Olink Analytical Validation

Mt Sinai CIMAC

Version 1.0

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The table below lists the nature of initial results produced (primary output), QC steps and analyses, and eventual output.

Assay type	Primary assay outputs	Pre-processing/ Normalization /QC	Initial analyses	Derived data outputs
O-Link	92 parameter targeted soluble factor immunoassay	Normalized protein eXpression (NPX)	Multivariate coexpression analyses	Quantification and correlation of multiplexed cytokine levels

OLINK PROTEIN SOLUBLE ANALYTES	
(i) accuracy	Accuracy determined by replicate analysis of spiked in known amounts of analytes in multiple assays (see Figure 2).
(ii) precision: Inter-assay	Depending on the level of protein detected, % of CV varies such that 63 analytes with standard detection have a CV value of <10% and 16 analytes with low detection have a CV value <30%. Other analytes were not detected and CV was not calculated (see Figures 4, 9 and 10). Every Olink assay plate has “inter-plate control” wells to be used in monitoring the inter-assay variation and normalization of the data.
(iii) analytical sensitivity	For the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ), see Table 1 and Figure 3.
(iv) analytical specificity including interfering substances	Olink uses two monoclonal antibodies targeting two different epitopes of the target. (see Figure 5)
(vi) reference intervals (normal values) with controls and calibrators	Varies by analytes and sample matrices, i.e., plasma, serum, bone marrow aspirate plasma etc.
(vii) standardization, harmonization, reproducibility and ruggedness	Assay standardization is established at levels of assay, operators and equipment, and Mt Sinai CIMAC has been recognized as a “Certified Service Provider of Olink Analysis” by the Olink company with strict training and following of SOPs. See Figures 1, 9, 10 and 11.
(viii) establishment of appropriate quality control and improvement procedures	MS-CIMAC measures inter- and intra-assay variations in regular intervals using in-house control samples (normal human plasma pooled from 15 subjects). MS-CIMAC was certified by Olink in June 2017 and the plan is to have annual refresher trainings by using Olink training kit.
(ix) any other performance characteristics required for assay performance	All of the required equipment, including Biomark, Juno and PCR thermal cycler, have biannual preventive service contracts to maintain optimal performance. All other small equipment such as multi-channel pippetman also has biannual calibration performed by certified vendors.

1. Purpose of assay

Measuring soluble analytes is an attractive way to relate changes to treatment and potentially to local tumor events, because secreted proteins or molecules shed through exosomes can be repeatedly sampled in the periphery. The Proseek Olink Proteomics platform operating on a Fluidigm Biomark HD microfluidic PCR is a simple yet quantitative and reproducible assay to measure levels of molecules related to inflammation in peripheral serum or plasma, and that can also be applied to tissue culture supernatants. Mount Sinai-CIMAC is the first center in the US that has been trained and certified to perform these assays in house. The Proximity Extension Assay (PEA) technology that underlies these assays relies on dual antibody recognition and incorporates several innovative QC steps to evaluate each step of the assay protocol, resulting in an extremely robust assay. Critically, in addition to profiling cytokines, chemokines and growth factors, this platform also allows detection of circulating immune co-stimulatory and inhibitory molecules. Plasma or serum will be collected and frozen and used for Olink multiplex immunoassay at Mount Sinai-CIMAC. Markers included into the panel are described in **Table 1.**

2. Assays performance characteristics based on Olink's internal validation

Olink® Immuno-Onc I^{96x96} is a reagent kit measuring 92 immuno-oncology related human protein biomarkers simultaneously. The Olink reagents are based on the Proximity Extension Assay (PEA) technology[1-2], where 92 oligonucleotide labeled antibody probe pairs are allowed to bind to their respective target protein present in the sample. A PCR reporter sequence is formed by a proximity dependent DNA polymerization event, amplified, and subsequently detected and quantified using real-time PCR. The assay is performed in a homogeneous 96-well format without any need for washing steps, see Figure 1.

2.1 Quality Controls

Internal and external controls have been developed by Olink for data normalization and quality control purposes. These controls 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 two Immunoassay controls, one Extension control and one Detection control. The Immunoassay controls (two non-human proteins) monitor all three steps starting with the immunoreaction. The Extension Control (an antibody linked to two matched oligonucleotides for immediate proximity 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 for which one or more of the internal control values deviate from a pre-determined range will be flagged and may be removed before statistical analysis.

An external control, inter-plate control (IPC), is included on each plate and used in a second normalization step. This control is made up of a pool of probes similar to the Extension control (Ext Ctrl), but generated with 92 matching oligonucleotide pairs. Furthermore, the improves inter-assay precision and allows for optimal comparison of data derived from multiple runs. The term "Normalized Protein eXpression (NPX)" refers to normalized data as described above.

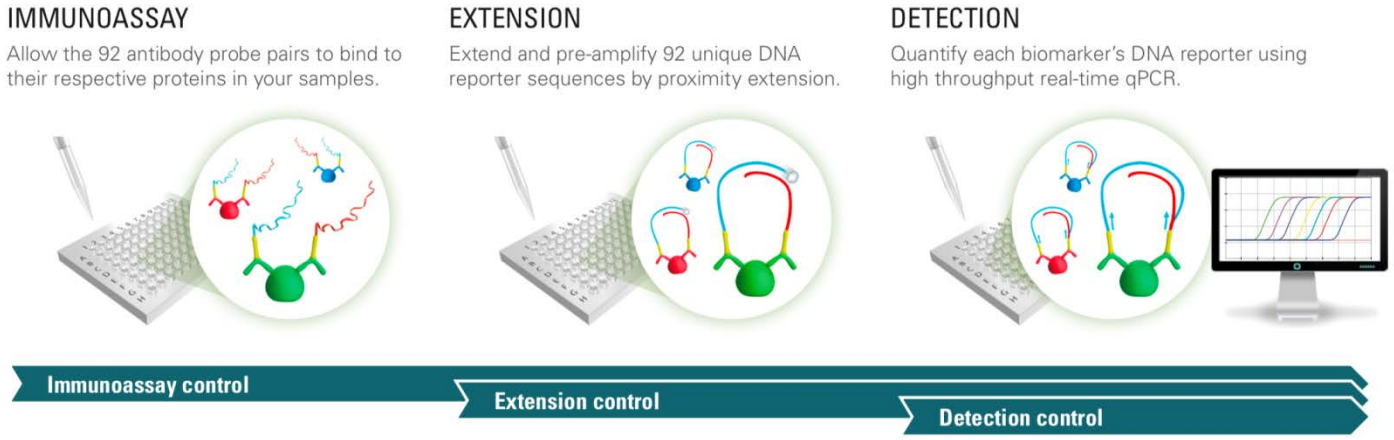

Fig 1. Olink assay procedure (above) and controls (below). The internal controls enables monitoring of the three core steps in the Olink assay and used for quality control and data normalization. Read out is performed by using the Fluidigm® Biomark™ or the Fluidigm® Biomark™ HD system.

2.2 Data Analysis

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 is then performed for each assay by subtracting the corresponding dCq-value for the Interplate Control (IPC) from the dCq-values generated. In the final step of the pre-processing procedure the values are set relative to a correction factor determined by Olink. The generated Normalized Protein eXpression (NPX) unit is on a log2 scale where a larger number represents a higher protein level in the sample, typically with the background level at around zero. Linearization of data is performed by the mathematical operation $2^{\wedge NPX}$. Coefficient of variation (CV) calculations were performed on linearized values.

2.3 Sample Types

The ability to use different sample types was evaluated with Olink Immuno-Onc I 96×96 by collecting matched serum, EDTA, acid citrate dextrose (ACD), and sodium heparin plasma samples from 4 healthy individuals. Table 1 summarizes response values for 32 normal EDTA plasma samples expressed in NPX, as well as relative differences 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 will therefore function without limitation in these sample types. In addition, cell lysates from 10 different cell lines were also evaluated.

2.4 Detection Limit

Calibrator curves were determined for 90 out of 92 biomarkers simultaneously in a multiplex format. Two protein biomarkers (IL-1alpha and CD8) lacked accessible recombinant antigen. Limit of detection (LOD) was defined as 3 standard deviations above background and reported in pg/mL for all assays where recombinant protein antigen was available, see Table 1 and Figure 2.

2.5 High Dose Hook Effect

The high dose hook effect is a state of antigen excess relative to the reagent antibodies, resulting in falsely lower values. In such cases, a significantly lower value can be reported which leads to misinterpretation of results. Therefore, the hook effect was determined for each analyte, here reported in pg/mL for 90 out of 92 assays, see Table 1.

2.6 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. The upper and lower limits of quantification, ULOQ and LLOQ, respectively were calculated with the following trueness and precision criteria; relative error ≤ 30% and CV ≤ 30%, of back- calculated values, and reported in pg/mL, see Table 1.

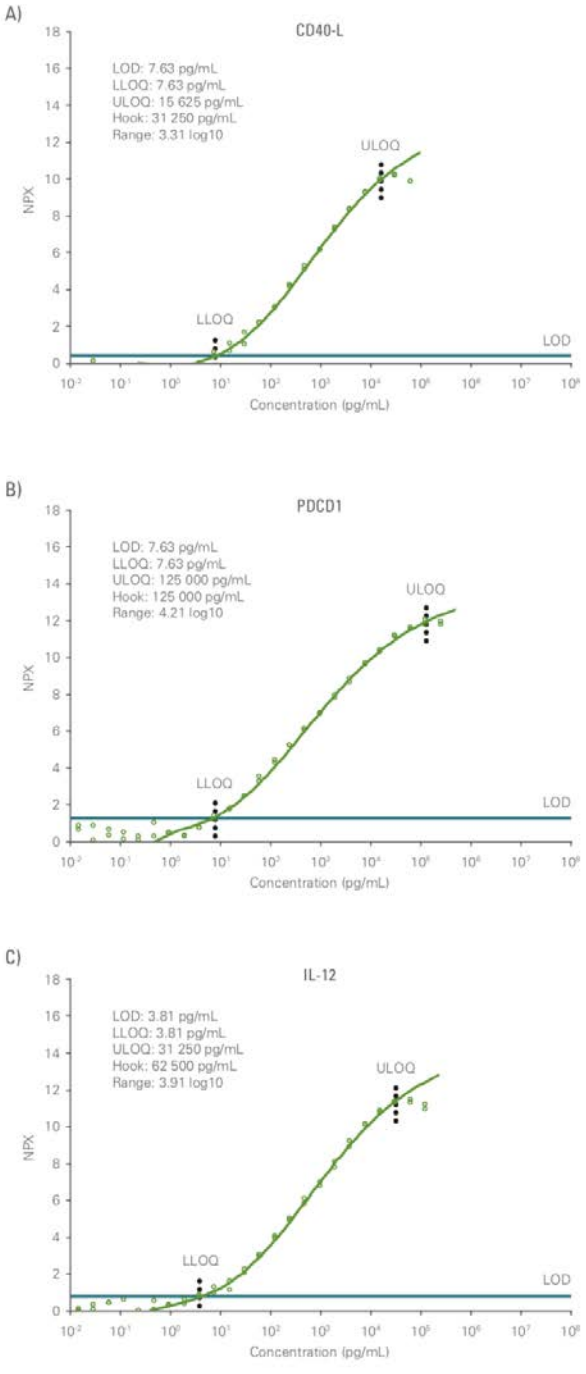

Fig 2. Calibrator curves from 3 assays and their corresponding analytical measurement data.

Three assays with their analytical data are shown in Figure 2 and the distribution of measuring ranges of 90 assays and endogenous plasma levels are shown in Figure 3. Separate calibrator curves established for each assay may be viewed at www.olink.com.

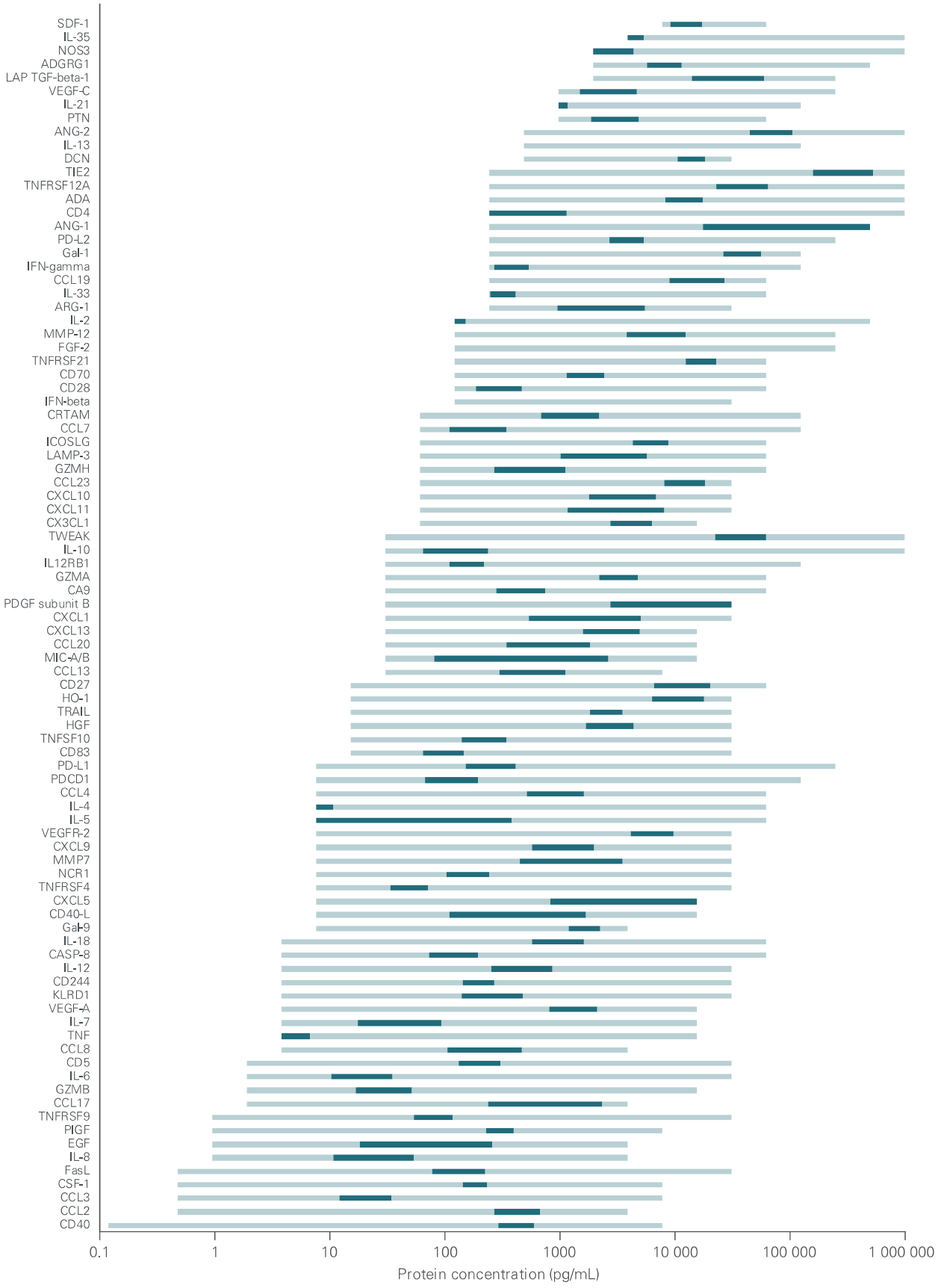

Fig 3. Distribution of analytical measuring range, defined by the lower and upper limits of quantification (LLOQ-ULOQ), and normal plasma levels (dark blue bars) for 90 out of 92 analytes.

Table 1. Sample Types; Normalized Protein eXpression (NPX), Endogenous Interference, Analytical Measurement; 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 92 analytes. Not available, NA

		Sample types						Endogenous Interference	Analytical measurement					Precision	
		Normal plasma levels (NPX)			Relative to EDTA plasma (%)			(mg/mL)	pg/mL				log10	% CV	
Target	UniProt No	10th %tile	Median	90th %tile	ACD	Heparin	Serum	Hemolysate	LOD	LLOQ	ULOQ	Hook	Range	Intra	Inter
Adenosine deaminase	P00813	3.3	3.8	4.5	94	90	103	0.2	244	244	1 000 000	1 000 000	3.6	5.8	13
Adhesion G-protein coupled receptor G1	Q9Y653	NA	1.5	1.9	94	86	120	15	1953	1953	500 000	1 000 000	2.4	8.0	15
Angiopoietin-1	Q15389	5.9	7.4	10	16	157	274	15	122	244	500 000	500 000	3.3	7.4	8.2
Angiopoietin-1 receptor	Q02763	7.6	8.1	8.5	97	92	105	15	244	244	1 000 000	1 000 000	3.6	7.2	8.4
Angiopoietin-2	O15123	4.7	5.3	6.1	90	84	107	15	488	488	1 000 000	1 000 000	3.3	7.4	12
Arginase-1	P05089	NA	1.6	4.6	33	52	163	0.0	244	244	31 250	250 000	2.1	6.5	7.0
Carbonic anhydrase 9	Q16790	3.3	4.1	4.8	90	100	110	15	15	30	62 500	125 000	3.3	7.7	7.3
Caspase-8	Q14790	4.2	4.8	5.7	72	130	174	0.2	3.8	3.8	62 500	62 500	4.2	7.2	9.7
C-C motif chemokine 13	Q99616	6.1	7.2	8.8	57	120	227	15	7.6	30	7812	7812	2.4	8.3	13
C-C motif chemokine 17	Q92583	5.7	7.4	9.4	37	134	276	15	1.9	1.9	3906	7812	3.3	8.7	14
C-C motif chemokine 19	Q99731	7.9	8.6	10	98	87	108	15	122	244	62 500	125 000	2.4	7.7	11
C-C motif chemokine 2	P13500	9.2	9.8	11	100	99	122	15	0.5	0.5	3906	3906	3.9	8.0	12
C-C motif chemokine 20	P78556	5.1	6.2	8.2	102	75	74	15	15	30	15 625	31 250	2.7	8.5	16
C-C motif chemokine 23	P55773	8.7	9.4	11	95	85	90	15	30	61	31 250	62 500	2.7	8.2	14
C-C motif chemokine 3	P10147	4.6	5.2	6.4	70	98	145	15	0.5	0.5	7812	3906	4.2	8.2	14
C-C motif chemokine 4	P13236	6.3	7.1	8.3	66	99	134	15	7.6	7.6	62 500	125 000	3.9	8.5	16
C-C motif chemokine 7	P80098	1.6	2.3	3.0	85	111	127	7.5	30	61	125 000	1 000 000	3.3	8.7	10
C-C motif chemokine 8	P80075	5.4	6.9	8.3	62	92	173	15	1.9	3.8	3906	3906	3.0	7.6	13
CD27 antigen	P26842	7.6	8.2	8.7	92	100	108	15	7.6	15	62 500	125 000	3.6	6.7	7.2
CD40 ligand	P29965	2.9	4.3	7.0	39	292	1042	15	7.6	7.6	15 625	31 250	3.3	7.7	9.4
CD40L receptor	P25942	10	11	11	95	105	126	15	0.1	0.1	7812	31 250	4.8	7.3	10
CD70 antigen	P32970	3.4	4.0	4.7	92	97	169	15	122	122	62 500	125 000	2.7	8.0	11
CD83 antigen	Q01151	2.5	3.0	3.5	88	83	104	15	2.8	15	31 250	62 500	3.3	7.8	11
C-X-C motif chemokine 1	P09341	7.2	8.7	11	37	157	285	7.5	7.6	30	31 250	31 250	3.0	7.2	12
C-X-C motif chemokine 10	P02778	6.7	7.5	9.5	88	87	112	15	30	61	31 250	31 250	2.7	11	14
C-X-C motif chemokine 11	O14625	4.6	5.8	8.9	29	147	281	0.5	30	61	31 250	31 250	2.7	8.8	12
C-X-C motif chemokine 13	O43927	8.1	8.8	9.8	106	81	131	15	30	30	15 625	15 625	2.7	6.2	11
C-X-C motif chemokine 5	P42830	7.8	10	13	13	190	271	7.5	7.6	7.6	15 625	31 250	3.3	7.9	11
C-X-C motif chemokine 9	Q07325	6.2	6.9	8.2	89	94	95	15	1.9	7.6	31 250	31 250	3.6	9.8	14
Cytotoxic and regulatory T-cell molecule	O95727	4.1	4.8	5.8	90	105	127	15	30	61	125000	125 000	3.3	7.2	15
Decorin	P07585	4.7	5.0	5.4	99	120	118	15	488	488	31 250	1 000 000	1.8	8.2	8.2
Fas antigen ligand	P48023	6.1	6.7	7.7	109	100	109	15	0.5	0.5	31 250	31 250	4.8	7.7	11
Fibroblast growth factor 2	P09038	NA	NA	2.0	95	56	89	15	30	122	250 000	1 000 000	3.3	6.3	9.0
Fractalkine	P78423	6.1	6.6	7.1	94	110	143	15	30	61	15 625	1 000 000	2.4	7.1	7.5
Galectin-1	P09382	6.4	6.7	7.2	94	102	111	15	244	244	125 000	250 000	2.7	6.0	8.0
Galectin-9	O00182	7.7	8.1	8.5	102	102	110	0.5	3.8	7.6	3906	15 625	2.7	5.3	9.1
Granzyme A	P12544	5.1	5.5	6.2	90	88	109	7.5	30	30	62 500	125 000	3.3	9.9	13
Granzyme B	P10144	2.2	3.0	3.6	89	76	88	0.5	1.9	1.9	15625	31 250	3.9	8.1	13
Granzyme H	P20718	3.5	4.5	5.7	104	103	116	0.5	30	61	62 500	125 000	3.0	8.5	12
Heme oxygenase 1	P09601	12	12	13	94	92	98	15	15	15	31 250	31 250	3.3	7.3	13
Hepatocyte growth factor	P14210	6.9	7.5	8.3	74	73	153	15	3.8	15	31 250	250 000	3.3	8.4	10
ICOS ligand	O75144	5.3	5.7	6.0	95	144	146	15	61	61	62 500	1 000 000	3.0	6.6	7.4
Interferon beta	P01574	NA	NA	NA	117	64	102	15	30	122	31 250	125 000	2.4	6.0	12
Interferon gamma	P01579	NA	NA	0.3	112	66	106	15	244	244	125 000	250 000	2.7	8.8	9.7
Interleukin-1 alpha	P01583	NA	NA	NA	88	81	69	15	NA	NA	NA	NA	NA	20	NA
Interleukin-10	P22301	2.1	2.6	3.5	87	85	111	15	15	30	1 000 000	1 000 000	4.5	9.7	12
Interleukin-12	P29459, P29460	4.9	5.8	6.8	84	75	109	15	3.8	3.8	31 250	62 500	3.9	7.5	11
Interleukin-12 receptor subunit beta-1	P42701	2.0	2.3	2.8	91	84	98	15	30	30	125 000	125 000	3.6	7.1	11
Interleukin-13	P35225	NA	NA	0.4	93	64	110	15	244	488	125 000	1 000 000	2.4	8.9	6.6

		Sample types						Endogenous Interference	Analytical measurement					Precision	
		Normal plasma levels (NPX)			Relative to EDTA plasma (%)			(mg/mL)	pg/mL				log10	% CV	
Target	UniProt No	10th %tile	Median	90th %tile	ACD	Heparin	Serum	Hemolysate	LOD	LLOQ	ULOQ	Hook	Range	Intra	Inter
Interleukin-18	Q14116	8.0	8.9	9.6	87	92	108	3.8	0.9	3.8	62 500	62 500	4.2	8.0	12
Interleukin-2	P60568	NA	NA	NA	107	60	122	15	122	122	500 000	1 000 000	3.6	12	7.2
Interleukin-21	Q9HBE4	NA	NA	NA	107	65	102	15	976	976	125 000	125 000	2.1	11	10
Interleukin-33	O95760	NA	NA	0.8	102	77	112	15	244	244	62 500	125 000	2.4	7.3	9.8
Interleukin-35	Q14213, P29459	NA	NA	NA	93	43	91	15	1953	3906	1 000 000	1 000 000	2.4	18	16
Interleukin-4	P05112	NA	NA	NA	100	76	105	15	7.6	7.6	62 500	62 500	3.9	7.1	13
Interleukin-5 (IL-5)	P05113	NA	0.9	5.2	94	94	102	15	7.6	7.6	62 500	125 000	3.9	12	15
Interleukin-6	P05231	3.4	4.1	5.2	106	106	119	15	0.9	1.9	31 250	31 250	4.2	7.3	11
Interleukin-7	P13232	3.3	4.2	6.1	45	102	307	15	1.9	3.8	15 625	31 250	3.6	7.6	11
Interleukin-8	P10145	4.5	5.3	7.1	71	116	175	7.5	0.5	0.9	3906	7812	3.6	7.9	13
Latency-associated peptide transforming growth factor beta-1	P01137	1.6	1.9	3.1	77	102	168	15	1953	1953	250 000	250 000	2.1	6.4	13
Lysosome-associated membrane glycoprotein 3	Q9UQV4	4.2	5.9	6.7	90	94	111	15	30	61	62 500	250 000	3.0	7.2	9.6
Macrophage colony-stimulating factor 1	P09603	7.9	8.2	8.5	94	92	111	15	0.2	0.5	7812	31 250	4.2	7.0	11
Macrophage metalloproteinase-12	P39900	6.1	7.1	8.0	138	117	125	15	30	122	250 000	500 000	3.3	8.1	9.8
Matrix metalloproteinase-7	P09237	6.7	8.4	9.8	442	421	472	15	7.6	7.6	31 250	62 500	3.6	7.0	11
MHC class I polypeptide-related sequence A/B	Q29983, Q29980	NA	4.6	5.5	88	95	109	15	30	30	15 625	1 000 000	2.7	6.5	10
Natural cytotoxicity triggering receptor	O76036	3.7	4.2	4.9	90	97	118	15	7.6	7.6	31 250	62 500	3.6	7.3	8.9
Natural killer cell receptor 2B4	Q9BZW8	5.7	6.1	6.6	86	97	113	15	1.9	3.8	31 250	31 250	3.9	7.4	9.8
Natural killer cells antigen CD94	Q13241	4.7	5.6	6.8	84	94	109	15	3.8	3.8	31 250	31 250	3.9	6.9	10
Nitric oxide synthase, endothelial	P29474	NA	0.9	2.4	128	59	76	15	976	1953	1 000 000	1 000 000	2.7	19	20
Placenta growth factor	P49763	7.9	8.3	8.8	88	95	109	15	0.9	0.9	7812	31 250	3.9	7.7	11
Platelet-derived growth factor subunit B	P01127	7.6	9.1	11	22	102	179	15	15	30	31 250	62 500	3.0	8.2	13
Pleiotrophin	P21246	NA	1.6	2.7	73	22	42	15	488	976	62 500	125 000	1.8	8.4	18
Pro-epidermal growth factor	P01133	3.9	5.4	8.4	28	145	710	15	0.9	0.9	3906	3906	3.6	8.0	9.6
Programmed cell death 1 ligand 1	Q9NZQ7	4.3	4.9	5.6	69	91	108	15	7.6	7.6	250 000	1 000 000	4.5	8.9	11
Programmed cell death 1 ligand 2	Q9BQ51	2.2	2.6	3.0	93	92	111	15	244	244	250 000	500 000	3.0	6.4	11
Programmed cell death protein 1	Q15116	3.3	4.0	4.7	89	98	111	15	7.6	7.6	125 000	125 000	4.2	9.7	13
Stromal cell-derived factor 1	P48061	NA	0.6	1.8	88	60	76	15	7812	7812	62 500	125 000	0.9	7.6	13
T-cell surface glycoprotein CD4	P01730	NA	NA	NA	109	88	81	15	244	244	1 000 000	1 000 000	3.6	7.1	9.6
T-cell surface glycoprotein CD5	P06127	4.6	5.3	5.9	93	94	105	15	2.9	1.9	31 250	31 250	4.2	6.9	17
T-cell surface glycoprotein CD8 alpha chain	P01732	8.4	9.6	10	100	86	89	15	NA	NA	NA	NA	NA	10	10
T-cell-specific surface glycoprotein CD28	P10747	1.2	1.5	2.0	97	81	106	15	61	122	62 500	125 000	2.7	5.8	13
TNF-related apoptosis-inducing ligand	P50591	7.7	8.3	8.8	100	99	114	15	7.6	15	31 250	31 250	3.3	7.6	8.9
Tumor necrosis factor	P01375	NA	NA	NA	93	75	108	15	3.8	3.8	15 625	31 250	3.6	10	16
Tumor necrosis factor ligand superfamily member 12	O43508	7.6	8.3	9.9	81	92	129	15	30	30	1 000 000	1 000 000	4.5	13	11
Tumor necrosis factor ligand superfamily member 14	O43557	3.1	3.8	4.6	71	125	341	7.5	15	15	31 250	31 250	3.3	7.8	8.9
Tumor necrosis factor receptor superfamily member 12A	Q9NP84	5.8	6.5	7.3	96	98	92	15	244	244	1 000 000	1 000 000	3.6	9.2	13
Tumor necrosis factor receptor superfamily member 21	O75509	7.7	8.1	8.6	85	90	115	15	30	122	62 500	125 000	2.7	7.4	11
Tumor necrosis factor receptor superfamily member 4	P43489	2.9	3.4	4.0	90	97	124	15	3.8	7.6	31 250	31 250	3.6	7.1	12
Tumor necrosis factor receptor superfamily member 9	Q07011	5.4	5.9	6.6	96	96	110	15	0.9	0.9	31 250	31 250	4.5	7.5	10
Vascular endothelial growth factor A	P15692	7.9	8.4	9.2	75	92	142	15	3.8	3.8	15 625	31 250	3.6	7.8	10
Vascular endothelial growth factor C	P49767	NA	2.2	4.0	40	124	307	15	488	976	250 000	250 000	2.4	7.1	12
Vascular endothelial growth factor receptor 2	P35968	7.1	7.7	8.1	100	96	111	15	7.6	7.6	31 250	31 250	3.6	6.9	12

*U/µl

2.7 Precision: Repeatability

Intra-assay variation (within-run) was calculated as the mean %CV for 6 individual samples run in triplicates within each of 8 separate runs during the validation studies. Inter-assay variation (between runs) was calculated between experiments with the same operator. The reported inter-assay %CV is the average of three operators' %CV. Variation calculations were performed on linearized values for 92 analytes for which response levels could be measured in serum and normal plasma, see Table 1.

Across all 92 assays, the mean intra-assay and inter-assay variations were observed to be 8.3% and 11.5%, respectively. The distribution of both intra- assay and inter-assay variations are shown in Figure 4.

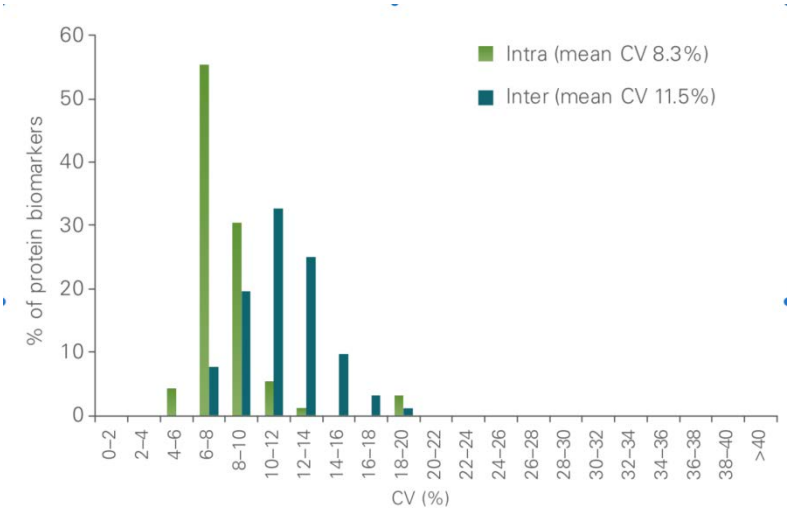

Fig 4. Distribution of intra-assay and inter-assay variations of Olink Immuno-Onc I [96×96]

2.8 Precision: Reproducibility

Inter-site variations (between-site) were investigated during the validation of previous panels in beta-site studies to estimate the expected variations in values between different laboratories, with different operators and using different equipment. The beta- site studies have previously shown reproducibility and repeatability in line with Olink Proteomics results. Additional information is provided in section 3.

2.9 Analytical Specificity: Assay Specificity

The antibodies selected for use in Olink Immuno-Onc I have previously been evaluated against 92 panel-specific proteins as well as against additional 107 proteins. In principle, the specificity is tested by creating a test sample, consisting of a pool of antigens, which is then incubated with all 92 antibody probe pairs from 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. Ten sub-pools of antigen are evaluated to cover the 92 assays in Olink, see Figure 5.

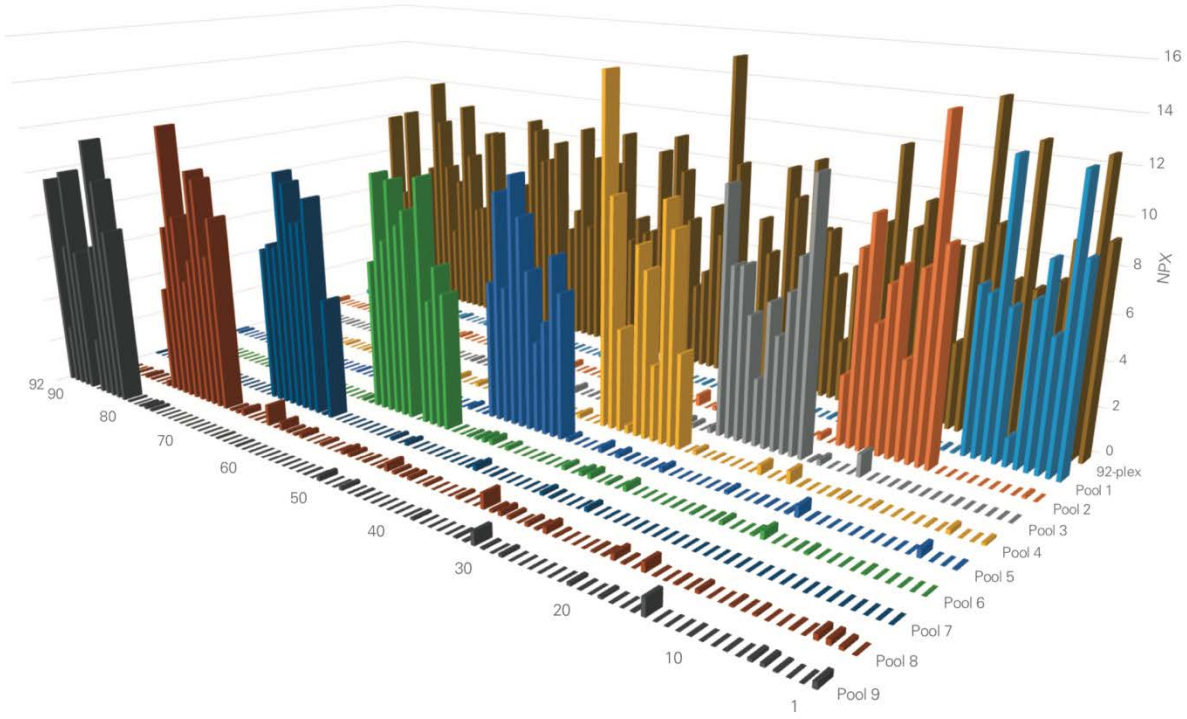

Fig 5. Assay readout specificity of the Olink platform. For each assay, specificity is confirmed by testing antigen sub-pools against the complete 92-plex pool as to each sub-mix.

2.10 Analytical Specificity: Endogenous Interference

Endogenous interference from heterophilic antibodies, e.g. human anti-mouse antibody (HAMA), and rheumatoid factor are 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 could be detected for any of the samples in previously tested panels, indicating sufficient blocking of these agents (data not shown).

The potential impact of bilirubin, lipids and hemolysate, known interfering plasma and serum components, were evaluated at different added concentrations. An example of hemolysate levels tested is shown in Figure 6. These additions represent different patient health conditions and/or sample collection irregularities. Interference by bilirubin and lipids has previously been evaluated, and disturbance has only been observed at extreme levels corresponding to 8 or 10 times normal values[3, 4] and therefore not performed for Olink Immuno-Onc I. In 14 out of 92 assays, altered signal was observed by the addition of hemolysate. The reason is most likely due to actual analyte 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.

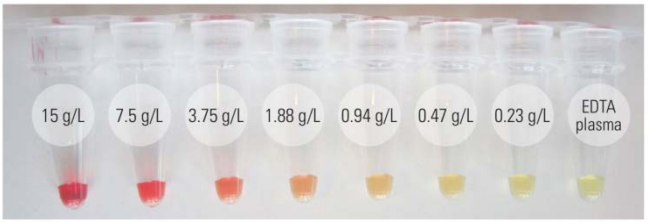

Fig 6. Endogenous interference. Levels tested for hemolysate were 0.23-15 g/L hemoglobin. The highest hemolysate concentration translates to about 10% hemolysis.

2.11 Scalability

Assay performance was further evaluated with regard to scalability, meaning the capability of the Olink technology to maintain the same quality of performance irrespective of multiplex level. Previously, we have shown that a step-wise increase of multiplex grade (8, 24, 48, 72 and 96) does not compromise assay performance (data not shown). To further strengthen that Olink provides consistent results, single assays for Growth Hormone (GH) and Matrix Metalloproteinase (MMP-7) were compared when run in a full 96-plex reaction. The results for each assay and their observed dCq-values were plotted against the entire 96-plex reaction. The square of the correlation coefficient (R^2) value was generated by linear regression.

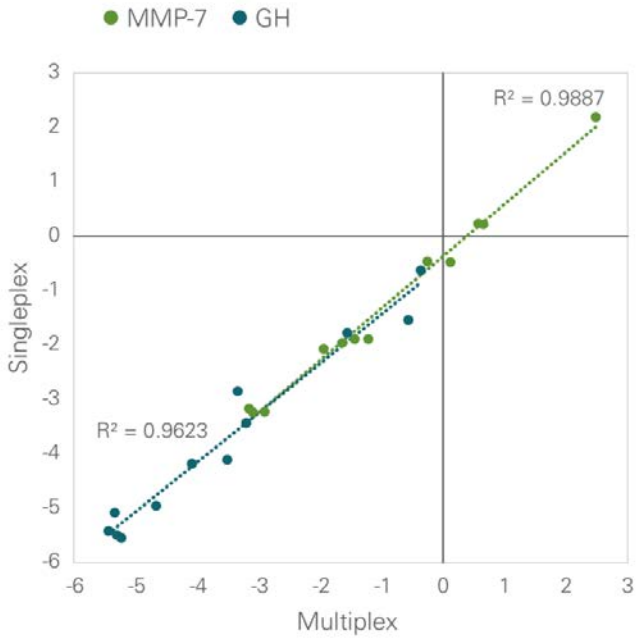

Fig 7. Scalability of the Olink technology platform. The experiment was performed using the Olink CVD II 96×96 panel. Human plasma samples were analyzed in singleplex for Growth Hormone (GH) and Matrix Metalloproteinase (MMP-7) with the equivalent assays performed in a full 96-plex reaction. The observed dCq (log2) values were plotted, and the correlation coefficient R^2 value was generated by linear regression.

3. Assays performance characteristics based on Mt Sinai CIMAC's internal validation

In addition to characteristics provided by manufacturer above, Mt Sinai CIMAC has independently performed a series of analytical validations, as required for proficiency testing to be considered a certified user with Olink, as well as for internal documentation and comparisons to other standard assays such as ELISA and Luminex multiplex.

- *Analyte(s)*

The list of 92 analytes in the Immuno-Oncology panel are shown in Table 1. Additional panels are routinely used by MS-CIMAC, such as an inflammation specific panel, or cardiovascular panel, which may be added on an ad-hoc basis depending on study endpoints. The list of 11 panels offered by Olink are provided at olink.com.

- *Technical platform(s)*

The Olink platform runs on Fluidigm Biomark, and uses Juno and PCR thermal cyclers. Instruments are calibrated and serviced at least once a year. Other small instruments such as automated pipetters are also serviced regularly.

- *Specimens & methods of for specimen acquisition, fixation, stabilization, and processing.*

The Olink platform can be used with only 1µl per sample of a variety of soluble biospecimen sources: peripheral blood plasma or serum, bone marrow plasma or serum, ascites supernatant, spinocerebral fluid, cell culture supernatant, urine, etc. The use of biospecimens however may not be interchangeable, as each of these have different characteristics based on processing (with or without heparin, addition of cell culture medium, etc).

MS-CIMAC has performed a comparison of peripheral blood plasma vs. bone marrow plasma in 20 paired samples from multiple myeloma patients. Figure 8 shows that there are notable differences in specific analytes when using bone marrow vs. peripheral blood plasma, even if the majority of samples has similar ranges of detection. In particular, peripheral blood plasma has greater levels of T cell related markers and cytokines, such as IL-7, IL-12, CD8a, CD40-L, while bone marrow plasma has greater detection of endothelial, stromal or tumor markers such as ADA, ARG1, CASP8, HGF, NOS3, or GAL9.

Therefore, it is recommended to annotate type of samples analyzed (serum, plasma, etc.) and ensure that differences observed in these analytes are to be studied within each sample type if comparing longitudinal samples. Additional studies are being conducted to assess effect of multiple freeze-thaw cycles upon analyte detection, even though a majority of studies conducted so far have been done using serum or plasma thawed once (as recommended usage).

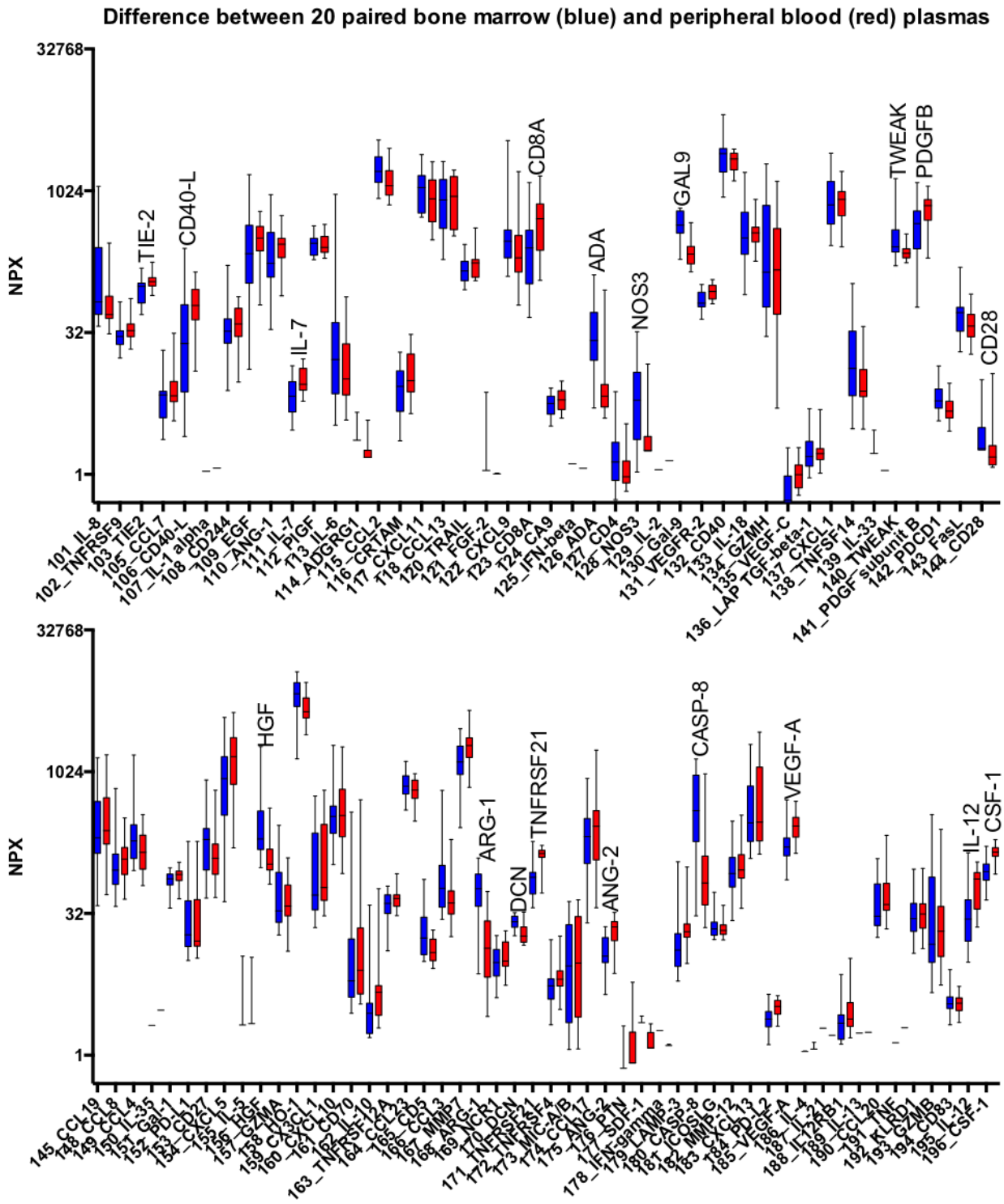

Figure 8. Box plots of each analyte in the I-O Olink kit showing min, max, 25% and 75% percentile, and median of peripheral blood plasma (red) or bone marrow plasma (blue) in 20 paired samples from multiple myeloma patients (representing 8 patients with up to 3 time points). Analytes with the largest differences are indicated with their names above them.

- ***Assay validation data**, including:*
  - *Current status of studies defining the sensitivity, specificity, accuracy, precision, reportable range, reference ranges/intervals (normal values), turn-around time and failure rate of the assay as it is to be performed in the trial.*

Besides assay validation performed by Olink, a series of validation assays are underway to compare data generated with Olink to those from Luminex multiplex assay and from standard ELISA titrations on a

subset of common targets. In figures 9 to 11 below are a range of validation assays done at MS-CIMAC to assess reproducibility and robustness of data across assays and operators.

- *Use of positive and negative controls, calibrators, and reference standards.*

1. **INTERNAL CONTROLS added to each individual sample**
    a. Two incubation controls: non-human antigens which monitor potential variation in all three steps. GFP and PE.
    b. One extension control: an antibody with both DNA-tags always in proximity which monitors the extension step and is used for normalization across samples.
    c. One detection control: a complete double-stranded amplicon which controls the amplification/detection steps.
2. **EXTERNAL CONTROLS added to separate wells on the plate (6 wells out of 96)**
    a. Inter-plate control (IPC) – 3 wells: a pool of 92 antibodies, each with one of the pairs of unique DNA tags on it positioned in fixed proximity (i.e. 92x extension control). Used for normalization, and compensates for potential sequence biases and variation between runs/plates.
    b. Negative control – 3 wells: Buffer with no antigens. Sets the background levels (LOD) for all proteins
3. **HIMC internal control sample**: whenever possible we include 1 or 2 pooled human normal donor plasma (N=12) in all the plates when relevant, as an additional comparator and calibrator.

- *How run-to-run variation (Coefficient of Variation; CV) was assessed and handled.*

In the figures below, a series of experiments comparing variation observed across assays and operators are provided (Figure 9). Generally, a majority of analytes had a CV <10% (Figure 10), regardless of operator or date of test, except for those with the lowest detection range where variability had a greater effect due to differences in relative lower limit of detection after normalization in each assay. There was a high degree of similarity observed in the 2 independent assays (Figure 11).

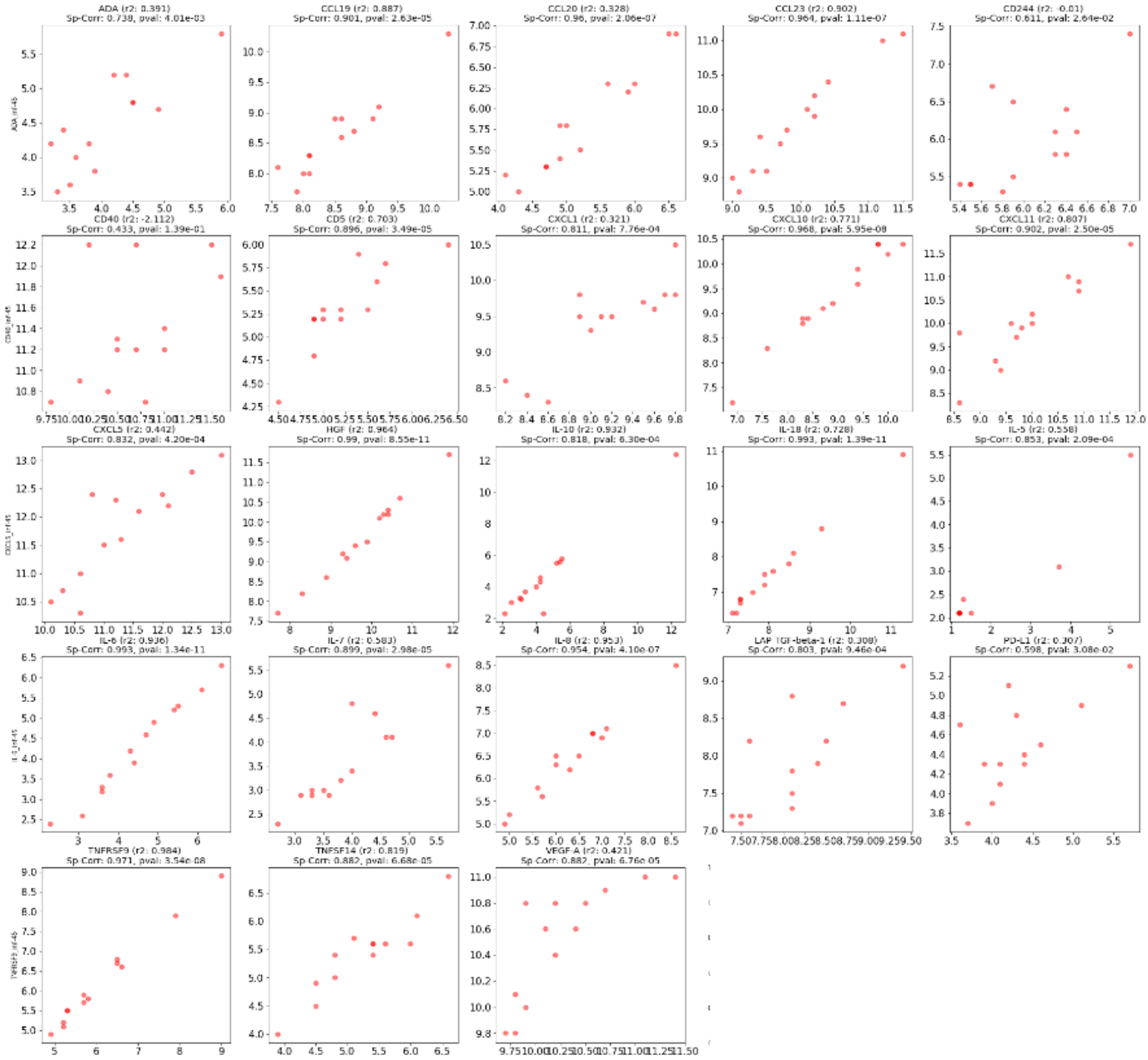

Figure 9. O-Link Soluble analyte (protein) measurement in sera from 13 patients to establish inter-assay variation. Assays were performed at 2 different dates by 2 different operators using two different kit lot numbers. Protein expression (NPX relative value) correlation across the 13 common samples. R^2 and Spearman correlation values are shown.

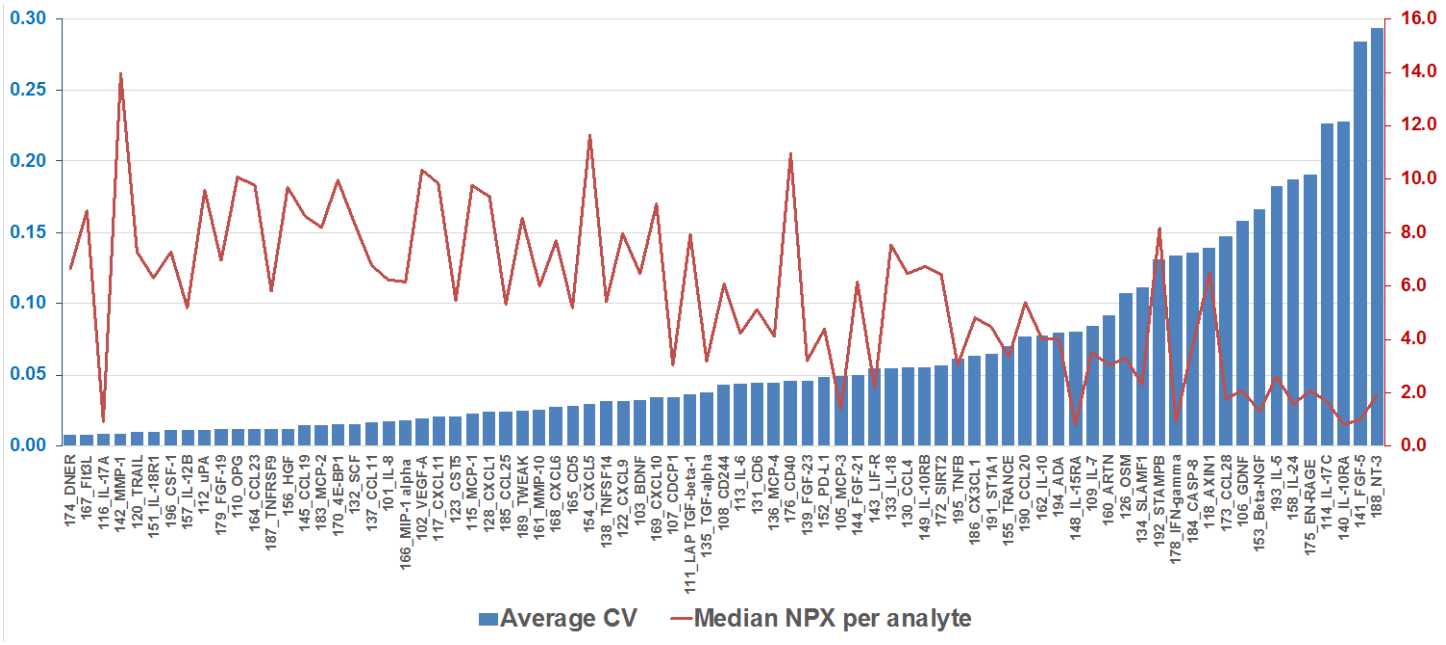

Figure 10. Inter-assay correlation expressed by Coefficient of Variation (CV). The mean CV of individual samples (blue columns, left Y-axis) across all analytes was 6.4% (interquartile range 1.8-7.9). Data shows reproducibility of NPX values above limit of detection from 13 human plasma samples over 2 consecutive assays with different operators using the Olink Inflammation panel (X-axis). Analytes with CV greater than 10% are nearly all for analytes with the lowest detection range in these samples, as shown by median NPX values <3 (red line, right Y-axis), close to the limit of detection with only very weak reactivity, thereby introducing greater standard deviation over average.

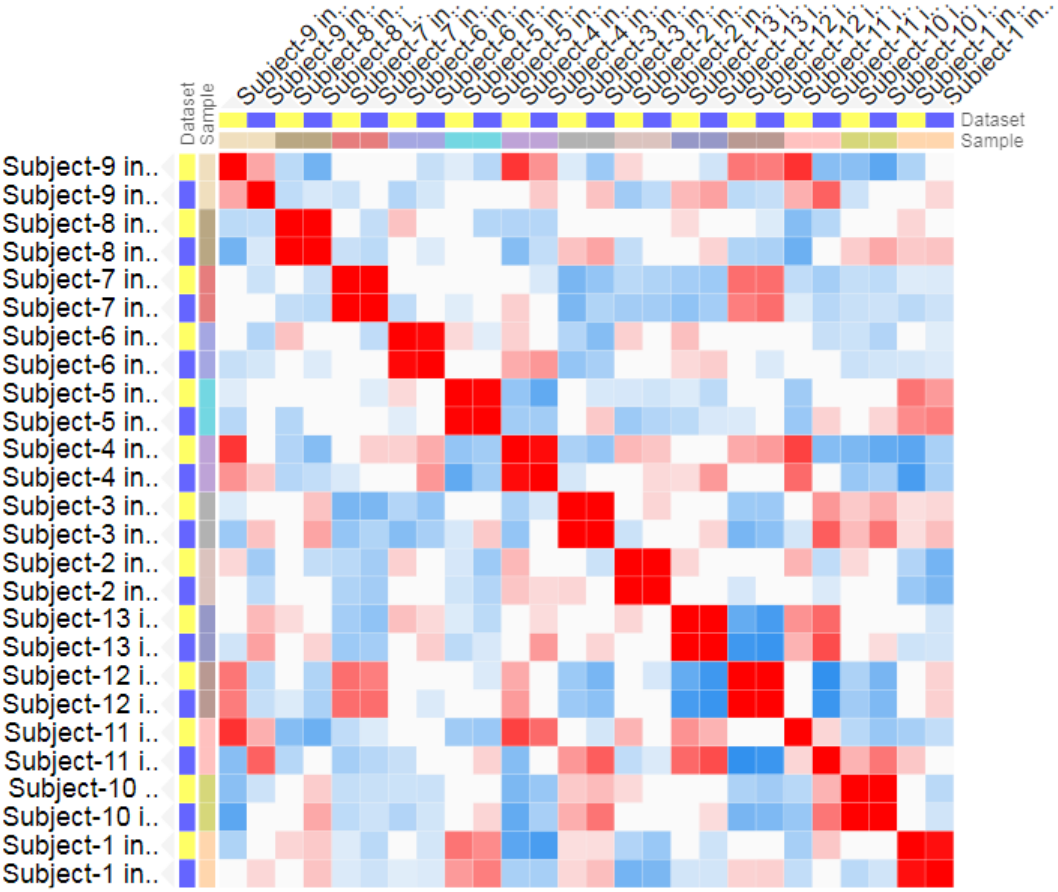

Figure 11. Similarity matrix of Olink samples in 2 assays. Sample correlation across common proteins shown as a similarity heatmap. Rows and columns have been grouped by sample id (each sample is measured in two different panels). Measurements from the same sample are more similar than measurements from different samples

- *How inter-laboratory variability in the measurements was assessed and how these sources of variation were minimized to maintain performance at all sites within acceptable limits and to prevent drift or bias in the assay.*

Inter-laboratory variability was handled by proficiency samples shared between Olink and Mt Sinai CIMAC as part of the certification of the assay. Kits for proficiency are run twice a year. However, because Olink is proposed at a single CIMAC site, the inter-laboratory variation is for now not required.

- *Describe proficiency testing and results.*

We have been trained by Olink scientist to perform the assay based on the strict Olink's training policy from the sample processing to the final data analysis using the NPX manager software. Data quality assurance is guided throughout the assay and report form is generated describing the assay pass and fail criteria in detail ensuring the final data quality. Bi-yearly proficiency tests are performed based on kits provided by Olink.

- *Scoring procedures and type of data to be acquired:*
  - *quantitative/continuously distributed*
  - *semi-quantitative/ordered categorical*
  - *qualitative/non-ordered categorical*

Scoring procedure are described in sections 2.2-2.6, and data output is a QCed relative score known as NPX (normalized protein expression) on a Log2 scale. It is a continuous variable with upper and lower limits of detection. Using the continuous data is recommended, as relative differences can be measured more precisely. Alternatively, using the lower limit of detection, it is also possible to qualify each analyte as a categorical dataset (present/absent). Finally, data can also be considered as falling within high/low categories, based on cutoffs from cohort analyses, such as median or quantiles – however, in contrast to limit of detection, these cutoffs are not pre-defined based on assay performance and would need to be decided based on biological significance instead.

- *Criteria and metrics for defining significant changes (e.g., between timepoints, between responders and non-responders).*

Defining significant changes will require additional validation based on expected biological variations between time points and between individuals. These assays are planned but require collection of longitudinal samples from individuals in a variety of clinical settings. There is currently no pre-set cutoff for significance, and statistical models, including paired pre-post analysis, unpaired cross-cohort analyses, and mixed model including both, will be used, based on variance of data and sample numbers. From a biological significance, it is unlikely that anything below 2x change will be relevant, so filters may need to be applied to ensure a minimum threshold is set for significance.

4. References

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