Olink®

White paper

Ensuring quality with flexibility: Olink® Flex validation & verification

Introduction

Olink® Flex is a mix-and-match, made-to-order product that enables the selection and combination of up to 21 human proteins into one customized biomarker panel.

Quality, rigor and transparency are very important values for us at Olink, and this document transparently presents some of the results from our verification and validation tests carried out during the development of Olink Flex.

With other methodologies, customized panels may require compromises on performance and data quality, but Olink's unique PEA™ technology and commitment to rigorous validation overcome these limitations. Consequently, customers can combine their assays of choice into one kit from the full library of ~200 with 99 % combinability, and full confidence that their panel will perform with high quality. The estimated mean CV for lot-to-lot variation is <10 %.

Verification

The verification was conducted on matched serum and plasma samples from 15 healthy, adult donors and 68 plasma samples from adult patients diagnosed with any of the following conditions: Asthma, Crohn's Disease, Atopic Dermatitis, Rheumatoid Arthritis, Ulcerative Colitis, Systemic Lupus Erythematosus, Cystic Fibrosis or Multiple Sclerosis. Analytical performance data from the verification, such as sensitivity, precision, biological range and specificity, can be found in the Olink Flex Validation data document. Additional stability studies were performed and the results are reported below.

Component stability study

The Calibrator and Sample Control have a new composition for Olink Flex and were thoroughly tested. The stressed stability study was set up to investigate the Calibrator, Sample Control, Negative Control and antigen pool (a pool with recombinant proteins for all protein targets in the Olink Flex library), during different conditions. Their functionality after multiple freeze-thaw cycles, and after 1 and 3 weeks stored at +4 °C was tested. Untreated components were used as reference in all stability tests.

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The **Sample Control** is a pooled human plasma sample, with spiked in recombinant antigens for low abundant proteins, to ensure that all proteins are detected. The Sample Control is used to assess potential variation between runs and plates, as well as quality control of the quantification of data in pg/mL.

The **Calibrator** is a pooled human plasma sample with spiked in recombinant antigens for proteins with low endogenous levels to ensure that all proteins are detected within Limit of Quantification (LOQ). The calibrator is used to adjust the predefined standard curve along the y-axis during development at Olink. The Calibrator is used to normalize the data.

Freeze-thaw cycles

It is important to be aware of how our components behave during repeated freeze-thaw cycles to be able to use them to their maximal potential. Five repeated freeze-thaw cycles of the Calibrator, Sample Control, Negative Control and antigen pool were carried out and an Olink Flex panel was run after the freeze-thaw cycles. The resulting NPX values are plotted in Figure 1.

When plotting the results, it could be seen that neither the Calibrator, Sample Control nor the antigen pool were significantly impacted by the freeze-thaw cycles. The same result could be seen when looking at the NPX difference between the test and the reference.

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NPX™ is an arbitrary, relative quantification unit. Olink normalizes the Ct values from the qPCR into the relative quantification unit NPX, using a series of computations. These operations are designed to minimize technical variation and improve interpretability of the results. With Olink Flex, the measured NPX value can then be translated to protein concentration in pg/mL.

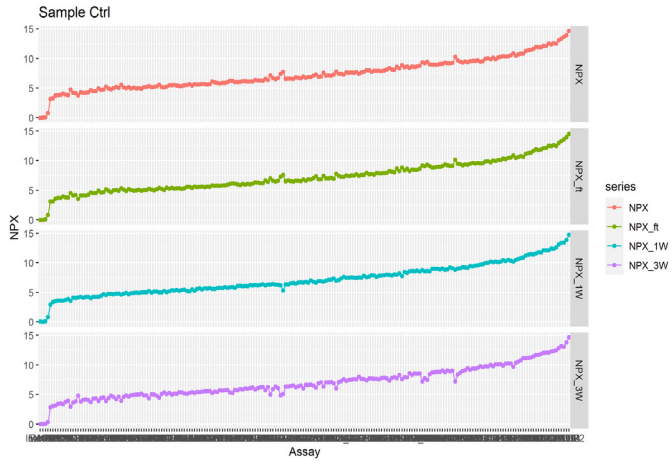

Figure 1: NPX results of the Sample Control for all assays, sorted by NPX value. Red: Before experiment. Green: After five freeze-thaw cycles. Blue: After storage at +4 °C for 1 week. Purple: After storage at +4 °C for 3 weeks.

The red and green curves in Figure 1 show the NPX results for the Sample Control before and after five freeze-thaw cycles, respectively.

The result showed that the freeze-thaw cycles did not significantly affect the performance of the kit components. All assays had an accuracy within +/- 30 %, calculated between reference and test. The highest calculated inter-CV for an assay was 19 %, which is still within the accepted limits. The data was evaluated using both relative (NPX) and absolute (pg/mL) quantification. The results showed that neither the Calibrator, Sample Control, Negative Control nor the recombinant proteins in the Olink Flex library were sensitive to repeated freeze-thawing and could still be processed with high quality.

Storage at +4 °C

The Olink Flex Control kit including the Sample Control and Calibrator is recommended to be stored at -80 °C. One part of the stability study included storage of the kit components at an incorrect temperature. After 3 weeks storage of the Calibrator and Sample Control at +4 °C, a total of 40 assays (for the Calibrator) and 37 assays (for the Sample Control) out of the complete Olink Flex library displayed an accuracy outside of the +/- 30 % criteria calculated between reference and test.

The number of assays with an accuracy below this limit more than doubled between 1 and 3 weeks of storage. Only a few assays had a very low accuracy in the test. The data showed that more antigen spiked assays than assays that were not spiked were affected by storage at +4 °C. One explanation for this observation is that native proteins are in general more stable than recombinant proteins. It is important to know that extended storage of the Calibrator and Sample Control at temperature around +4 °C can have an impact on the data for many of the assays in the library. In Figure 1 the blue and purple curves show the results after storage for 1 and 3 weeks at +4 °C.

Validation

To validate the performance of Olink Flex, sample plates were distributed to six laboratories with previous experience of running other types of Olink products, together with 20-plex Olink Flex reagent kits. The sample plates contained triplicates of 10 samples and a duplicate of a pooled plasma sample. Selected samples were run in 1:4 dilutions. The total number of assays were 20 per site, with an overlap of 14 assays that were run on all sites.

Two operators per site performed the analysis of the samples on Olink® Signature Q100 according to instructions. The intra and inter assay CVs are listed in Table 1 and the correlations between the labs for the 14 overlapping assays are listed in Table 2. For CSF2 the calculated correlation was very poor due to the low spread of data with a small difference between the sample with the highest and the sample with the lowest concentration of CSF2. Including a few samples with higher and lower concentrations of CSF2 would likely increase the correlation.

Table 1. Inter-site variation seen during beta-site validation study.

	Pooled sample (plasma)	Sample control (spiked plasma)
Intra CV	5.8 %	7.1 %
Inter CV	10.9 %	9.7 %
Inter-site CV	10.9 %	5.6 %

Table 2. Between-site correlation seen during beta-site validation study.

Protein name	Correlation (*r*-value)
IFNG	0.960
IL19	0.981
IL1B	0.903
IL6	0.993
VEGFD	0.938
CXCL10	0.967
IL10	0.968
TNF	0.996
CXCL8	0.986
CSF2	0.348
VEGFA	0.961
CCL2	0.926
IL18	0.941
HAVCR1	0.986

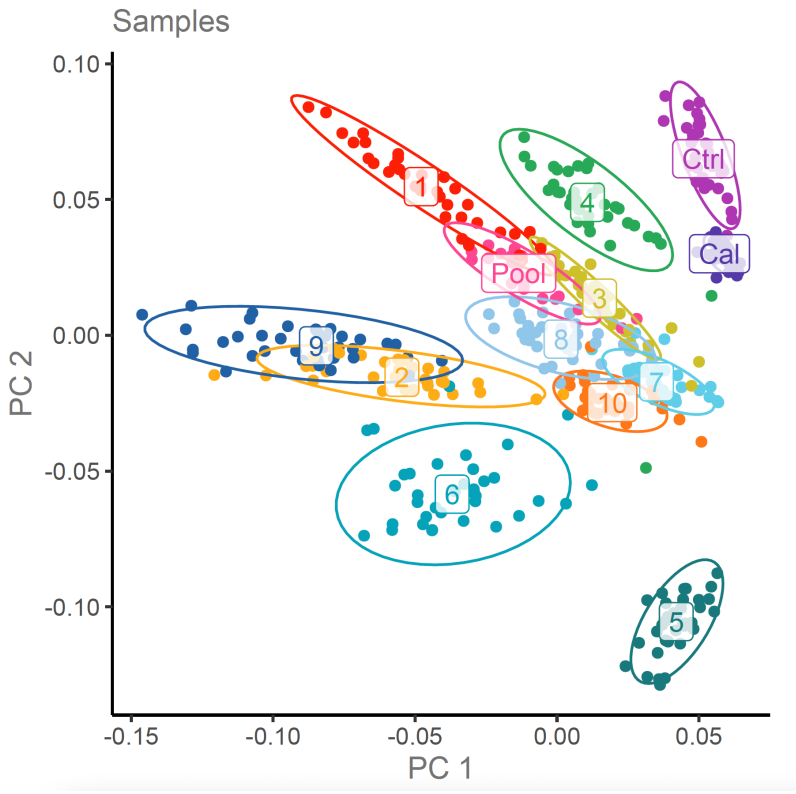

Figure 2: A PCA projection of sample replicates into a 2-dimensional space. Data from 14 assays shared between sites was used and each sample replicate is shown as a point. Only undiluted samples as well as Sample Control and Calibrator are shown. Color and label by sample ID.

Principal Component Analysis

A PCA was created to evaluate how sample types clustered across sites. The 14 shared protein biomarkers were included in the plot shown in Figure 2.

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Principal Component Analysis (PCA) is a statistical technique that is used to analyze and interpret data. It is a method for reducing the dimensionality of large data sets, while still retaining as much of the information as possible. PCA transforms the data into a new coordinate system, where the first coordinate represents the direction of maximum variance in the data, and subsequent coordinates represent directions of successively lower variance.

It is apparent that overall replicates of the same sample cluster together while there is no obvious separation by site in the dataset. This can be seen as indication that for the totality of the 14 assay fingerprint, the biological signal originating from the different samples prevails over the variance between sites.

There are many laboratories around the world trained by Olink to run panels (see www.olink.com/service for details). Our experience over several years is that inter-site reproducibility is very good provided that operators are properly trained, although technical variation between sites must be considered in experimental designs. For more information please contact support@olink.com.

Absolute quantification with Olink®

In absolute quantification using the standard curve method, you quantify unknowns based on a known quantity. First you create a standard curve; then you compare unknowns to the standard curve and extrapolate a value. Common practice is to produce a standard curve to run on each plate, using a standard provided by the vendor that then is diluted into the correct concentration by the operator. Olink has developed a different way of performing absolute quantification using a calibrator system and pre-defined calibration curves. A calibrator in triplicate is run on each sample plate and the median value of that calibrator is used to adjust the predefined standard curve along the y-axis as described in Figure 3.

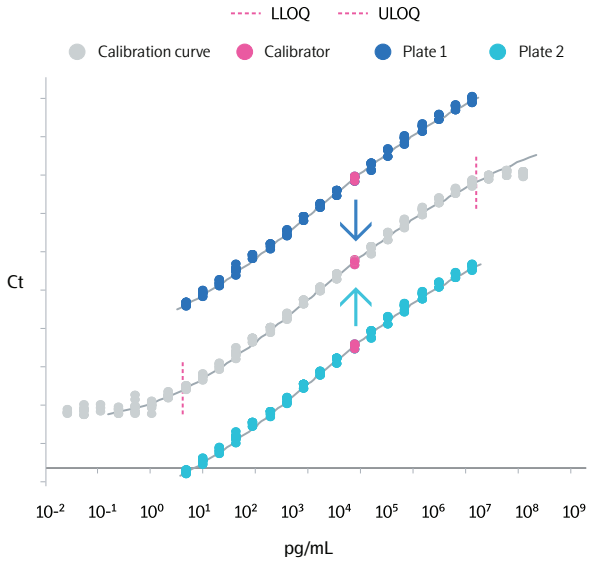

Figure 3: An example of a point standard curve defined for each assay during development.

Standard curve at Olink

During product development, a thorough fine-tuned 24-point standard curve is developed for each protein biomarker (see Figure 4). Multichannel pipetting and numerous replicates of the curves are used to minimize errors and establish an accurate immunoassay curve fitting. The pre-defined standard curves avoid operator dependent reconstitution and pipetting of standard curves at each lab and for each run. Standard curves for each assay can be found via the panel product page (www.olink.com/flex).

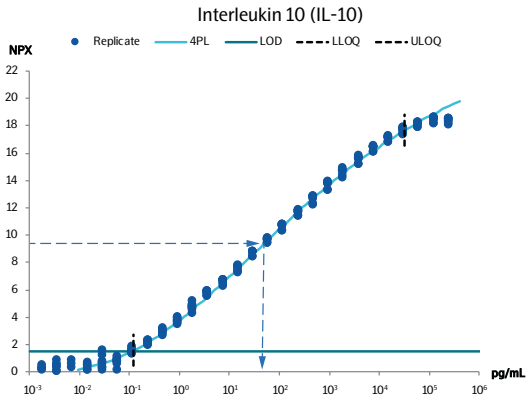

Figure 4: An example of a point standard curve defined for each assay during development.

4PL model

A 4PL model fit is performed to define the standard curve mathematically in the measurement range for each protein in the panel. A 4PL model curve fit is used to describe the immunoassay standard curve, indicated by the turquoise line in Figure 4.

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Four parameter logistic (4PL) curve is a regression model often used to analyze bioassays. They follow an s-shaped curve. This type of curve is particularly useful for characterizing bioassays because they are often only linear across a specific range of concentration magnitudes.

When running a project, the measured patient sample value (represented by the dotted blue arrows in Figure 4) is related back to the adjusted standard curve model using the Calibrator run in triplicate on each plate, indicated by the pink dot in Figure 3, which translates the measured value to the protein concentration in pg/mL (see Figure 3). Repeated testing and validation show that the 4PL curve fitting describes the standard curve well, and can be used to correctly estimate the concentration in analyzed samples within the limits of quantification (LOQ). The lower and upper limits of quantification (LLOQ and ULOQ) are defined during the development of the panel, see the panel specific Validation Data document available on the Olink website (www.olink.com) for details.

QC of assays in pg/mL

The QC of each assay is performed utilizing the Sample Controls provided with each kit. They are assessed for both accuracy and precision (CV%) for each individual assay to confirm that the concentration values in pg/mL are within the expected limits (±30 % of expected value) and assure the correct functionality of the quantification method.

Quality Control of each panel

When a customer orders an Olink Flex panel, all components have already been quality controlled and are ready to use. To make sure that the custom panel maintains the expected quality after the chosen assays have been pooled, however, each Olink Flex order is also quality controlled before shipment to customer.

The quality control is a test run of the produced kits which evaluates that:

- Correct protein biomarker assays have been combined and are measured
- Quantification is accurate, and accuracy and precision lie within expected ranges.

The Sample Controls, Calibrator and Negative Controls are included in the evaluation and the acceptance criteria have been set so that Olink Flex matches the same renowned prestanda as for all our other products. The Certificate of Analysis provided with each Olink Flex order states that this quality control has been performed.

Lot-to-lot monitoring

Olink has implemented extensive QC procedures to minimize lot-to-lot variation and to help ensure the generation of reliable data that customers can trust.

A calibrator curve, a dilution series and reference samples are analyzed simultaneously with each new batch and a fixed reference batch of PEA probes to address any differences in for example dynamic range, sensitivity or detectability. The fixed reference batch is used to avoid drift and simplify trending of data. For panels with absolute quantification, the lot-to-lot variation is additionally minimized by harmonizing the output data by calculating a bridging factor for each assay that will be applied in the data analysis software. This will give absolute quantification in standard concentration units (pg/mL).

If the performance of any assay is deemed affected, the assay is failed and re-manufactured.

Correlation with Olink® Target panels

To ensure scalability between Olink's products, the correlation of Olink Flex with Olink Target 48 Cytokine, Olink® Target 96 Inflammation and Olink® Target 96 CVD II has been evaluated. Three example plots are shown in Figure 5 and the correlation for the measured biomarkers is shown in Figure 6 .

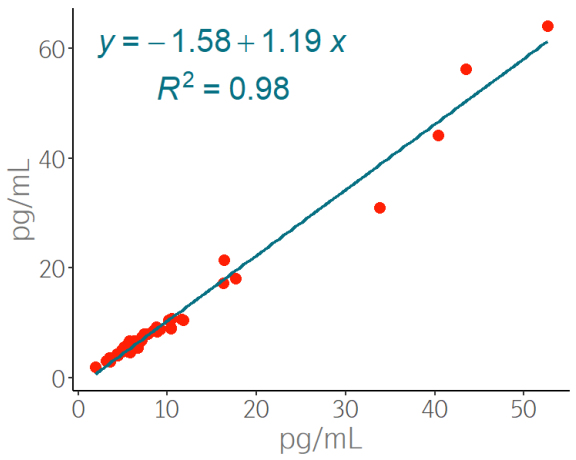
The correlation plots only contain data for which the mean of replicates is within the quantifiable range (above the lower limit of quantitation, LQL), and if there were fewer than three samples that had data within the quantifiable range for the two compared panels, no plot was generated.

Overall the correlation between products was very good with the exception of some assays where there were limited samples available with data within the quantifiable range.

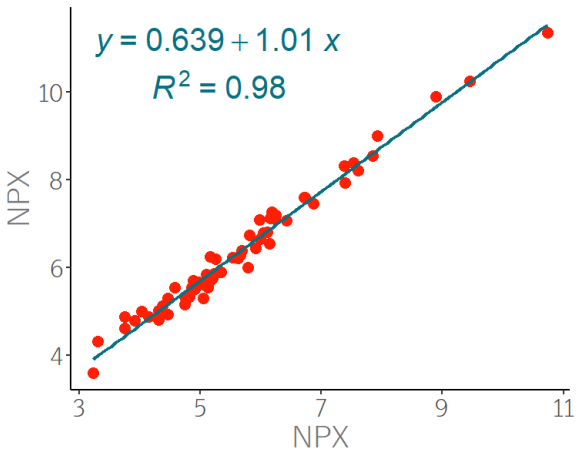
Summary

The data presented in this white paper demonstrates Olink's commitment to rigorous quality control and validation of our products, as well as our proven track record of transparently sharing this information with our customers. You can be confident that Olink Flex offers a unique combination of flexibility and data quality for the measurement of your protein biomarkers.

(A) Olink® Flex vs Target 48 Cytokine for C-C motif chemokine 3 (CCL3)



(B) Olink® Flex vs Target 96 Inflammation for Interferon gamma (IFNG)



(C) Olink® Flex vs Target 96 CVD II for Macrophage metalloelastase (MMP12)

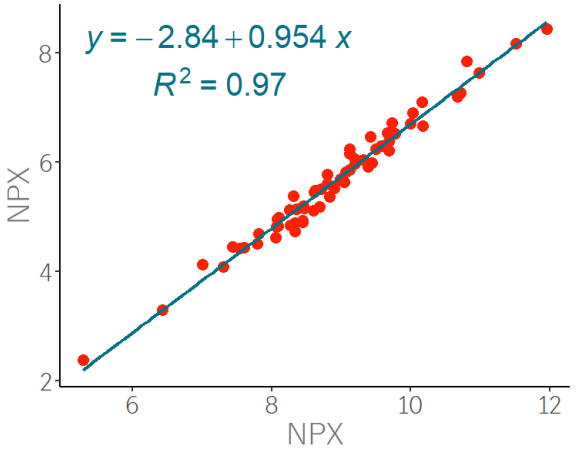
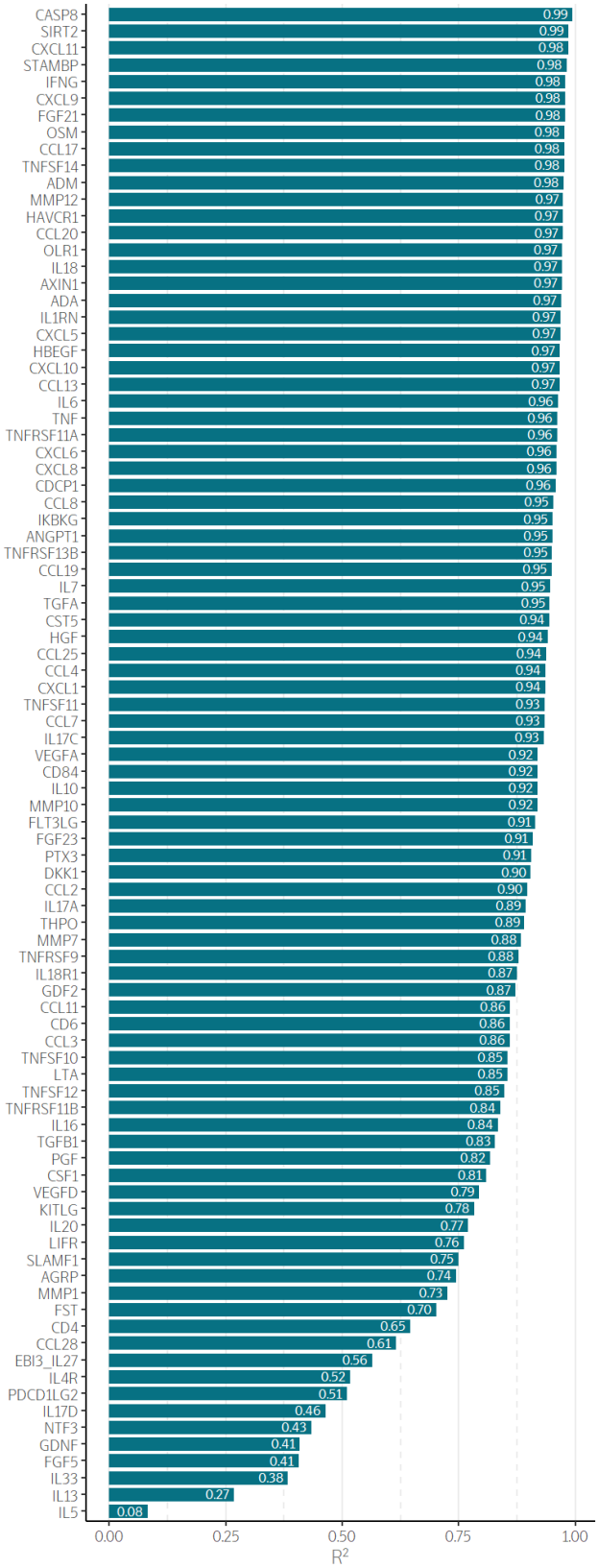


Figure 5 Correlation plots for three example assays in Olink® Flex vs Target 48 Cytokine, Target 96 Inflammation and Target 96 CVD II. R^2, the coefficient of determination, is stated in each plot.

(A) Olink® Target 96 Inflammation and Target 96 CVD II



(B) Olink® Target 48 Cytokine

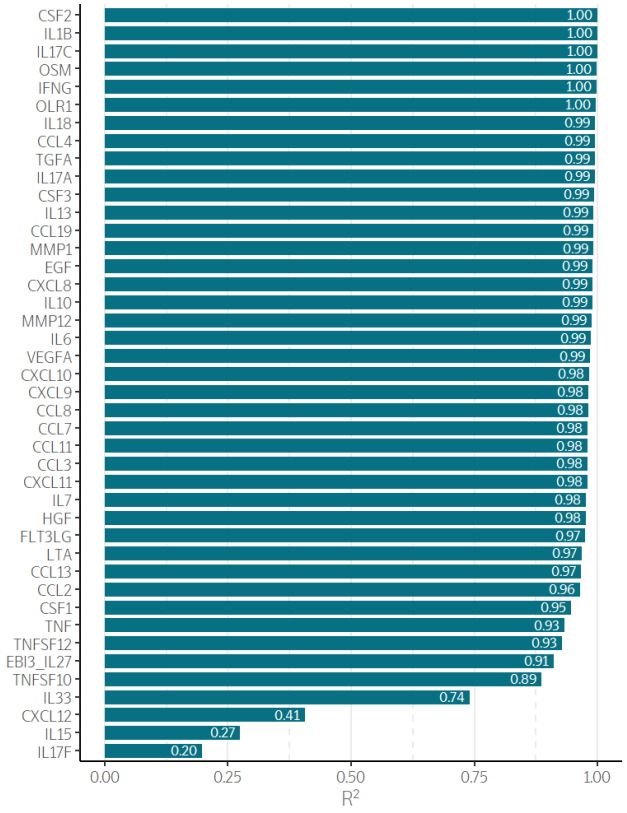


Figure 6 Correlation between Olink® Flex and (A) Olink® Target 96 Inflammation and Target 96 CVD II and (B) Olink® Target 48 Cytokine. The x-axis shows the R^2 (coefficient of determination).

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