Validation data

Olink® Target 48 Mouse Cytokine

Introduction

Olink® Target 48 Mouse Cytokine is a reagent kit measuring 43 well-established protein biomarkers relevant in inflammation, immune-response and immuno-oncology 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 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

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-mammal 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 NPX™ Signature software.

Eight controls are applied to each run. 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-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 of the three Calibrator replicates from the dCq-values generated. The next step in the pre-processing procedure was to set the

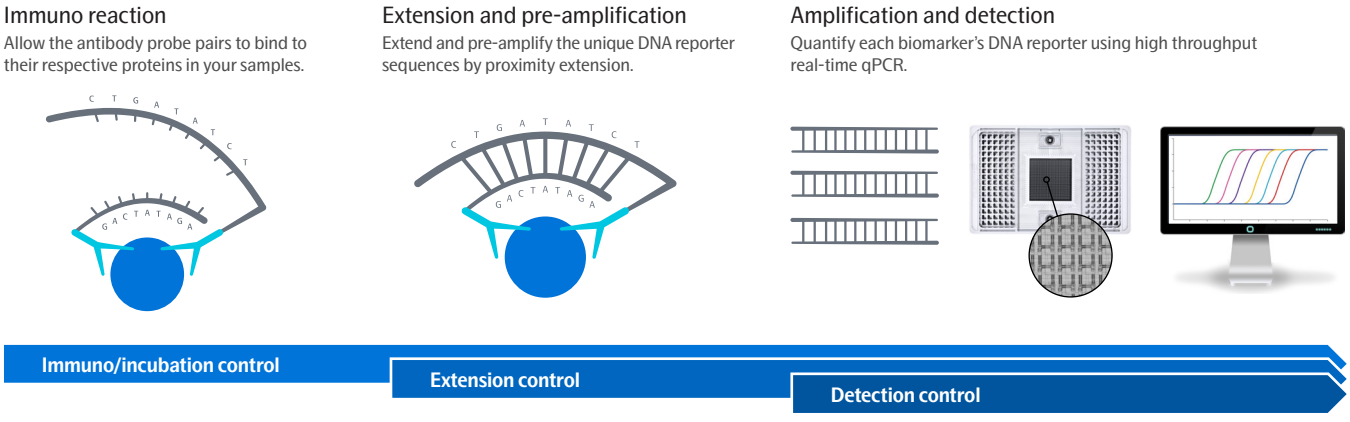

Figure 1. Olink assay procedure (above) and controls (below). The internal controls enable monitoring of the three core steps in the Olink assay and are used for quality control and data normalization. Readout is performed by using Olink® Signature Q100.

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 published on the respective biomarker page. Three examples are shown in Figure 2.

Performance characteristics

Sample information

Verification and validation of Olink Target 48 Mouse Cytokine was executed using 162 (45 control, 117 disease) plasma and 126 (37 control, 89 disease) serum samples. The plasma and serum samples covered different murine disease models differing in genetic background, genotype, age and/or applied treatment. The disease models used include three different cancer models, Sjögren's disease, obesity, multiple sclerosis, rheumatoid arthritis, allergic rhinitis, systemic lupus erythematosus (SLE), inflammation, asthma, and Alzheimer's disease. Plasma and serum samples from healthy controls came from several different mouse strains: BALB/C, CD-1 (ICR), C57BL/6, FVB/NCRL and DBA/1 mice.

Analytical measurement

Detection limit

Standard curves were determined for the 43 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 conjugated antibodies used, resulting in decreasing values with increasing concentration. In such cases, the values reported do not reflect the biomarker concentration, 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 the Range is reported in log10 scale, 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). As seen in Table 2, the majority of observed values for many of the assays fall within the quantifiable range. Some of the targeted

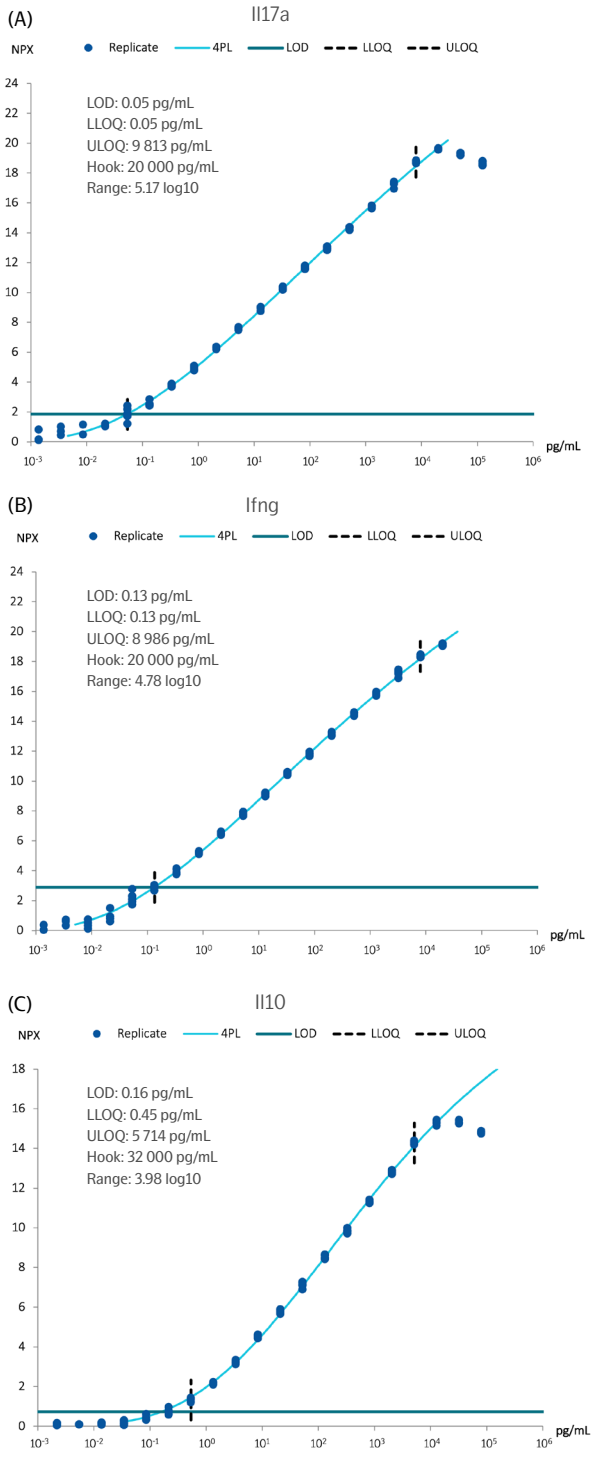

Figure 2. Calibrator curves from three assays and their corresponding analytical measurement data.

biomarkers showed low or very low abundance in the mouse and disease models used, but might show higher expression in other disease models. Calibrator curves were defined for each biomarker and they can be accessed via the panel product page together with the analytical data for the assay. Three examples of assays and their analytical data are shown in Figure 2. The measuring ranges of the 43 assays and endogenous biomarker levels in control animals are shown in Figure 3.

Table 1. Analytical measuring range; Limit of Detection (LOD), Lower Limit of Quantification (LLOQ), Upper Limit of Quantification (ULOQ), High Dose Effect (Hook). Interference; Hemoglobin, Mouse IgG. Precision indicative of assay performance are shown for the protein biomarkers.

Target		Analytical measuring range					Interference		Precision (CV)	
		(pg/mL)				(log10)	(mg/mL)	(mg/mL)	(%)	
Protein name (gene name)	UniProt No	LOD	LLOQ	ULOQ	Hook	Range	Hemoglobin	Mouse IgG	Intra	Inter
C-C motif chemokine 12 (Ccl12)	Q62401	0.64	1.97	9310	20000	3.58	15.0	15.0	4	5
C-C motif chemokine 17 (Ccl17)	Q9WUZ6	11.3	53.0	15188	32000	2.39	15.0	15.0	3	4
C-C motif chemokine 22 (Ccl2)	P10148	0.10	0.26	8830	20000	4.38	15.0	15.0	4	2
C-C motif chemokine 22 (Ccl22)	O88430	0.06	0.06	9079	20000	5.17	15.0	15.0	4	4
C-C motif chemokine 4 (Ccl4)	P14097	0.32	0.90	527	8000	2.79	15.0	15.0	5	8
C-C motif chemokine 5 (Ccl5)	P30882	2.46	6.04	3896	8000	2.79	15.0	15.0	7	8
C-X-C motif chemokine 11 (Cxcl11)	Q9JHH5	0.23	0.71	3561	20000	3.58	15.0	15.0	4	7
C-X-C motif chemokine 2 (Cxcl2)	P10889	0.06	0.06	220	8000	3.58	15.0	15.0	5	3
C-X-C motif chemokine 9 (Cxcl9)	P18340	0.11	0.23	9341	20000	4.38	15.0	15.0	4	4
Cytotoxic T-lymphocyte protein 4 (Ctla4)	P09793	0.08	0.11	9774	20000	4.78	15.0	15.0	4	4
Eotaxin (Ccl11)	P48298	0.96	2.71	71766	160000	4.38	15.0	15.0	4	3
Fibroblast growth factor 21 (Fgf21)	Q9JJN1	0.12	0.22	8612	50000	4.38	15.0	15.0	4	2
Granulocyte colony-stimulating factor (Csf3)	P09920	0.24	0.72	9595	20000	3.98	15.0	15.0	4	6
Granulocyte-macrophage colony-stimulating factor (Csf2)	P01587	0.12	0.27	9263	20000	4.38	15.0	15.0	4	5
Growth-regulated alpha protein (Cxcl1)	P12850	0.06	0.06	1326	20000	4.38	15.0	15.0	5	3
Hepatocyte growth factor (Hgf)	Q08048	0.17	0.43	36826	80000	4.78	15.0	15.0	4	5
Interferon alpha-2 (Ifna2)	P01573	0.02	0.02	3400	20000	5.17	15.0	15.0	5	3
Interferon gamma (Ifng)	P01580	0.13	0.13	8986	20000	4.78	15.0	15.0	5	4
Interferon lambda-2 (Ifnl2)	Q4VK74	0.05	0.12	3583	20000	4.38	15.0	15.0	4	2
Interleukin-1 alpha (Il1a)	P01582	0.40	0.40	11980	64000	4.38	3.8	0.5	5	4
Interleukin-1 beta (Il1b)	P10749	0.32	0.32	9679	20000	4.38	3.8	15.0	4	3
Interleukin-10 (Il10)	P18893	0.16	0.45	5714	32000	3.98	15.0	15.0	4	4
Interleukin-12 (Il12a, Il12b)	P43431, P43432	0.23	0.44	14869	80000	4.38	15.0	15.0	5	5
Interleukin-17A (Il17a)	Q62386	0.05	0.05	9813	20000	5.17	15.0	15.0	4	4
Interleukin-17F (Il17f)	Q7TNI7	0.24	0.75	9191	20000	3.98	15.0	15.0	3	5
Interleukin-21 (Il21)	Q9ES17	20.4	46.9	210540	1000000	3.58	15.0	15.0	5	4
Interleukin-22 (Il22)	Q9JJY9	0.25	0.74	3306	20000	3.58	15.0	15.0	4	4
Interleukin-2 (Il2)	P04351	0.26	0.77	9250	50000	3.98	15.0	15.0	5	6
Interleukin-27 subunit alpha (Il27)	Q8K3I6	0.61	3.18	35793	200000	3.98	15.0	15.0	4	6
Interleukin-3 (Il3)	P01586	0.23	0.46	35452	80000	4.78	15.0	15.0	5	4
Interleukin-31 (Il31)	Q6EAL8	2.19	2.19	76125	160000	4.38	15.0	15.0	5	5
Interleukin-33 (Il33)	Q8BVZ5	0.43	1.12	13907	80000	3.98	15.0	15.0	4	5
Interleukin-4 (Il4)	P07750	0.10	0.31	3521	8000	3.98	15.0	15.0	4	5
Interleukin-5 (Il5)	P04401	0.07	0.07	1400	8000	4.38	15.0	15.0	3	7
Interleukin-6 (Il6)	P08505	0.95	2.87	40620	80000	3.98	15.0	15.0	4	4
Interleukin-7 (Il7)	P10168	0.94	3.45	37416	80000	3.98	15.0	15.0	5	7
Interleukin-9 (Il9)	P15247	0.13	0.13	3347	20000	4.38	15.0	15.0	4	6
Macrophage colony-stimulating factor 1 (Csf1)	P07141	58.6	58.6	94670	200000	3.18	15.0	15.0	6	5
Pro-interleukin-16 (Il16)	O54824	3.95	11.9	24809	50000	3.18	15.0	15.0	4	4
Programmed cell death 1 ligand 1 (Cd274)	Q9EP73	2.26	7.80	14190	200000	3.18	15.0	15.0	5	3
Programmed cell death 1 ligand 2 (Pdcd1lg2)	Q9WUL5	0.75	2.29	26012	50000	3.98	15.0	15.0	6	13
Stromal cell-derived factor 1 (Cxcl12)	P40224	23.0	252	70947	1000000	2.39	15.0	3.8	8	6
Tumor necrosis factor (Tnf)	P06804	0.63	2.50	3536	20000	3.58	15.0	15.0	4	6

Table 2. Detectability; Control, Disease models and Human normal plasma. Plasma and serum ranges indicative of assay performance are shown for the protein biomarkers. Not available, NA.

Target	Detectability					Biological range plasma						Biological range serum					
	(%)					(pg/mL)						(pg/mL)					
	Control		Disease models		Human	Control			Disease models			Control			Disease models		
Protein name (gene name)	Plasma (n=45)	Serum (n=37)	Plasma (n=117)	Serum (n=89)	Normal plasma (n=8)	10th %tile	Median	90th %tile	10th %tile	Median	90th %tile	10th %tile	Median	90th %tile	10th %tile	Median	90th %tile
C-C motif chemokine 12 (Ccl12)	100	100	100	99	0	126	190	530	132	201	691	62.4	169	301	66.0	401	1079
C-C motif chemokine 17 (Ccl17)	36	84	23	82	0	55.6	66.5	155	53.9	71.7	161	100	168	248	69.1	139	243
C-C motif chemokine 22 (Ccl2)	100	100	99	100	0	254	471	1387	324	672	1793	255	578	1492	298	932	2792
C-C motif chemokine 22 (Ccl22)	100	100	100	100	0	97.9	255	466	35.4	186	364	69.4	362	734	153	401	770
C-C motif chemokine 4 (Ccl4)	100	97	95	97	0	14.0	21.5	53.8	15.5	33.3	69.5	4.67	21.8	45.6	15.0	35.5	119
C-C motif chemokine 5 (Ccl5)	100	97	100	100	0	19.3	38.2	71.8	16.5	37.1	145	19.6	50.3	88.3	23.3	65.3	125
C-X-C motif chemokine 11 (Cxcl11)	20	30	3	54	0	31.8	96.7	180	NA	126	NA	3.23	7.39	47.2	12.7	31.6	84.1
C-X-C motif chemokine 2 (Cxcl2)	100	100	100	100	0	0.15	0.70	2.09	0.47	1.40	4.12	0.58	1.49	3.63	0.57	1.89	7.34
C-X-C motif chemokine 9 (Cxcl9)	100	100	98	100	0	71.6	155	452	86.7	164	589	109	196	685	133	403	1209
Cytotoxic T-lymphocyte protein 4 (Ctla4)	100	97	100	100	0	0.22	0.34	1.00	0.25	0.39	0.77	0.29	0.57	1.13	0.40	0.73	1.78
Eotaxin (Ccl11)	100	100	100	100	0	155	250	483	86.8	222	599	97.7	316	507	225	397	575
Fibroblast growth factor 21 (Fgf21)	100	100	100	96	0	95.1	200	770	105	270	594	52.6	213	1742	120	478	1730
Granulocyte colony-stimulating factor (Csf3)	100	100	99	98	0	40.5	78.5	334	53.6	96.7	549	34.9	55.9	402	25.7	62.8	1848
Granulocyte-macrophage colony-stimulating factor (Csf2)	9	16	15	11	0	NA	0.33	NA	0.29	0.35	4.17	NA	0.29	NA	0.29	0.51	0.91
Growth-regulated alpha protein (Cxcl1)	100	100	100	99	0	3.41	19.5	69.3	8.90	28.3	153	1.59	32.8	81.6	23.9	61.2	167
Hepatocyte growth factor (Hgf)	100	100	97	100	0	161	228	176	146	234	1162	1245	4595	9044	498	3278	6296
Interferon alpha-2 (Ifna2)	100	97	100	99	0	0.11	0.24	5.90	0.15	0.25	0.74	0.08	0.19	4.50	0.07	0.29	0.70
Interferon gamma (Ifng)	100	97	97	89	0	0.21	0.34	14.5	0.22	0.45	2.60	0.42	0.86	39.9	0.39	1.42	5.00
Interferon lambda-2 (Ifnl2)	78	97	93	98	0	0.14	0.21	17.8	0.15	0.43	1.01	0.23	0.41	11.1	0.22	0.47	3.68
Interleukin-1 alpha (Il1a)	100	100	100	100	0	1.41	4.44	17.7	2.29	6.24	44.9	2.96	28.0	611	3.93	25.4	297
Interleukin-1 beta (Il1b)	53	92	73	97	0	0.36	0.55	2.11	0.39	0.73	2.52	0.37	1.46	4.06	0.61	1.95	8.66
Interleukin-10 (Il10)	100	97	100	100	0	5.26	7.83	14.1	5.72	10.5	25.8	2.00	3.96	10.1	2.68	6.48	87.9
Interleukin-12 (Il12a, Il12b)	58	62	42	74	0	0.49	1.33	3.23	0.48	1.00	3.87	0.57	1.58	2.89	0.57	1.31	7.03
Interleukin-17A (Il17a)	100	97	100	96	0	0.19	1.16	12.0	0.31	1.37	7.57	0.12	1.48	12.3	0.25	1.63	5.67
Interleukin-17F (Il17f)	96	92	99	98	0	2.01	6.35	166	1.71	6.98	52.8	1.62	14.8	245	4.82	18.2	173
Interleukin-21 (Il21)	0	0	0	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Interleukin-22 (Il22)	100	100	99	100	0	2.47	7.32	105	1.57	7.21	49.1	5.49	16.7	184	3.72	14.2	41.7
Interleukin-2 (Il2)	64	84	72	89	0	0.82	1.23	6.01	0.86	1.66	2.95	1.04	2.04	6.12	1.05	1.76	4.38
Interleukin-27 subunit alpha (Il27)	56	43	85	57	0	3.37	6.74	39.0	3.70	5.59	19.7	3.81	5.98	18.7	3.51	6.63	50.2
Interleukin-3 (Il3)	24	24	38	52	0	0.62	0.83	2.39	0.52	0.93	1.94	0.46	0.65	1.28	0.56	1.22	5.24
Interleukin-31 (Il31)	0	3	4	2	0	NA	NA	NA	NA	3.10	NA	NA	4.18	NA	NA	2.89	NA
Interleukin-33 (Il33)	80	62	91	97	0	1.41	4.56	14.3	1.64	3.88	9.70	2.32	13.2	54.0	1.92	11.8	67.8
Interleukin-4 (Il4)	11	22	30	48	0	NA	0.45	NA	0.35	0.53	2.40	NA	0.43	NA	0.37	0.51	1.17
Interleukin-5 (Il5)	100	97	100	98	0	0.26	0.57	1.66	0.29	0.61	1.70	0.31	0.68	2.26	0.19	0.69	2.18
Interleukin-6 (Il6)	91	95	100	96	0	5.31	24.0	109	7.58	47.2	286	9.83	32.91	149	12.6	45.9	296
Interleukin-7 (Il7)	0	3	3	8	0	NA	NA	NA	NA	3.57	NA	NA	5.01	NA	NA	4.28	NA
Interleukin-9 (Il9)	100	97	100	97	0	0.33	0.84	4.82	0.39	0.78	2.31	0.81	1.47	8.81	0.46	1.08	3.54
Macrophage colony-stimulating factor 1 (Csf1)	100	100	100	100	0	2921	4642	9449	3754	5262	9254	2228	4995	17412	1853	4856	7159
Pro-interleukin-16 (Il16)	100	100	100	100	0	1954	2398	4526	2057	3132	5377	900	1823	3433	1457	4083	9170
Programmed cell death 1 ligand 1 (Cd274)	100	100	100	100	0	54.6	71.9	120	69.2	105	235	62.7	113	229	71.9	113	294
Programmed cell death 1 ligand 2 (Pdcd1lg2)	100	100	99	100	0	916	1805	3219	1006	1523	2503	784	1902	3760	884	2220	4970
Stromal cell-derived factor 1 (Cxcl12)	18	11	9	16	13	NA	459	NA	261	652	9545	NA	585	NA	280	585	29417
Tumor necrosis factor (Tnf)	100	95	100	98	0	6.59	9.43	33.3	8.49	16.8	35.0	4.66	8.95	32.5	4.77	12.7	40.8

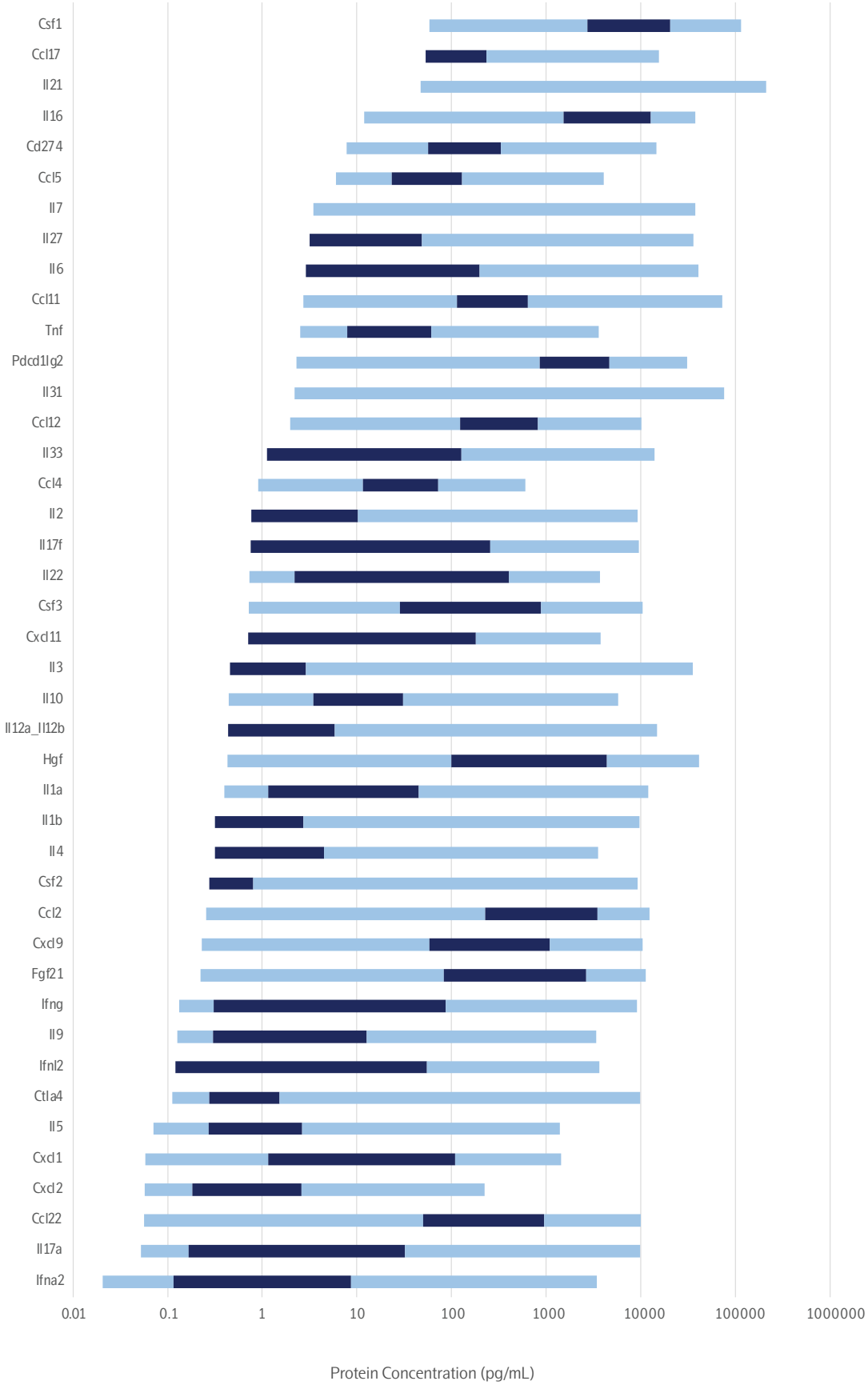

Figure 3. Distribution of analytical measuring range, defined by the lower and upper limits of quantification (LLOQ-ULOQ), and normal plasma levels (darker bars) for the protein biomarkers.

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 thirteen 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 43 analytes for which response levels within LOQ were detected, see Table 1.

Across the 43 assays, the mean intra-run and inter-run variations observed were 4 and 5 %, respectively. The distribution of both intra-assay and inter-assay variations are shown in Figure 4.

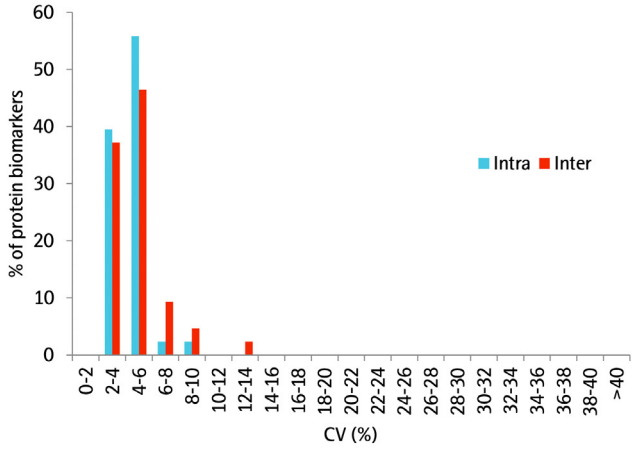

Figure 4. Distribution of intra-run and inter-run variations of Olink Target 48 Mouse Cytokine.

Reproducibility

To determine CV inter-operator (between operators) and CV inter-site (between sites), identical sample plates were sent to 5 laboratories (sites) together with Olink Target 48 Mouse Cytokine kits. Ten individual murine plasma samples (in triplicates) and a pooled plasma sample (in duplicate) 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 3).

Table 3. The average CV intra-run was determined for each assay on each run (n=10), 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	8.9	6.6	5.6
Inter-operator	11.1	10.4	9.7
Inter-site	8.7	9.3	5.9

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 specificity of the PEA probes of the Olink Target 48 Mouse Cytokine panel, all antibodies used were tested for cross reactivity against all proteins targeted. To confirm that the antibodies implemented into Olink Target 48 Mouse Cytokine are specific for their targets, detection of the 43 proteins were determined applying recombinant proteins solitary to the multiplex. These tests revealed that only two assays showed minimal cross-binding to other proteins, with 1.02% (Csf2 detecting Il16) and 1.19% (Cxcl2 binding Cd274) respectively.

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 3 out of 43 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.

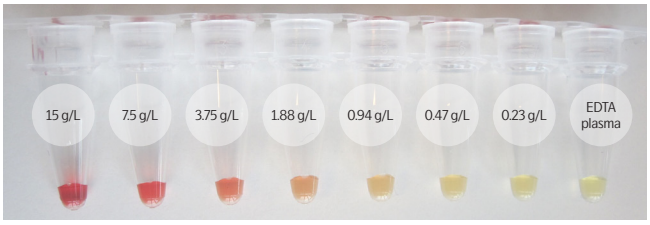

Figure 5. Endogenous interference. Levels tested for hemolysate were 0.23–15 g/L hemoglobin. The highest hemolysate concentration translates to about 10% hemolysis.

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]. Therefore, this test was not repeated in the context of the development of the Olink Target 48 Mouse Cytokine panel.

Linearity

Linearity was assessed in true matrix conditions by diluting one sample in another sample. A native sample containing a relatively high endogenous level of the target protein was mixed with a native 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 experiment was done using six sample set pairs, from which five pairs were plasma samples and one pair was serum samples. 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 an example in Figure 6).

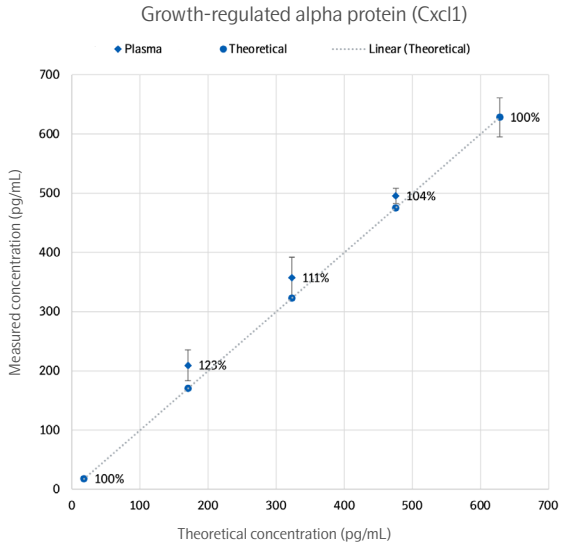

Figure 6. The difference between the "theoretical" concentration and the "measured" concentration.

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Technical support

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

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