White paper

Multiplex analysis of inflammatory proteins: A comparative study across multiple platforms

Introduction

Many of the best-selling drugs in the world today are not effective in all patients, largely due to our lack of understanding of the biology of disease and its variation among individuals. Proteins directly control and regulate most of the body's biological functions and are crucial for health, disease and drug efficacy. It is therefore essential that healthcare moves on from the current "one size fits all" approach, towards more precise and better-informed solutions. To support this change, accurate and precise proteomics platforms are crucial. To be able to measure multiple proteins simultaneously in biological samples, several antibody-based assay platforms have been developed. It is not straightforward to compare these multiplex platforms in terms of correlations or quality parameters. Definition of measurements can also differ slightly between providers, as in the case of detection limits for example. Multiplexing technologies often have problems with cross-reactive binding or interference contributing to the signal readout, which leads to poor specificity. This problem usually escalates with the degree of multiplexing. Olink's proprietary Proximity Extention Assay (PEA) technology, circumvents these problems by use of a dual recognition approach with matched pairs of antibodies labeled with complimentary DNA oligos (see Figure 1).

White paper content
- A comparative study between three multiplex proteomics platforms
- A correlation study between Olink Target 48 Cytokine and Olink Explore 1536 in collaboration with Massachusetts General Hospital

Aim of the study

The aim of this study was to compare the Olink® Target 48 Cytokine panel head-to-head with two commonly used commercial multiplex proteomics platforms (Mesoscale Discovery (MSD) and BioRad/Luminex). With this comparative study, we aimed to verify the quality of the Olink technology relative to similar products widely used in protein biomarker research. The aim is not to investigate or explain potential differences between platforms and no criticism of the other technologies involved is implied or intended. In a follow-up study, the correlation between Olink Target 48 Cytokine and Olink Explore 1536 was examined.

Methods

This was a comparative study with three antibody-based platforms with maximum overlapping protein targets compared to Olink Target 48 Cytokine. The three technology platforms were Olink, MSD and Luminex. Three identical sample plates were developed and run on each of the platforms for head-to-head comparison of the 20 overlapping assays. Each platform was run at separate laboratory locations in the Stockholm-Uppsala area in Sweden.

Table 1. Information about platforms, samples and analysis laboratories. The Olink technology uses 1 µL/panel, BioRad/Luminex normally consumes 12.5 µL/panel and MSD needs 20-40 µL/panel. MSD can measure a maximum of 10 assays per panel, which explains the increased sample volume required.

	Olink	MSD	Luminex
	Olink R&D Uppsala	SciLife Lab Uppsala	SciLife Lab Stockholm
Number of samples/plate run	80	80	80
Number of proteins/plate run	45	10	27
Total number of proteins measured	45	43	27
Sample volume requested	20 µL	150 µL	50 µL

Results were evaluated and compared for linearity, dilution series, detectability, measurement range and precision for the overlapping assays. All three panels had an overlap of 20 proteins and Olink and MSD also overlapped on an additional 13 proteins. The data generated was extensive, and cannot be presented in its entirety in this white paper, but all data can be found in the white paper data appendix.

Technology platforms

Olink

Olink panels are able to achieve a high level of multiplexing while maintaining data quality thanks to the proprietary PEA technology. Each protein is addressed by a matched pair of antibodies, coupled to unique, partially complementary oligonucleotides and measured either by quantitative real-time PCR (qPCR) or Next Generation Sequencing (NGS) depending on the specific Olink readout platform used. The dual antibody recognition and DNA-coupled method provide exceptional specificity (1).

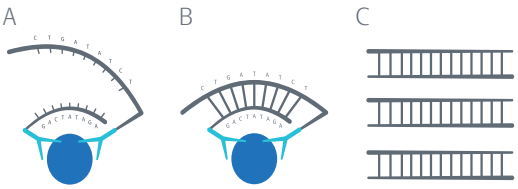

Figure 1. Main pre-readout steps in PEA. (A) Antibody pairs, labelled with DNA oligonucleotides, bind target protein in solution. (B) Oligonucleotides that are brought into proximity hybridize and are extended by a DNA polymerase. (C) This newly created piece of DNA barcode is amplified by PCR ready for readout by NGS or qPCR.

MSD (MesoScale Discovery)

MSD uses an electrochemiluminescent detection technology. The technology offers multi-spots with a maximum of 10 assays, one per spot per well on a plate. The binding carbon electrodes at the bottom of the proprietary plates allow for easy attachment of biological reagents. The sandwich immunoassay in each spot consists of a capture antibody specific for the target analyte, the target analyte, and an analyte-specific detection antibody conjugated with an electrochemiluminescent label called a sulfo-tag. For detection, a buffer creates the appropriate chemical environment for electrochemiluminescence. The instrument applies a voltage to the plate and the electrodes causes the captured labels to emit light, which is detected using a plate reader instrument (2).

Biorad/Luminex

The Luminex technology is based on magnetic color-coded beads. Each bead in the multiplex assay has its own ratio of a green and a red signal. The beads are conjugated to a capture antibody incubated with a sample. Then a secondary detection antibody binds the analyte. Luminex uses an amplification method where the detection antibody is biotinylated, and the final detection step is an addition of streptavidin that emits the fluorescent signal. After the incubation with pools of different beads and different captured antibodies, the beads are sorted according to their spectral address. The instrument and software then extrapolate the associated fluorescent signal with each spectral address for each specific labeled bead (3).

Overlapping protein assays

When selecting the platforms for comparison, the aim was to have as many overlapping proteins as possible.

For MSD, multiplex V-plex assays were used when possible, which were for 23 biomarkers. Ten additional biomarkers were analyzed using U-plex assays. The MSD panels used were V-PLEX Chemokine Panel 1 (human), V-PLEX Proinflammatory Panel 1 (human), V-PLEX Cytokine Panel 1 (human), V-PLEX Cytokine Panel 2 (human), and one custom 10-plex U-PLEX (G-CSF/CSF3, IL-17F, IL-33, FLT3L, TRAIL/TNFSF10, SDF-1α/CXCL12, MIP-3β, MCP-2/CCL8, MCP-3/CCL7, I-TAC).

For the Luminex assays the Bio-Plex Pro Human Cytokine 27-plex panel from BioRad was used. The total overlap between Olink and MSD was 33 proteins and between Olink and Luminex there were 20 overlapping proteins. Between all the three panels there was an overlap of 20 proteins. These proteins are listed in Table 2.

Table 2. Protein assays that were overlapping between Olink, MSD and Luminex.

UniProt ID	Protein	UniProt ID	Protein
P51671	Eotaxin	P22301	IL-10
P09919	G-CSF	P35225	IL-13
P04141	GM-CSF	P40933	IL-15
P01579	IFN-γ	Q16552	IL-17A
P01584	IL-1β	P02778	IP-10
P60568	IL-2	P13500	MCP-1
P05112	IL-4	P10147	MIP-1α
P05231	IL-6	P13236	MIP-1β
P13232	IL-7	P01375	TNF-α
P10145	IL-8	P15692	VEGF-A

Olink vs MSD

All three platforms had 20 overlapping assays. MSD however, had 13 additional overlapping assays with the Olink panel. The assays are listed in the table below, and the results for those assays can be found in the white paper data appendix.

Table 3. The 13 additional proteins that were overlapping between Olink and MSD.

UniProt ID	Protein
P49771	FL3LG
Q9P0M4	IL-17C
Q96PD4	IL-17F
O95760	IL-33
O14625	I-TAC
P80075	MCP-2
P80098	MCP-3
Q99616	MCP-4
Q99731	MIP-3β
P48061	SDF-1α
P01374	TNF-β
P50591	TRAIL
Q969D9	TSLP

Samples

The samples were de-identified and commercially sourced from a biobank. Three sample plates were prepared and run on each platform to evaluate the performance in linearity, dilution series in buffer, detectability, measurement range, precision, correlation and interference. All samples used were EDTA plasma. Eight samples from healthy adult donors and 24 samples from patients with diagnosed diseases associated with inflammation were

included, according to Table 4.

Table 4. Sample information.

Number of samples	Sample information
3	Alzheimer's disease
2	Atopic dermatitis
3	Coronary artery disease
3	Crohn's disease
1	Liver disease
2	Multiple sclerosis (MS)
2	Psoriasis
4	Rhematoid arthristis (RA)
3	Systemic lupus erythematosus (SLE)
1	Ulcerative colitis (UC)

Dynamic range and sample distribution

The dynamic range and sample distribution were measured, using 32 undiluted samples (eight healthy and 24 pathological samples), see Figure 2. The limit of detection (LOD) and the lower and upper limits of quantification (LLOQ and ULOQ) for each individual platform were used to estimate the percentage of quantifiable samples within the limit of quantification (LOQ). The LOD and LOQ for MSD and Luminex were received in the result report provided by SciLife Lab. The LOD and LOQ for Olink were obtained from the validation data available on the Olink website. A complete table of all the data can be found in the white paper Appendix on the Olink website.

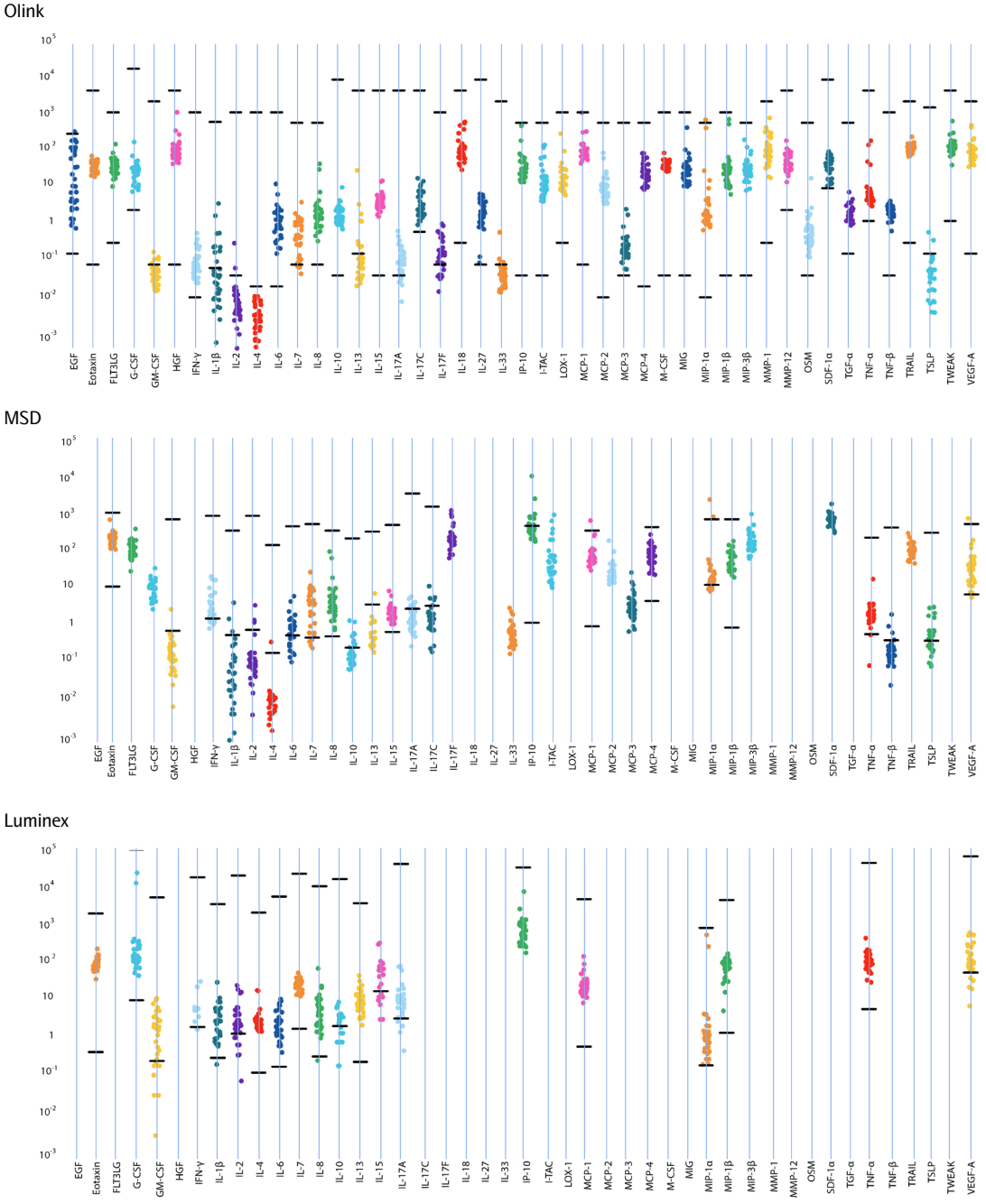

Figure 2. Dynamic range and sample distribution for Olink, MSD and Luminex. The horizontal bars represent the LLOQ and ULOQ per assay. Some MSD assays were run on their U-plex platform and therefore lack LOQ information, the others were run on their V-plex platform.

Precision CV (%)

Four samples, one healthy and three pathological, were run in triplicates to estimate the precision. Intra-assay %CV was calculated by the standard deviation for each sample divided by the mean for the same sample (see Figure 3 and Table 5).

> Definition
> The Coefficient of Variation (CV) for a sample is the standard deviation of the observations divided by the mean.

Table 5. Intra-assay %CV for each technology.

	Olink	MSD	Luminex
Intra-assay %CV (mean)	10 %	5 %	13 %

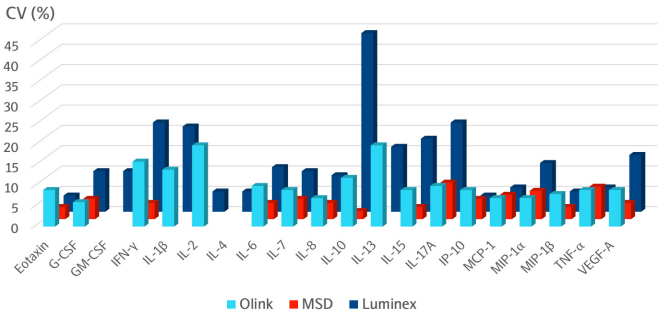

Figure 3. Intra-assay % CV for the three proteomics platforms. Assays with results under LLOQ are shown as blank in the diagram.

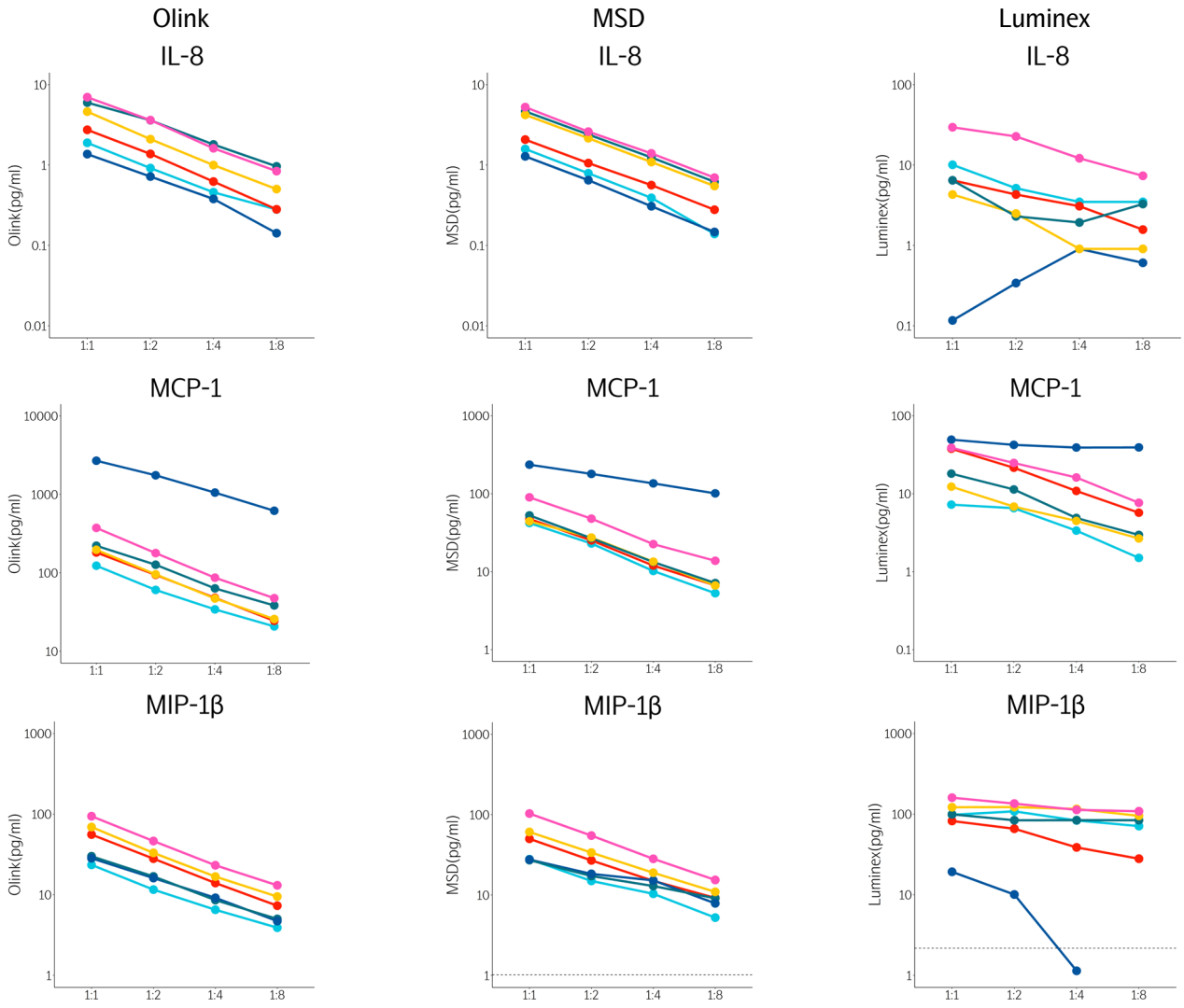

Figure 4. Parallelism between Olink, MSD and Luminex, calculated using dilution series in buffer. Olink results are shown in the left-hand column, MSD in the middle and Luminex to the right. Each sample is displayed with the same color in the plot throughout all charts. Visually the dilution series should be linear. Note that Luminex displays higher concentrations and changed order of samples compared to Olink and MSD for several assays. Concentrations below the dotted line are under LLOQ.

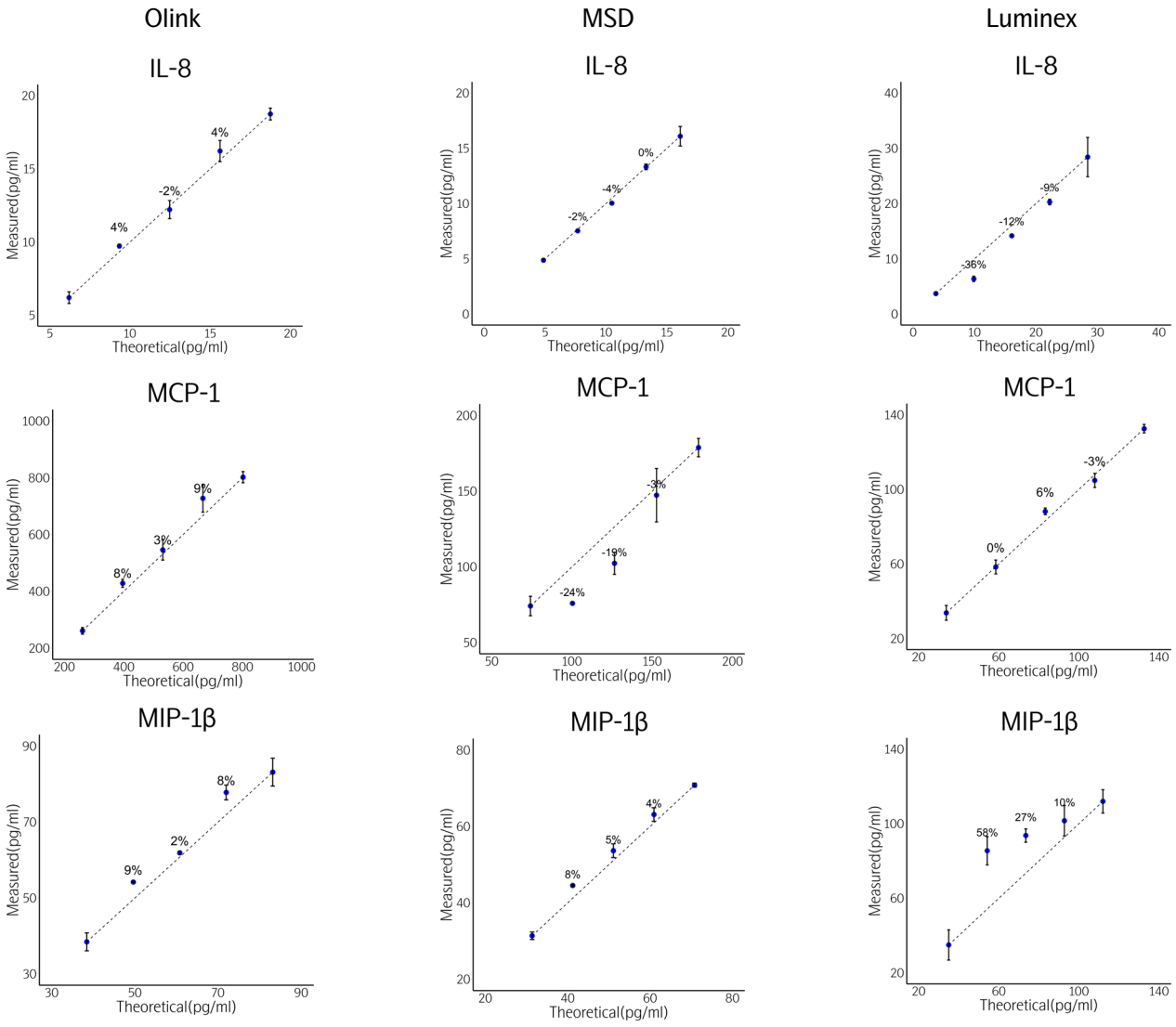

Figure 5. Platform linearity on the three example assays IL-8, MCP-1 and MIP-1β. Graphs display measured and theoretical concentrations. Bars indicate standard deviation and the percentage represents accuracy for each point.

Dilution series in buffer (parallelism)

Six samples, three healthy controls and three pathological samples, were diluted individually in four steps with a 1:2 dilution. Olink's sample diluent for Target 48 was used as diluent accross all three platforms. Information about the samples is shown in Table 6. From the data presented in Figure 4 we can conclude that Olink displays excellent parallelism.

Table 6. Information about samples used in the dilution series.

Number of samples	Sample information
1	Atopic dermatitis
1	Crohn's disease
1	SLE, Pulmonary embolism
3	Healthy adult donors

Linearity

Two samples were chosen, here referred to as HIGH and LOW, where one sample displayed high concentrations and the other low concentrations for as many proteins as possible. The linearity was evaluated by taking the high sample and the low sample and diluting them together in different predefined proportions.

The sample HIGH was diagnosed with diabetic nephropathy, Type 2 Diabetes, asthma, hypertension, coronary artery disease, multiple sclerosis, psoriasis and chronic obstructive pulmonary disease. The sample LOW was taken from a healthy donor.

Table 7. Information about samples used for linearity comparisons.

Number of samples	Sample information
100%	100% HIGH sample
75%	75% HIGH+ 25% LOW
50%	50% HIGH + 50% LOW
25%	25% HIGH +75% LOW
0%	100% LOW sample

The difference between the theoretically calculated value and the measured value is shown as percentages in the charts in Figure 5, where the bars indicate the standard deviation..

Five low-detection proteins, GM-CSF, IL-1β, IL-2, IL-4, IL-13, were excluded from the analysis since all results were below LLOQ for all platforms.

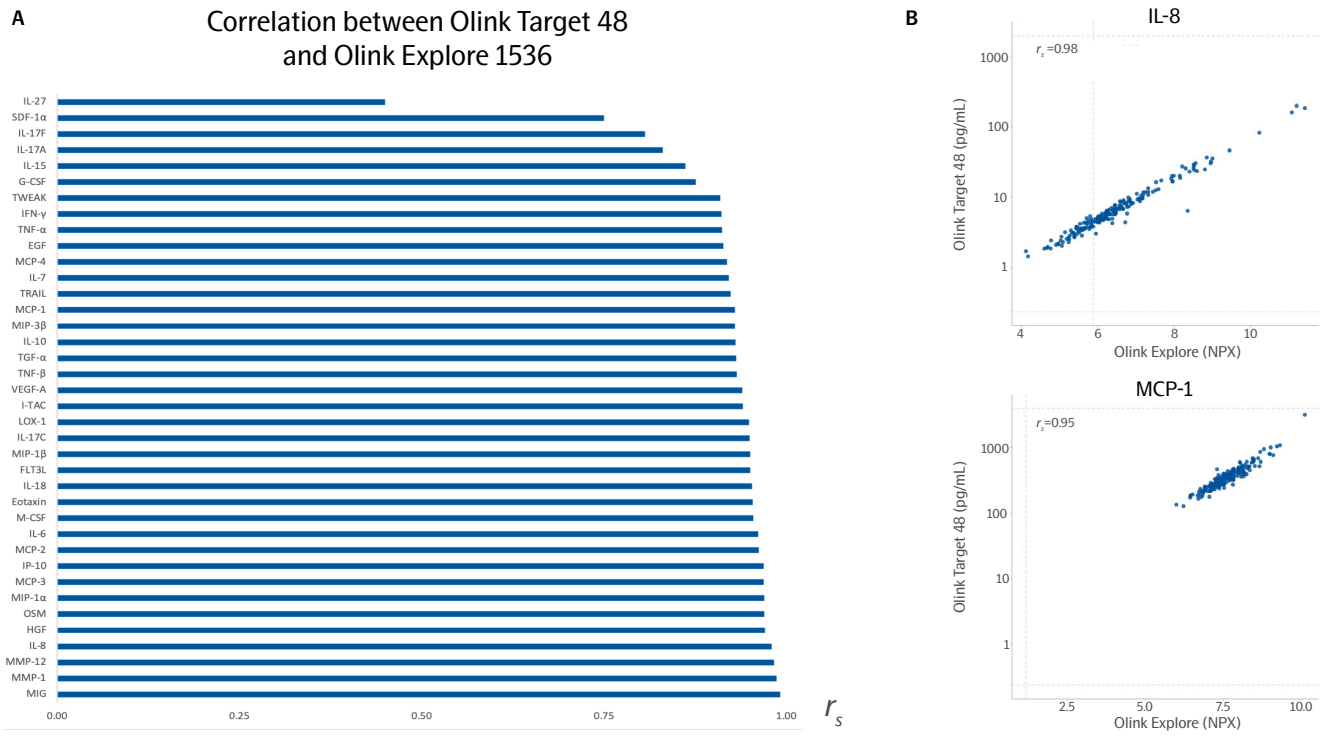

Figure 6. Results of correlation between Olink Target 48 and Olink Explore 1536. A) The x-axis shows the Spearman correlation coefficient (r_s). B) The graphs show the correlation between Olink Target 48 Cytokine and Olink Explore 1536 for IL-8 and MCP-1.

Higher concentrations above ULOQ of IL-6 and IL-7 were detected for all three platforms. The hook effect was seen for IL-6 and IL-7. Plots for these proteins can be seen in the white paper data appendix.

From the graphs in Figure 5, we can conclude that Olink displays excellent linearity.

Follow-up study: Olink panel comparisons

In the follow-up study, the correlation between Olink Target 48 Cytokine and Olink Explore 1536 was examined. 44 out of 45 assays on Target 48 are overlapping with Explore 1536.

About the correlation study in collaboration with MGH

Immune Checkpoint Blockade (ICB) has transformed the treatment of metastatic melanoma and other cancers.

> Definition
> Immune Checkpoint Blockade works by using immune checkpoint inhibitors to block checkpoint proteins from binding with their partner proteins. This prevents the "off" signal from being sent, allowing the T cells to kill cancer cells.

However, the response to ICB is very heterogeneous and prognosis for most patients remains dire. Non-invasive biomarkers to predict response to ICB remains an emerging area of investigation.

In collaboration with MGH Cancer Center, we used Olink Explore 1536 to perform comprehensive proteomic screening for ~1500 proteins directly in the plasma obtained from 200 metastatic melanoma patients at baseline and on ICB treatment at 6-weeks and 6-months.

The study demonstrated potential of circulating protein biomarkers to predict response during ICB treatment. Specifically, NPX levels measured of several cytokines using Explore 1536 were significantly differentially expressed during treatment and between ICB responders and non-responders in melanoma.

Results

To establish optimal threshold for several of the key markers identified, we performed analyses on a subset of the same melanoma cohort using Olink Target 48 Cytokine panel, reporting the protein concentration in pg/mL. For correlation purposes, 200 samples derived from 84 melanoma patients receiving ICB treatment previously analyzed by Olink Explore 1536 were analyzed with Olink Target 48 Cytokine at Olink Analysis Service in Boston.

Overall, data confirmed very strong correlation for the biomarkers measured on both the Explore 1536 (NPX levels) and Target 48 (pg/mL) platforms as shown in Figure 6.

Six out of 45 assays only showed results above LLOQ for five or less samples and were therefore excluded for further analysis. The six assays with too few measurements within LOQ were: IL-33, IL-4, IL-13, IL-2, TSLP and IL-1β.

For the remaining 38 assays, the median Spearman correlation coefficient (r_s) ranged between 0.50-0.99 (median 0.95).

Definition

Spearman's correlation coefficient, r_s, measures the strength and direction of association between two ranked variables. The closer r_s is to 1, the stronger the association between the ranks.

r_s was over 0.9 for 32 assays. The following biomarkers were significantly differentially expressed during treatment and between ICB responders and non-responders in melanoma patients: IL-6, IL-8, IL-10, MIG (CXCL9), IP-10 (CXCL10) and I-TAC (CXCL11).

Target 48 and Explore 1536 identified the same significant biomarkers and the correlation between the two platforms for these biomarkers are shown in Table 8..

Table 8. Correlation for cytokines of specific interest.

Cytokine	Spearman correlation (r_s)
IL-6	0.96
IL-8	0.98
IL-10	0.93
MIG	0.99
IP-10	0.97
I-TAC	0.94

The correlation of this data on the two Olink platforms supported the robust association of these key cytokines with response to ICB treatment responses. In conclusion, NPX levels of 44 proteins measured by the highly multiplexed Explore 1536 discovery platform correlated strongly with pg/mL concentration measured by the 48-plex platform demonstrating both scalability and reproducibility.

NPX differences identified for cytokines that significantly changed in melanoma patients during ICB treatment were consistent with fold-change estimates calculated using quantitative data in pg/mL, further verifying the findings.

Conclusion

MSD and Luminex have been considered by many to be the go-to antibody-based technologies for multiplex proteomics research. This reflects the relatively long and successful history of these technologies on the market. Science is forever moving forwards, however, and novel technologies are developed that may offer researchers new solutions that can better serve their needs.

Olink is a fast emerging technology with unparalleled specificity and scalability. In this white paper we have presented results demonstrating that Olink results show excellent parallellism when performing a dilution series and displays excellent linearity. Olink results are more consistent with MSD results, than any of MSD or Olink vs Luminex. Olink data is therefore largely consistent with well-established low- to mid-plex methods, and offers a much broader, scalable solution with unmatched specificity at high multiplexing levels, with uniquely low sample consumption.

The follow-up study showed that protein biomarkers on different Olink panels are highly correleated independent of the readout method used (qPCR or NGS), the multiplex degree, or protein concentration unit (pg/mL or NPX).
Olink Explore 1536 and Olink Target 48 data show excellent correlation and comparable results. This shows that the readout technology used (NGS or qPCR) does not influence the end results.

Olink solutions range from small custom panels (www.olink.com/custom) to our high throughput Olink Explore platform, enabling ~1500 proteins to be measured from <3 µL sample, with NGS readout (www.olinkexplore.com).

References

1. E. Assarsson *et al.*, Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. *PLOS ONE* 9, e95192 (2014).
2. https://www.mesoscale.com/en/technical_resources/our_technology/
3. https://www.bio-rad.com/en-se/applications-technologies/multiplex-detection-analytes-using-bio-plex-system and https://www.bio-rad.com/en-se/applications-technologies/multiplex-immunoassays

www.olink.com

Olink Proteomics, Dag Hammarskjölds väg 52B , SE-752 37 Uppsala, Sweden

1146, v1.1, 2021-03-11