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) technology[1-2], 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)

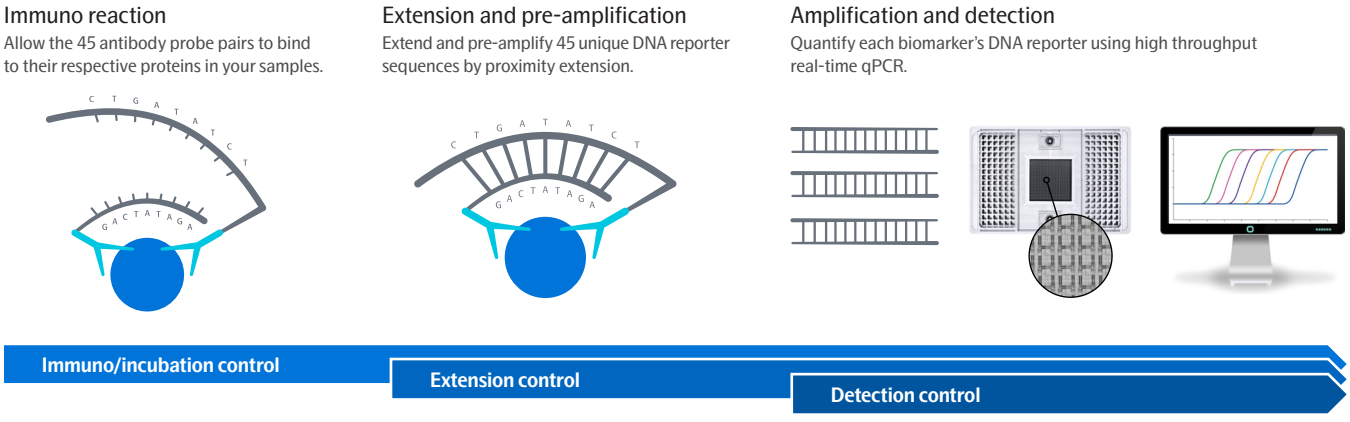

Figure 1. Olink assay procedure (above) and controls (below). The internal controls enable monitoring of the three core steps in the Olink assay and are used for quality control and data normalization. Readout is performed by using the Fluidigm® Biomark™ or the Fluidigm Biomark HD system.

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 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.

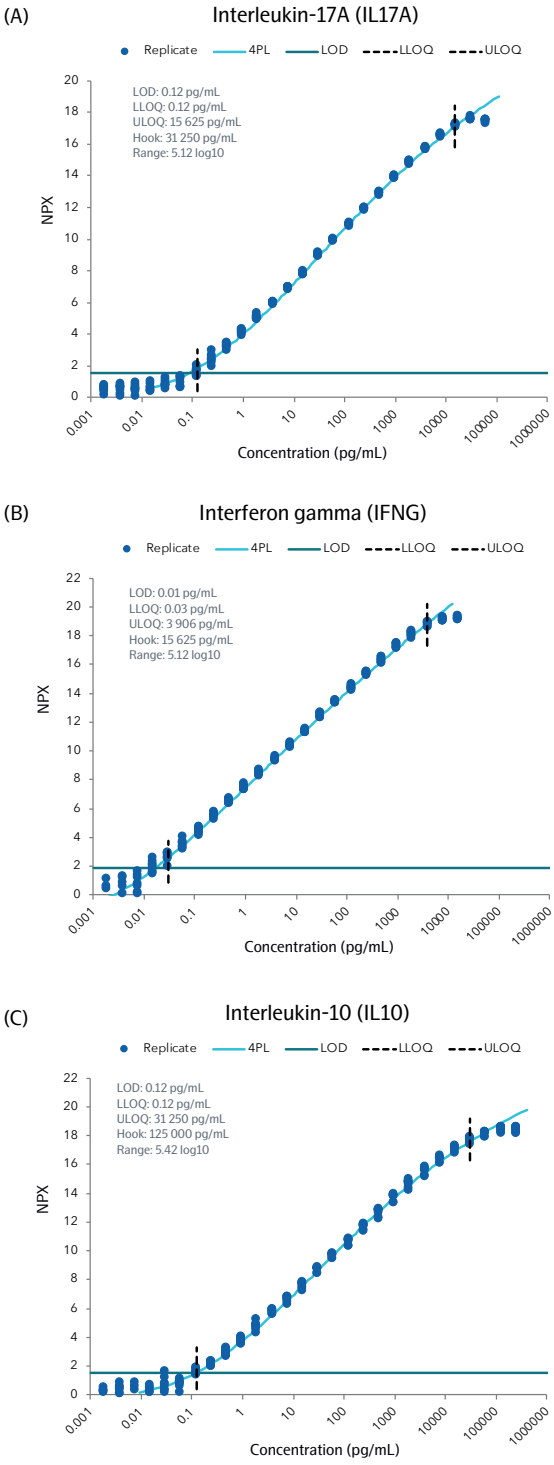

Figure 2 Calibrator curves from 3 assays and their corresponding analytical measurement data.

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	
		Normal plasma levels (pg/mL)			Relative to EDTA plasma (%)			(mg/mL)	(pg/mL)				log10	% CV	
Protein name (gene name)	UniProt No	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)	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

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.

Precision

Repeatability

Inter (between run) and intra (within run) CV were assessed by evaluating triplicate measurements of the Sample Control on each plate, based on 12 plate runs performed by four different operators.

Inter assay variation (between runs) was calculated between experiments with the same operator. The inter assay %CV reported here is the average of the four operators' %CV. CV calculations were performed on data in pg/mL for the 45 analytes for which response levels within LOQ were detected, see Table 1.

Across the 45 assays, the mean intra assay and inter assay variations observed was 4% and 6%, respectively. The distribution of both intra assay and inter assay variations are shown in Figure 4.

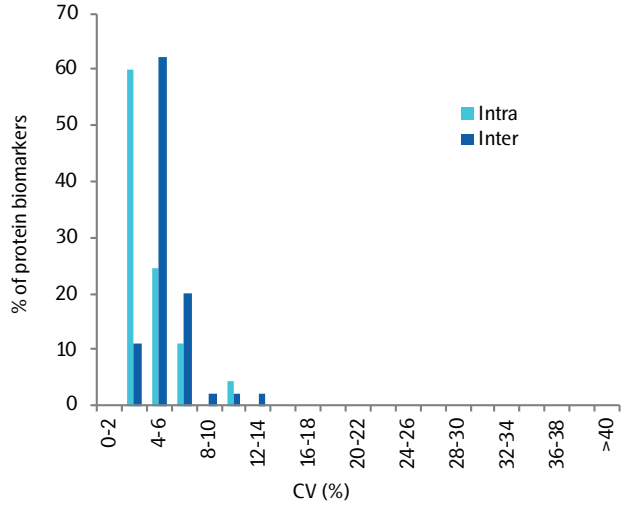

Figure 4. Distribution of intra-assay and inter-assay variations of Olink Target 48 Cytokine.

Reproducibility

Inter-site (between sites) and lot-to-lot (between batches) variation were investigated during the validation in a beta-site study. 15 individual samples were distributed to two laboratories together with two batches of Olink Target 48 Cytokine reagent kits. Each site performed the analysis of the 15 individual samples according to instructions for two independent runs, one with each kit batch and run by two operators. The intra assay mean CV value results for beta-site 1 and 2 were 7% and 7%, and the mean inter assay CVs were 10% and 8%, respectively. Overall, Olink Target 48 Cytokine showed good reproducibility and repeatability. In addition to Olink Analysis Service laboratory in Uppsala, Sweden and in Boston, US, there are many Olink-certified core laboratories around the world running Olink 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. For more information please contact support@olink.com.

Analytical Specificity

Assay specificity

To test that the antibodies and assays in Olink Target 48 Cytokine are specific for their desired targets, we measured each assay response to all of the 45 proteins (recombinantly produced) in 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. Each assay should only recognize its own target and no other protein in the panel, which is demonstrated as elevated levels along the entire diagonal in Figure 5. The last row contains a positive control sample where all proteins should be elevated as shown in Figure 5.

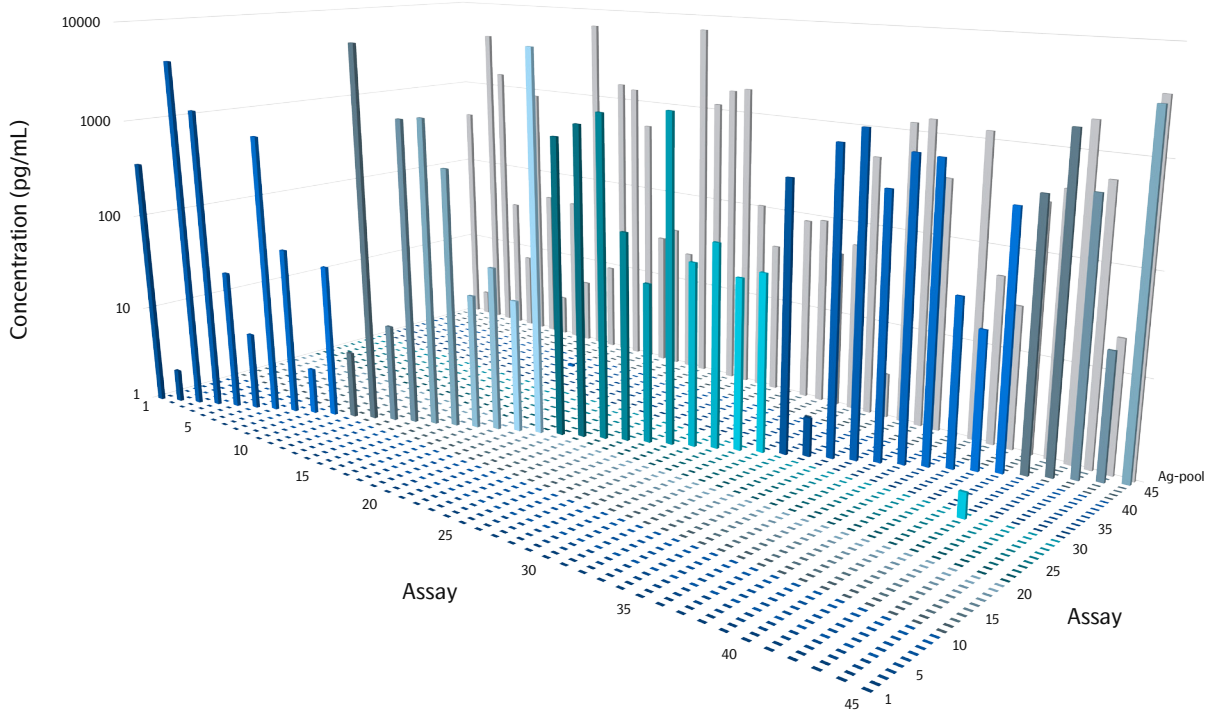

Figure 5. Assay readout specificity of the Olink platform. For each assay, specificity is confirmed by testing single antigens at endogenous levels against the complete 45-plex pool compared to each single antigen.

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).

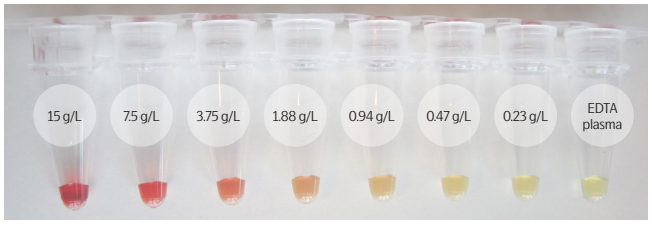

Figure 6. Endogenous interference. Levels tested for hemolysate were 0.23–15 g/L hemoglobin. The highest hemolysate concentration translates to about 10% hemolysis.

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%.

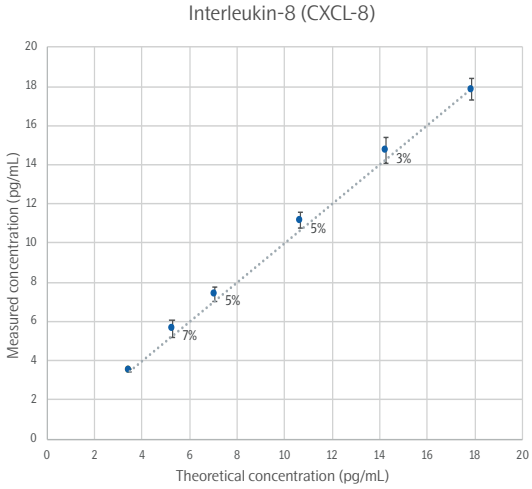

Figure 7. The difference between the "theoretical" concentration and the "measured" concentration.

References

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Technical support

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

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Validation data

Olink® Target 48 Immune Surveillance

Introduction

Olink® Target 48 Immune Surveillance is a reagent kit measuring 44 well-established protein biomarkers relevant in inflammation, immune response, immuno-oncology and infectious diseases simultaneously. Olink Target 48 Immune Surveillance has been developed without any human plasma components. 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™) technology[1-2], where 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. These reporter sequences are 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 or dilution 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

Plasma-free internal and external controls have been developed by Olink to enable data normalization and quality control. These have been designed to enable monitoring of the technical performance of each run, as well as the individual performance of each sample, providing information at each step of the Olink protocol (see Figure 1). The internal controls are added to each sample and include one Incubation Control, one Extension Control and one Detection Control. The Incubation 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 readout 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 warning in the Olink® NPX Signature software.

Eight controls are applied to each run. Triplicate of the Sample Control, duplicate of the Negative Control and triplicate of the Calibrator. The Calibrator is used in a second normalization step and is designed to improve inter-run precision, enabling optimal comparison of data derived from multiple runs and batches. The Sample Control is used to monitor and control the quality of reported output data by evaluating both accuracy and intra-run precision for all assays. Both the Sample Control and the Calibrator are composed of a pool of recombinant proteins, equivalent to the biomarkers targeted by the panel.

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

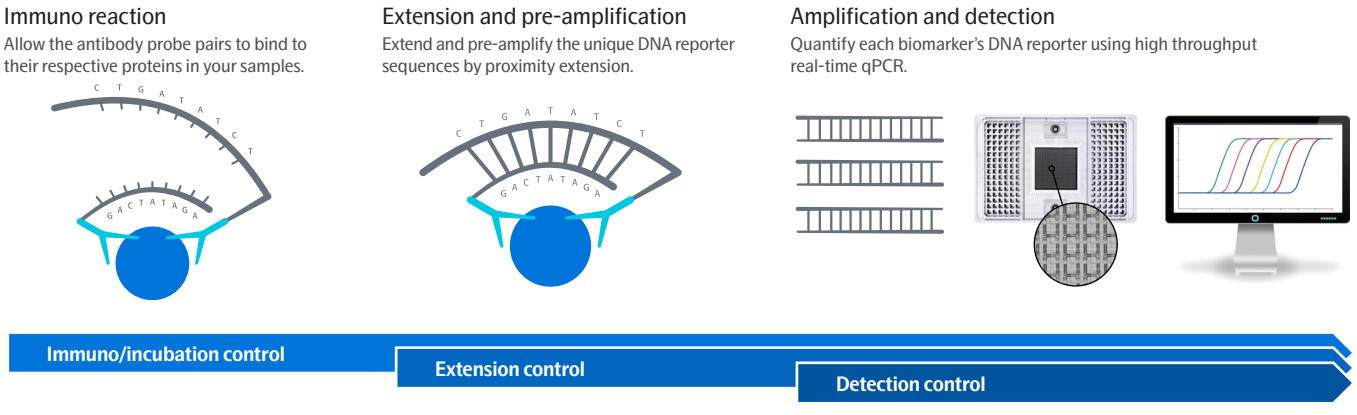

Figure 1. Olink assay procedure (above) and controls (below). The internal controls enable monitoring of the three core steps in the Olink assay and are used for quality control and data normalization. Readout is performed by using Olink® Signature Q100.

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) 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 product page (olink.com/target48human). Three examples are shown in Figure 2.

Performance characteristics

Sample information

Olink Target 48 Immune Surveillance was validated using 15 plasma samples from healthy, adult donors and 68 plasma samples from adult patients with diagnosed diseases like: Asthma, Atopic Dermatitis, Chronic Ischemic Heart Disease, Chronic Kidney Disease, Congestive Heart Failure, Coronary Artery Disease, COVID-19, Crohn's Disease, Hypertension, Leukemia, Lymphoma, Multiple Sclerosis, Myocardial Infarction, Neuroendocrine Carcinoma, Psoriasis, Rheumatoid Arthritis, Scleroderma, Stroke, Systemic Lupus Erythematosus and Type 2 Diabetes.

Sample types

The ability to use different sample types was evaluated by collecting matched serum and EDTA from 15 healthy individuals, acid citrate dextrose (ACD) from 4 healthy individuals, and sodium heparin plasma samples from 4 healthy individuals. Table 1 summarizes the response values for 15 normal EDTA plasma samples expressed in pg/mL, 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 44 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.

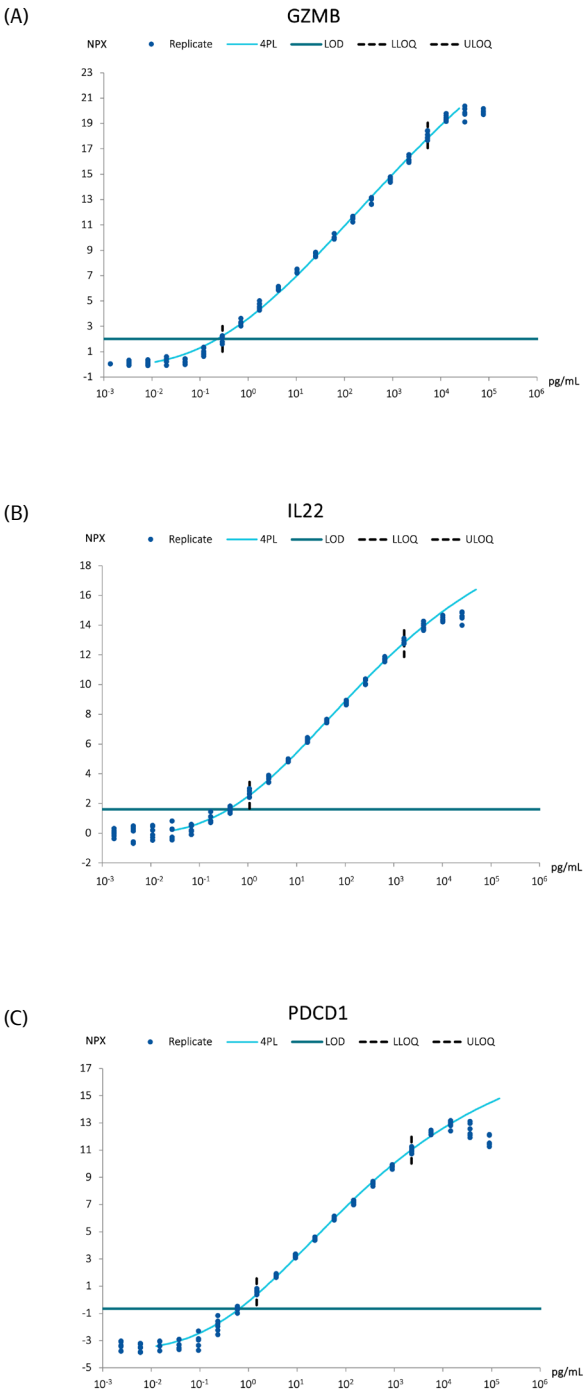

Figure 2 Calibrator curves from 3 assays and their corresponding analytical measurement data.

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 validation results during product development. The upper and lower limits of quantification (ULOQ and LLOQ, respectively) were

Table 1. Sample types; Normal and pathological plasma levels, relative to EDTA plasma, 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 44 protein biomarkers. Not available, NA.

Target		Sample types									Endogen. interference	Analytical measuring range					Precision	
		Normal plasma levels (pg/mL)			Pathological plasma levels (pg/mL)			Relative to EDTA plasma (%)			(mg/mL)	(pg/mL)				log10	% CV	
Protein name (gene name)	UniProt No	10th %tile	Median	90th %tile	10th %tile	Median	90th %tile	ACD	Heparin	Serum	Hemolysate	LOD	LLOQ	ULOQ	Hook	Range	Intra	Inter
		n=15			n=68			n=4	n=4	n=15								
Amphiregulin (AREG)	P15514	6.01	7.58	14.60	5.03	7.86	15.80	71	82	112	15	0.43	0.43	4680	10150	3.99	5	5
C-C motif chemokine 17 (CCL17)	Q92583	35.07	72.65	382.50	34.60	116.71	436.99	26	121	439	15	0.03	0.07	797	4260	3.98	4	6
C-C motif chemokine 20 (CCL20)	P78556	10.03	13.98	26.69	4.93	11.26	27.36	80	72	57	15	0.04	0.13	1603	8551	4.02	5	6
C-C motif chemokine 25 (CCL25)	O15444	73.39	107.15	171.98	69.64	113.18	156.93	77	85	102	15	0.56	0.99	1472	8105	3.11	5	6
C-C motif chemokine 26 (CCL26)	Q9Y258	101.73	148.78	191.28	67.57	135.52	298.95	61	94	66	15	22.98	22.98	99690	1317825	3.59	7	6
C-X-C motif chemokine 13 (CXCL13)	O43927	48.08	57.53	98.84	27.42	60.91	202.26	75	53	60	15	0.11	0.11	2080	34151	4.24	6	5
Carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5)	P06731	40.65	117.89	470.94	32.51	100.38	284.09	79	96	100	15	1.61	1.61	5469	35394	3.55	5	5
Erythropoietin (EPO)	P01588	3.64	6.31	20.34	4.17	9.49	22.19	81	99	104	15	0.08	0.25	1097	2496	3.59	4	4
Fibroblast growth factor 2 (FGF2)	P09038	1.81	3.90	7.02	4.27	20.37	82.64	65	NA	283	15	0.47	1.25	3838	21096	3.50	6	5
Fibroblast growth factor 21 (FGF21)	Q9NSA1	59.33	120.40	585.74	42.11	115.21	329.91	74	81	66	15	0.46	0.92	4485	54779	3.55	4	4
Fibroblast growth factor 23 (FGF23)	Q9GZV9	25.68	42.33	173.29	19.28	31.44	110.20	75	96	31	15	0.37	0.92	3942	22592	3.56	6	4
Granzyme A (GZMA)	P12544	158.83	217.50	259.90	173.48	245.52	429.20	78	95	103	7.5	0.08	0.08	10255	121626	5.03	4	6
Granzyme B (GZMB)	P10144	2.39	3.83	6.39	1.17	7.73	22.62	106	140	95	0.9	0.23	0.23	6137	77773	4.26	3	8
Growth-regulated alpha protein (CXCL1)	P09341	29.57	95.60	134.63	43.84	141.71	371.69	40	281	587	15	0.37	1.26	9455	63503	3.86	5	5
Hepatitis A virus cellular receptor 1 (HAVCR1)	Q96D42	20.67	44.23	117.16	19.23	56.89	144.88	81	94	105	15	0.32	0.99	3966	22978	3.61	5	6
Interferon alpha-2 (IFNA2)	P01563	NA	NA	NA	4.75	5.91	54.54	NA	NA	NA	15	1.01	1.01	3789	52179	3.57	5	5
Interferon beta (IFNB1)	P01574	NA	NA	NA	7.81	13.89	19.96	NA	NA	NA	15	1.47	4.58	12047	76863	3.38	6	7
Interferon lambda-1 (IFNL1)	Q8IU54	1.79	1.96	2.57	1.81	2.48	9.27	NA	NA	104	15	1.53	1.53	4478	23452	3.50	5	5
Interleukin-1 alpha (IL1A)	P01583	NA	NA	NA	0.40	1.25	2.19	237	NA	NA	15	0.12	0.36	1535	8828	3.57	4	5
Interleukin-1 receptor antagonist protein (IL1RN)	P18510	147.28	293.16	621.88	164.82	298.43	737.53	70	104	119	7.5	8.18	18.50	1944	345494	1.95	5	4
Interleukin-17D (IL17D)	Q8TAD2	1.99	2.63	4.59	1.73	2.69	4.41	93	49	75	15	0.16	0.47	1113	6022	3.37	4	5
Interleukin-19 (IL19)	Q9UHD0	90.25	154.41	324.80	81.32	152.96	494.83	77	112	123	15	0.84	2.90	28975	535368	4.10	5	5
Interleukin-20 (IL20)	Q9NYY1	1.15	1.47	3.01	0.73	1.32	2.44	83	79	95	15	0.05	0.05	2226	29709	4.65	8	10
Interleukin-22 (IL22)	Q9GZX6	1.58	3.11	6.27	1.93	4.30	13.27	80	89	100	15	0.40	1.18	1693	64000	3.18	6	7
Interleukin-25 (IL25)	Q9H293	NA	NA	NA	NA	NA	NA	NA	NA	NA	15	0.16	0.16	4494	26182	4.39	5	4
Interleukin-31 (IL31)	Q6EBC2	19.29	20.87	24.60	20.76	25.35	66.56	97	98	97	15	6.13	17.36	27421	400000	3.18	5	5
Interleukin-32 (IL32)	P24001	23.91	34.21	51.04	18.28	31.21	73.53	67	95	99	15	0.76	0.76	6361	84510	3.89	5	4
Interleukin-4 receptor subunit alpha (IL4R)	P24394	227.42	348.69	438.73	221.18	348.33	839.65	112	111	107	15	7.91	7.91	37301	563270	3.60	7	3
Interleukin-5 (IL5)	P05113	8.37	8.70	9.03	7.14	15.34	100.21	82	124	105	15	6.88	6.88	29438	168881	3.61	5	5
Keratin, type I cytoskeletal 18 (KRT18)	P05783	22.61	41.88	430.19	32.79	62.18	196.45	72	70	96	15	1.05	1.05	22136	119980	4.31	7	7
Leukemia inhibitory factor (LIF)	P15018	0.31	0.31	0.31	0.19	0.43	1.20	86	110	119	15	0.06	0.17	1438	7992	3.90	5	6
Lymphocyte activation gene 3 protein (LAG3)	P18627	1551.66	1953.19	3189.30	1378.84	2300.43	4511.09	83	95	100	15	70.39	70.39	112334	1777613	3.17	4	17
Placenta growth factor (PGF)	P49763	27.31	33.38	43.46	25.41	35.35	64.67	74	74	99	15	0.08	0.22	2327	34356	3.98	4	5
Pro-interleukin-16 (IL16)	Q14005	31.41	44.56	87.89	32.20	57.37	157.09	73	97	112	3.8	0.22	0.22	885	4902	3.57	7	6
Programmed cell death protein 1 (PDCD1)	Q15116	54.50	65.23	102.40	42.59	74.50	157.07	78	88	98	15	0.65	1.75	2245	14188	3.19	5	3
T-cell-specific surface glycoprotein CD28 (CD28)	P10747	394.29	583.44	926.56	386.38	567.16	959.71	66	92	109	15	70.68	70.68	51195	693717	2.76	5	6
T-lymphocyte activation antigen CD80 (CD80)	P33681	158.01	203.62	266.81	119.85	228.31	478.02	75	95	103	15	0.17	0.17	4361	24441	4.39	6	7
Triggering receptor expressed on myeloid cells 1 (TREM1)	Q9NP99	188.67	278.05	415.55	166.36	269.35	480.49	65	83	107	15	0.06	0.06	4371	64000	4.78	6	5
Tumor necrosis factor ligand superfamily member 14 (TNFSF14)	O43557	58.29	78.23	116.00	56.38	100.07	216.23	65	117	244	15	1.90	1.90	8189	112427	3.53	4	5
Tumor necrosis factor ligand superfamily member 6 (FASLG)	P48023	77.34	111.41	148.91	74.94	122.96	182.94	90	90	101	15	0.38	1.16	3788	54071	3.49	4	5
Tumor necrosis factor receptor superfamily member 4 (TNFRSF4)	P43489	61.77	82.35	107.11	57.63	88.93	195.10	78	96	108	15	0.27	0.71	6081	33479	3.88	4	5
Tumor necrosis factor receptor superfamily member 9 (TNFRSF9)	Q07011	14.27	21.41	33.18	14.14	22.37	97.84	68	86	109	15	1.13	3.42	5507	12234	3.14	4	5
Vascular endothelial growth factor receptor 1 (FLT1)	P17948	1065.61	1187.89	1397.03	1033.46	1330.58	2015.70	79	63	95	15	5.92	5.92	56454	400000	3.98	6	7
Vascular endothelial growth factor receptor 2 (KDR)	P35968	1005.22	1307.56	1612.40	1016.79	1344.98	1664.69	77	97	103	15	0.88	0.88	8289	43940	3.88	4	5

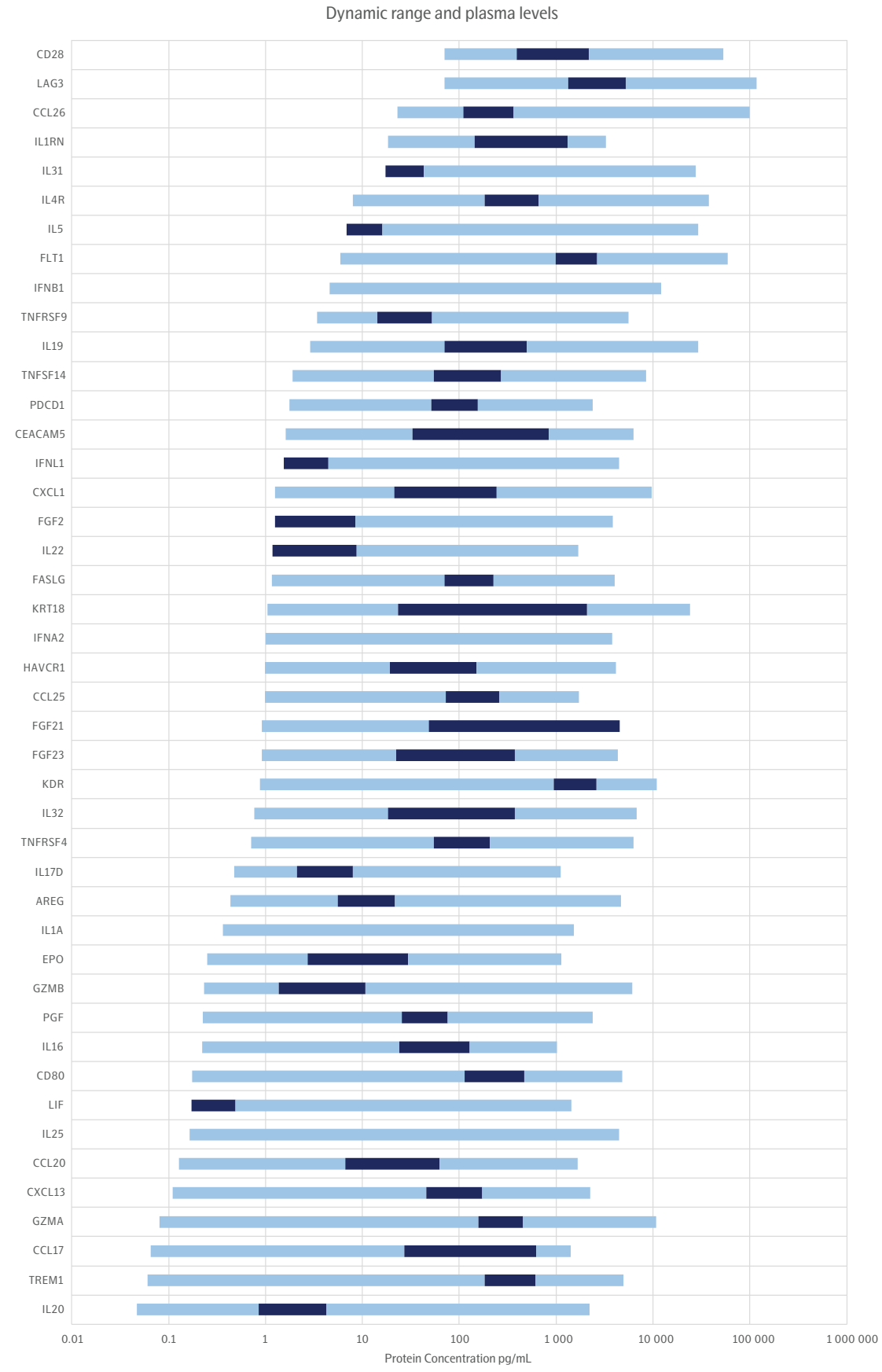

Figure 3 Distribution of analytical measuring range, defined by the lower and upper limits of quantification (LLOQ-ULOQ), and normal plasma levels (darker bars) for the 44 protein biomarkers.

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 product page (olink.com/target48human) 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 44 assays and endogenous plasma levels for healthy donors are shown in Figure 3.

Precision

Repeatability

Inter-run (between run) and intra-run (within run) CV were assessed by evaluating triplicate measurements of the Sample Control on each plate, based on 17 plate runs performed by three different operators. Each operator performed a minimum of three runs.

Inter-run CV values were calculated between runs done by the same operator. The inter-run CV reported here is the average of the three operators' CV. CV calculations were performed on data in pg/mL for the 44 analytes for which response levels within LOQ were detected, see Table 1.

Across the 44 assays, the mean intra-run and inter-run variations observed were 5% and 6%, respectively. The distribution of both intra-run and inter-run variations are shown in Figure 4.

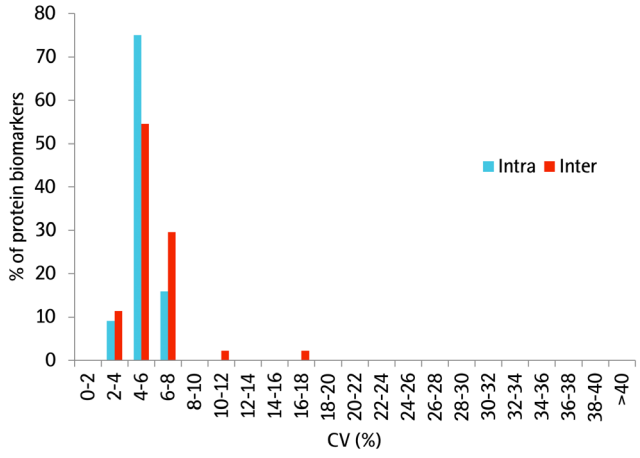

Figure 4. Distribution of intra-run and inter-run variations of Olink® Target 48 Immune Surveillance.

Reproducibility

To determine CV inter-operator (between operators) and CV inter-site (between sites), identical sample plates were sent to 6 laboratories (sites) together with Olink Target 48 Immune Surveillance kits. Ten individual plasma samples (in triplicate) and a pooled plasma sample were provided. Two operators per site executed one experiment each, using one sample plate each. Inter-operator and inter-site CV were calculated based on these samples and Olink's Sample Control, provided with the kit. All samples and controls showed good CV between operators and sites (see Table 2).

Table 2. The average CV intra-run was determined for each assay on each run (n=12), and values shown represent the average of all runs. CV inter-run is the average of all runs. Inter-operator CV was determined per site. CV inter-operator is the average of inter-operator CV from all sites. The CV inter-site was determined pairwise, between all sites. CV inter-site is the average of all pairwise calculations.

%CV	Plasma samples	Pooled plasma	Sample Control
Intra-run	7.7	8.2	7.2
Inter-operator	7.5	7.3	4.0
Inter-site	8.4	8.7	5.2

In addition to Olink Analysis Service laboratory in Uppsala, Sweden and in Boston, US, there are many Olink-certified core laboratories around the world running Olink 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. For more information please contact support@olink.com.

Analytical Specificity

Assay specificity

To test the specificity of the PEA probes of Olink Target 48 Immune Surveillance, all antibodies used were tested for cross reactivity against all proteins targeted. To confirm that the antibodies implemented into Olink Target 48 Immune Surveillance are specific for their targets, detection of the 44 proteins were determined applying recombinant proteins solitary to the multiplex. These tests revealed that only one assay showed minimal cross-binding to another protein, with 0.2% (CCL17 antibodies detecting CCL25). No other assays had any unspecific signal.

Endogenous interference

Bilirubin, lipids and hemolysate, are plasma and serum components that are known to interfere with some analytical assays. An example of the hemolysate levels tested is shown in Figure 5. These additions represent different health conditions and/or sample collection irregularities. In 4 out of 44 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

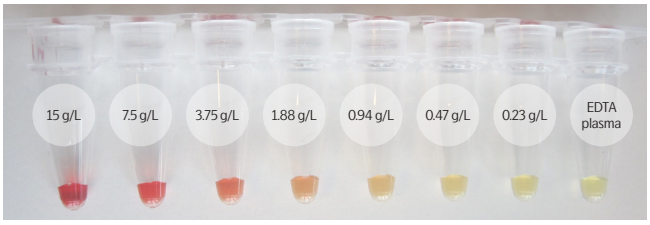

Figure 5. Endogenous interference. Levels tested for hemolysate were 0.23–15 g/L hemoglobin. The highest hemolysate concentration translates to about 10% hemolysis.

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 Immune Surveillance.

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 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 6).

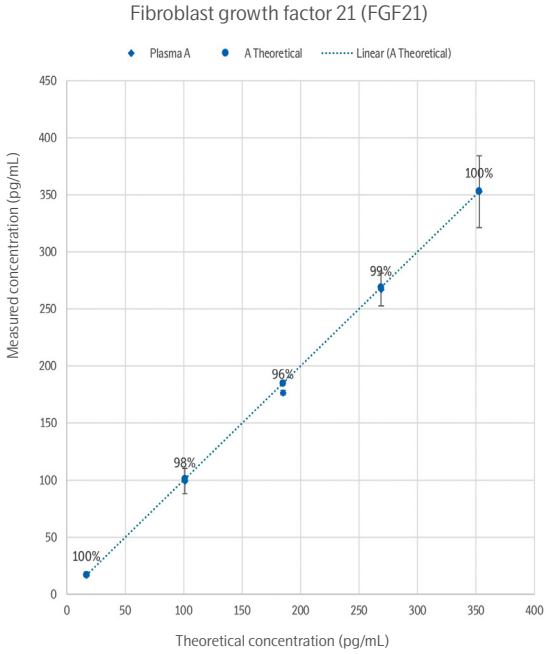

Figure 6. The difference between the "theoretical" concentration and the "measured" concentration.

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Technical support

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