

Validation data

Target 48 Cytokine

Introduction

Olink[®] Target 48 Cytokine is a reagent kit measuring 45 well-established, inflammation-related human protein biomarkers simultaneously. The analytical performance of the product has been carefully validated and the results are presented below.

Technology

The Olink reagents are based on the Proximity Extension Assay (PEA) technology¹⁻², where 45 oligonucleotide labeled antibody probe pairs are each 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. This is then amplified, and subsequently detected and quantified using real-time PCR. The assay is performed in a 48-plex format without any need for washing steps (see Figure 1), and results can be reported in both standard concentration units (pg/mL, default) and in relative concentration units (NPX, optional).

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 Detection control. The Immunoassay control (a non-human antigen) monitors all three steps starting with the immuno 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 Detection control (a synthetic double-stranded template) monitors the readout step. 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 software.

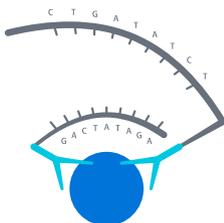
Each sample plate contains eight control samples. Triplicates of the Sample Control, duplicates of the Negative Control and triplicates of the Calibrator. The Calibrator is used in a second normalization step and is designed to improve inter assay precision, enabling optimal comparison of data derived from multiple runs. The Sample Control is used to monitor and control the quality of reported output data by evaluating both accuracy and intra assay precision for all assays. Both the Sample control and the Calibrator are composed of a pooled plasma from healthy donors spiked with recombinant proteins known to have low endogenous levels in normal plasma.

Data analysis and protein concentration calculation

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 median of the three Calibrator replicates from the dCq-values generated. The next step in the pre-processing procedure was to set the values relative to a bridging factor that bridges the data between different kit batches. The Normalized Protein eXpression (NPX)

Immuno reaction

Allow the 45 antibody probe pairs to bind to their respective proteins in your samples.



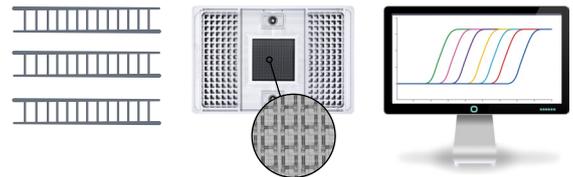
Extension and pre-amplification

Extend and pre-amplify 45 unique DNA reporter sequences by proximity extension.



Amplification and detection

Quantify each biomarker's DNA reporter using high throughput real-time qPCR.



Immuno/incubation control

Extension control

Detection control

Figure 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. Readout is performed by using the Fluidigm[®] Biomark[™] or the Fluidigm Biomark HD system.

unit generated is on a log₂ scale, where a larger number represents a higher protein level in the sample, typically with the background level at or close to zero. The protein concentration in standard concentration units (pg/mL) is obtained by fitting the NPX-value to a standard curve, describing the immunoassay shape, using four parameters in a non-linear logistic regression model. The standard curves are defined during the validation procedure and found via the panel product page (www.olink.com/t48cyt). Three examples are shown in Figure 2.

Performance characteristics

Sample information

Olink Target 48 Cytokine was validated using serum and plasma samples from 15 healthy, adult donors (15 serum samples and 15 plasma samples) and 60 plasma samples from adult patients with diagnosed inflammation-related diseases. The disease samples included 4-10 patients with each of the following diagnoses: Alzheimer's Disease, Atopic Dermatitis, Asthma, Coronary Artery Disease (CAD), Crohn's Disease, Ulcerative Colitis, Multiple Sclerosis (MS), Psoriasis, Rheumatoid Arthritis (RA), Systemic Lupus Erythematosus (SLE) or Type 2 Diabetes.

Sample types

The ability to use different sample types was evaluated by collecting matched serum, EDTA, acid citrate dextrose (ACD), and sodium heparin plasma samples from 4 healthy individuals. Table 1 summarizes the response values for 15 normal EDTA plasma samples expressed in NPX, as well as relative differences between the additional samples types 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 should therefore function without limitation in these sample types.

Analytical measurement

Detection limit

Standard curves were determined for the 45 biomarkers simultaneously in a multiplex format using recombinant proteins. Limit of detection (LOD) was defined as 3 standard deviations above background and reported in pg/mL (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 low values. In such cases, a significantly lower value can be reported, which leads to erroneous interpretation of results. Therefore, the hook effect was determined for each biomarker reported in pg/mL, 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. To ensure accurate quantification from lot to lot Olink establish release specifications for the limits of quantification (LOQ) for every manufactured lot. The analytical measuring data shown in Table 1 is based on the

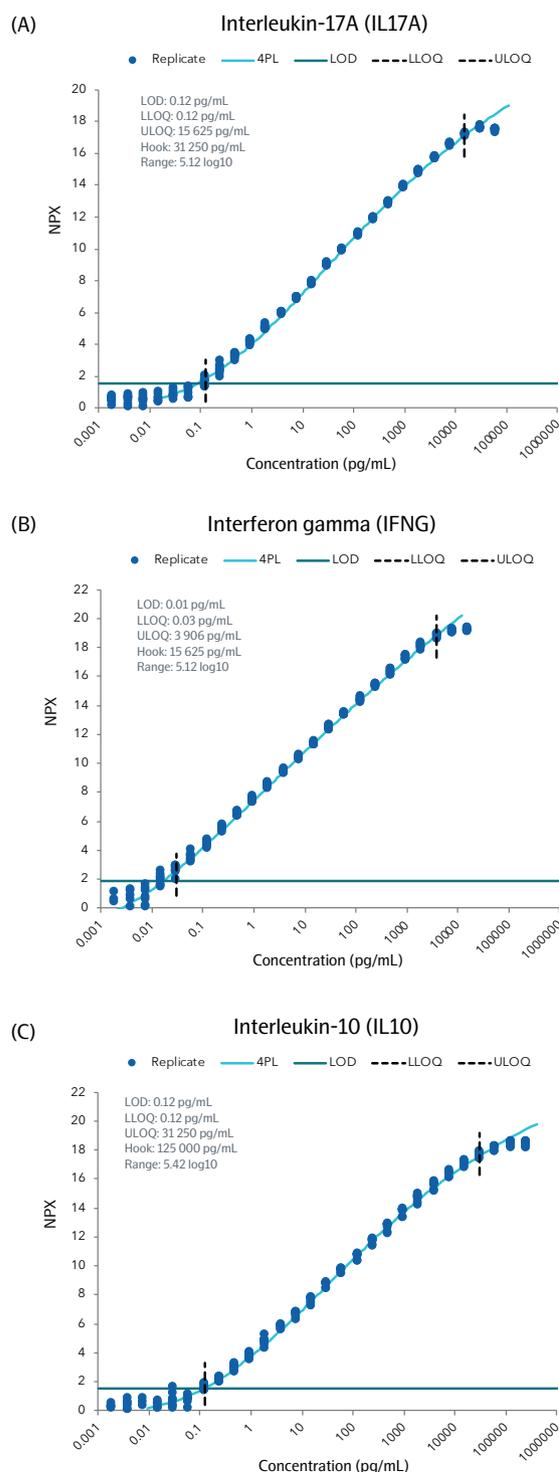


Figure 2 Calibrator curves from 3 assays and their corresponding analytical measurement data.

validation results during product development. The upper and lower limits of quantification (ULOQ and LLOQ, respectively) were calculated and reported in pg/mL with the following trueness and precision criteria relative error <30% and CV <30%, of back-calculated values (see Table 1). Separate calibrator curves were defined for each assay and can be accessed via the panel product page (www.olink.com/t48cyt) together with the analytical data for the assay. Three examples of assays and their analytical data are shown in Figure 2. The distribution of measuring ranges of the 45 assays and endogenous plasma levels for healthy donors are shown in Figure 3.

Table 1. Sample types; Normalized Protein eXpression (NPX), Endogenous interference, Analytical measuring range; Limit of Detection (LOD), Lower Limit of Quantification (LLOQ), Upper Limit of Quantification (ULOQ), High Dose Effect (Hook), Range and Precision indicative of assay performance are shown for the 45 protein biomarkers. Not available, NA

Target		Sample types						Endogenous interference	Analytical measuring range				Precision		
Protein name (gene name)	UniProt No	Normal plasma levels (pg/mL)			Relative to EDTA plasma (%)			(mg/mL)	(pg/mL)				log10	% CV	
		10th %tile	Median	90th %tile	ACD	Heparin	Serum	Haemolysate	LOD	LLOQ	ULOQ	Hook	Range	Intra	Inter
C-C motif chemokine 3 (CCL3)	P10147	2.6	4.4	7	78	116	153	15	0.030	0.03	1953	3906	4.8	4	4
C-C motif chemokine 4 (CCL4)	P13236	32	57	114	65	109	154	15	0.119	0.12	3906	3906	4.5	4	5
C-C motif chemokine 19 (CCL19)	Q99731	60	88	192	95	90	73	15	0.060	0.12	1953	3906	4.2	4	5
C-X-C motif chemokine 9 (CXCL9)	Q07325	27	52	145	87	108	96	15	0.119	0.12	3906	7812	4.5	3	4
C-X-C motif chemokine 10 (CXCL10)	P02778	37	65	271	84	92	104	15	0.119	0.12	1953	7812	4.2	4	4
C-X-C motif chemokine 11 (CXCL11)	O14625	10	39	183	44	210	275	1.9	0.119	0.12	1953	7812	4.2	3	4
Eotaxin (CCL11)	P51671	53	102	182	99	146	109	15	0.119	0.24	15625	62500	4.8	4	4
Fms-related tyrosine kinase 3 ligand (FLT3LG)	P49771	54	87	135	98	109	118	15	0.238	0.95	3906	7812	3.6	4	5
Granulocyte colony-stimulating factor (CSF3)	P09919	72	107	140	105	111	129	15	1.907	7.63	62500	250000	3.9	4	4
Granulocyte-macrophage colony-stimulating factor (CSF2)	P04141	0.06	0.14	0.20	NA	NA	NA	15	0.060	0.24	7812	15625	4.5	4	6
Hepatocyte growth factor (HGF)	P14210	160	212	428	74	59	193	15	0.238	0.24	15625	31250	4.8	3	4
Interferon gamma (IFNG)	P01579	0.06	0.15	0.31	92	120	117	15	0.015	0.03	3906	15625	5.1	10	11
Interleukin-1 beta (IL1B)	P01584	0.004	0.024	0.20	NA	NA	NA	3.8	0.095	0.19	3125	25000	4.8	5	5
Interleukin-2 (IL2)	P60568	0.006	0.014	0.033	NA	NA	NA	15	0.015	0.12	3906	15625	4.5	5	7
Interleukin-4 (IL4)	P05112	0.002	0.009	0.034	NA	NA	NA	15	0.015	0.06	3906	15625	4.8	5	6
Interleukin-6 (IL6)	P05231	1.3	2.5	5	104	111	132	15	0.030	0.06	3906	15625	4.8	5	7
Interleukin-7 (IL7)	P13232	0.4	1.2	3	39	110	385	15	0.119	0.24	1953	7812	3.9	3	4
Interleukin-8 (CXCL8)	P10145	2	4	12	76	153	211	7.5	0.119	0.24	1953	7812	3.9	4	5
Interleukin-10 (IL10)	P22301	2	4	9	152	116	108	15	0.119	0.12	31250	125000	5.4	3	8
Interleukin-13 (IL13)	P35225	0.10	0.27	4.1	NA	NA	NA	15	0.238	0.48	15625	500000	4.5	4	5
Interleukin-15 (IL15)	P40933	7.4	10.2	14.1	101	113	120	3.8	0.119	0.12	15625	125000	5.1	6	7
Interleukin-17A (IL17A)	Q16552	0.2	0.4	1.4	94	117	133	15	0.119	0.12	15625	31250	5.1	7	7
Interleukin-17C (IL17C)	Q9P0M4	4.3	7.5	22.0	118	98	98	15	0.238	1.91	15625	62500	3.9	4	5
Interleukin-17F (IL17F)	Q96PD4	0.09	0.33	2.6	102	119	130	15	0.238	0.24	3906	15625	4.2	5	7
Interleukin-18 (IL18)	Q14116	117	235	358	94	101	111	3.8	0.477	0.95	15625	31250	4.2	3	4
Interleukin-27 (IL27)	Q8NEV8, Q14213	1	5	13	146	144	108	15	0.060	0.24	31250	125000	5.1	6	7
Interleukin-33 (IL33)	O95760	0.03	0.08	0.16	NA	NA	NA	15	0.119	0.24	7812	31250	4.5	7	7
Oxidized low-density lipoprotein receptor 1 (OLR1)	P78380	22	41	84	111	317	777	1.9	0.238	0.95	3906	7812	3.6	3	4
Macrophage colony-stimulating factor 1 (CSF1)	P09603	103	116	140	98	108	114	15	0.060	0.12	3906	15625	4.5	3	3
Macrophage metalloelastase (MMP12)	P39900	57	122	271	115	92	132	15	1.907	7.63	15625	62500	3.3	4	6
Interstitial collagenase (MMP1)	P03956	31	161	511	212	986	1359	15	0.238	0.95	7812	31250	3.9	4	5
C-C motif chemokine 2 (CCL2)	P13500	129	196	339	111	120	123	15	0.119	0.24	3906	7812	4.2	4	5
C-C motif chemokine 7 (CCL7)	P80098	0.22	0.51	1.0	86	148	139	1.9	0.030	0.12	1953	7812	4.2	3	4
C-C motif chemokine 8 (CCL8)	P80075	7.9	24.3	60.3	71	107	194	15	0.030	0.03	1953	7812	4.8	4	6
C-C motif chemokine 13 (CCL13)	Q99616	25	70	181	57	142	237	15	0.030	0.06	1953	3906	4.5	4	5
Oncostatin-M (OSM)	P13725	0.5	1.4	4.6	45	110	293	15	0.030	0.12	1953	7812	4.2	6	8
Pro-epidermal growth factor (EGF)	P01133	4	17	53	42	171	1011	15	0.238	0.48	977	7812	3.3	4	5
Stromal cell-derived factor 1 (CXCL12)	P48061	68	190	246	77	68	80	15	30.518	30.52	31250	125000	3.0	6	7
Thymic stromal lymphopoietin (TSLP)	Q969D9	0.03	0.06	0.38	NA	NA	NA	15	0.119	0.48	7812	125000	4.2	3	4
Tumor necrosis factor ligand superfamily member 12 (TNFSF12)	O43508	129	374	574	93	106	135	15	0.954	3.81	15625	62500	3.6	4	5
Lymphotoxin-alpha (LTA)	P01374	6	10	12	96	99	141	15	0.119	0.12	3906	7812	4.5	4	6
Tumor necrosis factor ligand superfamily member 10 (TNFSF10)	P50591	264	314	544	100	113	98	15	0.477	0.95	7812	31250	3.9	4	4
Protransforming growth factor alpha (TGFA)	P01135	3	5	6	108	116	448	15	0.238	0.48	1953	7812	3.6	5	5
Tumor necrosis factor (TNF)	P01375	5	11	17	87	84	53	15	1.907	3.81	15625	62500	3.6	11	13
Vascular endothelial growth factor A (VEGFA)	P15692	129	202	329	70	97	166	15	0.238	0.48	7812	31250	4.2	4	5

Dynamic range and plasma levels



Figure 3 Distribution of analytical measuring range, defined by the lower and upper limits of quantification (LLOQ-ULOQ), and normal plasma levels (darker bars) for the 45 protein biomarkers.

The low-level single 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 for CCL4 on the other hand does not recognize CCL3. None of the biomarker assays in the Olink Target 48 Cytokine panel revealed cross-reactive signal contributions from any of the other proteins tested.

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. Evaluation of the potential impact of this specific interference was investigated during the validation of previous panels. No interference due to HAMA or RF was detected for any of the samples in previously tested panels, indicating sufficient blocking of these agents (data not shown).

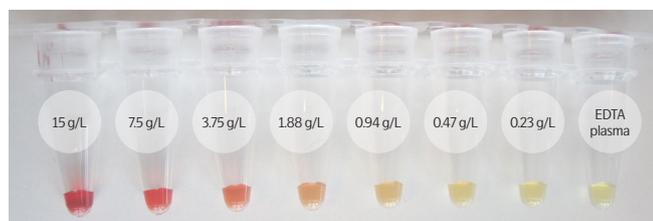


Figure 6. Endogenous interference. Levels tested for hemolysate were 0.23–15 g/L hemoglobin. The highest hemolysate concentration translates to about 10% hemolysis.

Bilirubin, lipids and hemolysate, are plasma and serum components that are known to interfere with some analytical assays. These were evaluated for potential impact on the Olink assays at different added concentrations. An example of the hemolysate levels tested is shown in Figure 6. These additions represent different patient health conditions and/or sample collection irregularities. In 7 out of 45 assays, altered signal was observed by the addition of hemolysate. The reason is most likely due to the specific analytes 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. 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^{3,4}. This test was therefore not repeated for Olink Target 48 Cytokine.

Linearity

Linearity was assessed in true matrix conditions by diluting one sample in another sample. A native plasma sample containing a relatively high endogenous level of the target protein is mixed with a native plasma sample containing a relatively low level of the protein, at different ratios, to give four equally spaced intermediate concentrations. Native samples were chosen to obtain as wide a range as possible, requiring several different sample combinations to be included in the test. The difference between the “theoretical” concentration and the “measured” concentration was analyzed. The expected (theoretical) concentrations were based on the measured concentration of the highest and lowest sample, and the theoretically calculated expected concentrations for the intermediate data points, (see Figure 7). For all but 7 assays, data within LOQ were obtained and the average accuracy over all assays was < 20%.

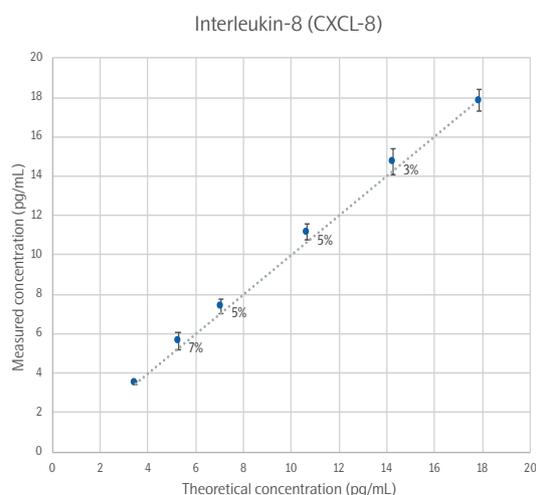


Figure 7. The difference between the “theoretical” concentration and the “measured” concentration.

References

1. Assarsson E, Lundberg M, Holmquist G, Björkesten J, Bucht Thorsen S, Ekman D, Eriksson A, Rennel Dickens E, Ohlsson S, Edfeldt G, Andersson AC, Lindstedt P, Stenvang J, Gullberg M, Fredriksson S. Homogenous 96-Plex PEA Immunoassay Exhibiting High Sensitivity, Specificity, and Excellent Scalability. *PLoS One* April (2014). doi: 10.1371/journal.pone.0095192.
2. Lundberg M, Eriksson A, Tran B, Assarsson E, Fredriksson S. Homogeneous antibody-based proximity extension assays provide sensitive and specific detection of low abundant proteins in human blood. *Nucleic Acid Res* June (2011). doi: 10.1093/nar/gkr424.
3. <http://emedicine.medscape.com/article/2074115-overview>
4. <http://www.nlm.nih.gov/medlineplus/ency/article/003479.htm>

Technical support

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



www.olink.com

For Research Use Only. Not for Use in Diagnostic Procedures.

This product includes a license for non-commercial use of Olink products. Commercial users may require additional licenses. Please contact Olink Proteomics AB for details. There are no warranties, expressed or implied, which extend beyond this description. Olink Proteomics AB is not liable for property damage, personal injury, or economic loss caused by this product.

The following trademarks are owned by Olink AB: Olink® and Olink Bioscience™.

This product is covered by several patents and patent applications including US 6,511,809, US 7,306,904 and related US and foreign patents.

© Copyright 2023 Olink Proteomics AB. All third party trademarks are the property of their respective owners.

1134, v1.2, 2023-12-22