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TECHNICAL SUPPORT
For technical support, please contact us at support@olink.com or +46 18 444 3970
1. Introduction

Proseek® Multiplex CVD I 96×96 is a reagent kit measuring 92 cardiovascular disease related human protein biomarkers simultaneously in plasma samples. The analytical performance of the product has been carefully validated and the results are presented below.

1.1 TECHNOLOGY
The Proseek reagents are based on PEA, a Proximity Extension Assay technology, where 92 oligonucleotide labeled antibody probe pairs are allowed to bind to their respective target present in the sample. A PCR reporter sequence is formed by a proximity dependent DNA polymerization event and is 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.

1.2 DATA ANALYSIS
Data analysis was performed by employing a pre-processing normalization procedure. For each data point, delta Cq (dCq) values were obtained by subtracting the value for the Extension control, thus normalizing each sample for technical variation within one run. Normalization between runs is then performed by subtraction of the Interplate Control (IPC) for each assay. In the final step of the pre-processing procedure the values are set relative to a fixed background level 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, although it might differ between runs. Linearization of data is performed by the mathematical operation $2^{\text{NPX}}$. Statistical analyses, e.g. coefficient of variation (CV) calculations were performed on linearized values.

Fig 1. Proseek Multiplex assay procedure employs three core steps: Incubation, Extension and Detection. High throughput real-time qPCR is performed by using the Fluidigm® Biomark™ or Fluidigm® Biomark™ HD systems.
2. Performance characteristics

2.1 SAMPLE TYPES
The ability to use different sample types was evaluated with the Proseek Multiplex CVD I 96×96 by collecting matched ethylenediaminetetraacetic acid (EDTA), acid citrate dextrose (ACD), and heparin plasma samples from 5 individuals. Table 1 shows signal to background values for each sample type and assay, as well as relative percentage differences compared to EDTA plasma. The results indicated that EDTA plasma is a suitable sample type for all assays. Variations observed between responses in heparin and citrate plasma, as compared to EDTA plasma, was generally small, and most of the assays will therefore function without limitation in these sample types.

2.2 ANALYTICAL MEASUREMENT

DETECTION LIMIT
Limit of detection (LOD) was defined as 3 standard deviations above background, and reported in pg/mL for 88 proteins out of 92, for which recombinant antigen was available, see Figure 2 and Table 1.

MEASURING RANGES
The analytical measuring range was defined by the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) and reported in pg/mL. Quantification limits of LLOQ and ULOQ were calculated with the following trueness and precision criteria; relative error ≤ 30% and CV ≤ 30%, of back-calculated values, respectively. Measuring ranges were reported in order of log10. See Figure 2 and Table 1.

Calibrator curves were determined for 88 protein biomarkers simultaneously in multiplex format. Two protein biomarkers lacked recombinant antigens and two were non-purified preparations. Representative assays with their analytical data are exemplified in Figure 2 and the distribution of their corresponding measuring range per assay is shown in Figure 3. Separate calibrator curves established for each assay may be viewed at www.olink.com/products/proseek-multiplex/proseek-multiplex-cvd-i.

HIGH DOSE HOOK EFFECT
A high dose hook effect is a state of antigen excess relative to the reagent antibodies resulting in falsely lower values. If undetected, a significantly lower value will be reported which can lead to misinterpretation of results. Therefore, the high dose hook effect was determined for each analyte, here reported in pg/mL, see Figure 2 and Table 1.

Fig 2. Calibrator curves from 4 representative assays and their corresponding analytical measurement data.
Fig 3. Distribution of analytical measuring range, defined by the limits of quantification LLOQ-ULOQ, for 88 out of 92 analytes.
<table>
<thead>
<tr>
<th>Target</th>
<th>UniProt No</th>
<th>Signal-to-background (2^20^) (%)</th>
<th>Relative 2^20^ to EDTA plasma (%)</th>
<th>LLOQ pg/mL</th>
<th>Log10</th>
<th>Precision (%)</th>
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<td>91% 87%</td>
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<td>UniProt No</td>
<td>Signal-to-background (2^(-ΔCt))</td>
<td>Analytical measurement</td>
<td>Precision</td>
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<td></td>
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**Sample types**

- EDTA plasma

**Analytical measurement**

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<th>Hook</th>
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**Precision**

- Intra-assay
- Inter-assay
- Inter-site
2.3 PRECISION

REPEATABILITY
Intra-assay variation (within-run) was calculated as the mean coefficient of variation (% CV) for 7 individual samples, within each of the 9 separate runs during the validation studies. Inter-assay variation (between-run) was calculated as the mean coefficient of variation (% CV), for the same 7 individual samples, between the 9 separate runs during the validation studies. Variation calculations were assessed on linearized values for 90 out of 92 analytes. Assays with values below limit of detection were not reported, see Table 1. Across 90 assays, the mean CV intra-assay and inter-assay variations were observed to be 8% and 15%, respectively. The distribution of both inter-assay and inter-assay variations per assay is shown in Figure 4.

Olink Bioscience
Intra: 8%
Inter: 15%

β 1
Intra 1: 6%
Intra 2: 5%
Inter: 17%

β 2
Intra 1: 9%
Intra 2: 5%
Inter: 13%

Fig 5. Validation of the Proseek Multiplex CVD I 96×96 at 2 (β1-β2) different laboratories. Larger boxes shows intra-assay and inter-assay variations for each site and small boxes represent the inter-site run variations in direct comparison to Olink Bioscience.

REPRODUCIBILITY
Inter-site variation (between-site) was also investigated during the validation in a β-site study, to estimate the expected variations in values between different laboratories, with different operators and using different equipment. Seven individual samples were distributed to each site together with Proseek Multiplex CVD I 96×96 reagent kits. Each site was instructed to perform the analysis of the 7 individual samples according to the same run design. Each site was also asked to perform two independent runs.

The overall design of the β-site study enabled the estimation of both the intra-assay and inter-assay variations for 3 sites including Olink Bioscience, and the inter-site variation for each site, here shown in Figure 5.

The mean % CV value in the first analysis ranged from 6% to 9% intra-assay. The mean % CV ranged from 13% to 17% inter-assay, and 10% to 16% inter-site, here shown in direct comparison to Olink Bioscience in Figure 5.

Overall, the Proseek Multiplex CVD I 96×96 showed very good reproducibility and repeatability with average inter-site variation of 15%.

2.4 ANALYTICAL SPECIFICITY

ENDOGENOUS INTERFERENCE
Endogenous interference from heterophilic antibodies, e.g. HAMA, and rheumatoid factor are known to cause problems in immunoassays. To evaluate the potential impact of this specific interference, a special “mismatch” system was designed. The only way to generate a signal here 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. Two different “mismatched” probe pairs of varying antibody host species origin were designed and evaluated with a Heterophilic Assessment Panel from Scantibodies Laboratory Inc. (part no. 3KG027) and two sets of samples known to contain rheumatoid factor (<20-320 International Units/mL (IU/mL)) and rheumatoid arthritis (375-600 arbitrary units (AU)). No interference could be detected for any of the panel samples, indicating a sufficient blocking ability in all assays in the Proseek Multiplex CVD I 96×96.
### Table 2. Performance characteristics. Endogenous interference was performed by addition of hemolysate, lipids and bilirubin in plasma EDTA matrix. Reported are the highest tested concentrations without impact on assay performance.

<table>
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<th>Targets 1-46</th>
<th>Endogenous interference</th>
<th>Targets 47-92</th>
<th>Endogenous interference</th>
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<td>g/L Hemolysate mg/mL Lipids µg/mL</td>
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<td>g/L Hemolysate mg/mL Lipids µg/mL</td>
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The potential impact of certain known interfering serum and plasma components was evaluated by using serial dilutions of bilirubin, hemolysate, and lipids, respectively in EDTA plasma, as shown in Figure 6. These additions represent different patient health conditions and/or sample collection irregularities. No interference was detected by addition of lipids while 2 assays were observed to be affected by bilirubin and 23 assays out 92 were altered by hemolysate.

The latter is probably due to actual analyte leaking out from the disrupted blood cells rather than disturbance of the assay mechanism. Table 2 shows the highest concentrations without impact on assay performance for each component.

2.5 SCALABILITY

Assay performance was further evaluated with regard to scalability, meaning the capability of the Proseek Multiplex technology to maintain the same quality of performance irrespective of multiplex grade. A step-wise increase of multiplex grade (24, 48, 72 and 96) was performed and the observed dCq values for the 24-plex were plotted against the 48-plex, 72-plex and 96-plex for each analyte. The correlation coefficient $R^2$ value generated by linear regression analysis reflects the correlation between the multiplex assays. The $R^2$ values were >0.99 for the different multiplex blocks, as shown in Figure 7, demonstrating the scalability of the system.

![Fig 6. Endogenous interference. Levels tested for hemolysate were 0.23-15 g/L hemoglobin, lipids 0.3-20 mg/mL and bilirubin 10-630 µg/mL. The highest hemolysate concentration translates to about 10% hemolysis.]

![Fig 7. Scalability of the Proseek Multiplex technology platform. This experiment was performed using the Proseek Multiplex Oncology I panel. Human serum samples were analyzed with a 24-plex, 48-plex and 72-plex assay and the complete Proseek Multiplex Oncology panel. The observed dCq (log2) values were plotted, and the correlation coefficient $R^2$ value was generated by linear regression.]
3. References
