets, we measured each assay response to all of the 92 panel-specific proteins, as well as against an additional 107 proteins (not shown). In principle, the specificity is tested by creating a test sample, consisting of a pool of antigens, which is then incubated with all 92 antibody probe pairs from the panel. Only if there is a correct match will a reporter sequence be created and serve as a template for subsequent real-time qPCR. Ten sub-pools of antigen are evaluated to cover the 92 assays, see Figure 5. None of the Target 96 Oncology III showed significant signal from the proteins tested.

Endogenous interference

Endogenous interference from heterophilic antibodies, e.g. human anti-mouse antibody (HAMA), and rheumatoid factor are known to cause problems in some immunoassays. Evaluation of the potential
impact of this specific interference has been performed previously using a special “mismatch” system. The only way to generate a signal in this system is by antibody probe pairs being brought into proximity, by cross-binding substances other than antigens, e.g. heterophilic antibodies and similarly acting rheumatoid factor. No interference due to HAMA or RF could be detected for any of the samples in any of the previously tested panels, indicating sufficient blocking of these agents (data not shown).

Fig 6. Endogenous interference. Levels tested for hemolysate were 0.23–15 g/L hemoglobin. The highest hemolysate concentration translates to about 10% hemolysis.

The potential impact of bilirubin, lipids and hemolysate, known interfering plasma and serum components, were evaluated at different added concentrations. An example of hemolysate levels tested is shown in Figure 6. These additions represent different patient health conditions and/or sample collection irregularities. Interference by bilirubin and lipids has previously been evaluated, and disturbance has only been observed at extrem levels corresponding to 8 or 10 times normal values and therefore not performed for Target 96 Oncology III. In 31 out of 92 assays, altered signal was observed by the addition of hemolysate. The reason is most likely due to actual analyte leaking out of the disrupted blood cells. A concentration of 15 g/L of hemolysate represents 10% hemolysis of a sample. Table 1 reports the highest concentration of hemolysate that does not have an impact on assay performance.

Scalability

Assay performance was further evaluated with regard to scalability, meaning the capability of the Olink technology to maintain the same quality of performance irrespective of multiplex level. Previously, we have shown that a step-wise increase of multiplex grade (8, 24, 48, 72 and 96) does not compromise assay performance (data not shown). To further strengthen that Olink provides consistent results, single assays for Growth Hormone (GH) and Matrix Metalloproteinase (MMP-7) were compared when run in a full 96-plex reaction. The results for each assay and their observed dCq-values were plotted against the entire 96-plex reaction. The square of the correlation coefficient ($R^2$) value was generated by linear regression.

Fig 7. Scalability of the Olink technology platform. The experiment was performed using the Olink CVD II panel. Human plasma samples were analyzed in singleplex for Growth Hormone (GH) and Matrix Metalloproteinase (MMP-7) with the equivalent assays performed in a full 96-plex reaction. The observed dCq (log2) values were plotted, and the correlation coefficient R2 value was generated by linear regression.

References


Technical support

For technical support, please contact us at support@olink.com.