Technical note

Compatibility of Olink® Explore with autoantibodies in autoimmune disease

Systemic Lupus Erythematosus (SLE) as a case study

Background

SLE is an autoimmune disease characterized by the near universal presence of autoantibodies against nuclear antigens (Antinuclear antibodies, ANA) (1). However, ANA can be found in a variety of other autoimmune and infectious diseases, as well as in healthy individuals. Lupus-specific autoantibodies, such as those directed against double-stranded DNA (anti-dsDNA), cardiolipin, and spliceosomal proteins (anti-Sm and anti-nRNP), may occur years before diagnosis or before associated clinical manifestations (2, 3). Anti-Sm is associated with increased disease activity and earlier mortality (4). Anti-dsDNA autoantibodies, especially at high concentrations, are associated with lupus nephritis/glomerulonephritis and their levels may increase at/after disease flare and decrease with disease improvement (5). The dysregulation of immune system in SLE, and the subsequent development of autoantibodies, affects the whole body and has clinical manifestations ranging from mild mucocutaneous lesions to severe, life-threatening, multi-organ damage.

Proof of Concept

Olink® Explore workflow includes steps where dsDNA is produced during analysis. Therefore, the aim of this study is to show that Olink® Explore platform is compatible with SLE samples and produces high quality data despite the presence of anti-dsDNA autoantibodies.

Study Design & Methods

Protein analysis was conducted on 88 serum samples comprising healthy controls (n=16) and SLE patients (n=72) from Oklahoma Medical Research Foundation (OMRF). The SLE cohort has four subgroups (n=18 each), stratified for varying levels of SLE-associated autoantibodies measured by standard clinical diagnostics The subgroups, however, were combined into two groups, based on the presence or absence of anti-dsDNA, to evaluate the effects of anti-dsDNA antibodies on assay performance (Figure 1). Anti-dsDNA antibody titers were measured using BioPlex™ 2200 ANA kits according to the provider's protocol. All samples were analyzed on Olink® Explore 384 Cardiometabolic, Inflammation, Neurology, and

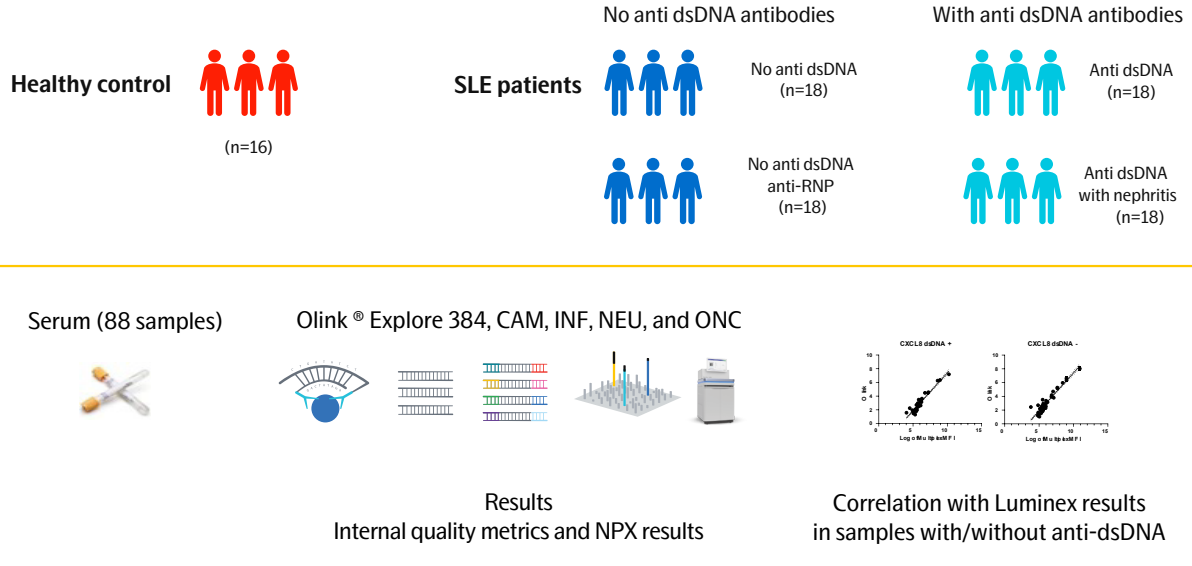

Figure 1. Schematic overview of the study design and computational analysis of the 51 biomarkers measured on both the Olink and Luminex platforms.

Oncology panels. Six control and all SLE samples were also analyzed using a custom-designed Luminex Multiplex panel with xMAP beads. Spearman correlation coefficients and Benjamini-Hochberg corrected P values from linear regression models were used to compare 51 protein biomarkers measured using both technologies.

Olink® Explore Technology

The Olink Explore platform uses Proximity Extension Assay (PEA) technology coupled with Next Generation Sequencing (NGS) to analyze proteins in biological samples. PEA relies on the dual recognition of a target protein by a pair of matched antibodies that are conjugated to complementary DNA oligonucleotides. Upon binding of the two antibodies to the target protein, the DNA oligonucleotides are brought into proximity, allowing them to hybridize and produce DNA amplicons after extension by DNA polymerase. The generated amplicons are then sequenced on Illumina NGS platforms to produce data on the abundance of up to 3,000 protein biomarkers in each sample. This dual-antibody protein recognition combined with protein-specific amplicon sequences results in highly specific protein assays. Combined with the incorporation of internal and external controls into each sample and run, high-quality data is produced. Read our white paper (6) on Olink® PEA technology to learn more.

Results

Quality Control (QC)

The Olink QC system contains internal controls designed to monitor the three main steps of the Olink protocol: Immunoreaction, Extension, and Amplification/Detection (6). The Incubation Control (for immunoreaction) and the Amplification Control (for amplification/detection) are specifically used to monitor assay performance, as well as the quality of individual samples. These controls are spiked into every sample at the same concentration, and thus, their signal in the form of barcoded NGS reads is expected to be the same across the plate. The reads are then translated into relative quantification units, referred to as Normalized Protein eXpression (NPX). Values deviating from the median NPX of all samples produce a QC warning in the software used to process the results (i.e., MyData Olink Cloud software) and potentially indicate pipetting errors or pre-analytical variation(s) in the samples affecting performance (e.g., matrix effects).

Definition

The matrix effect is the effect on an analytical assay caused by all other sample components except the specific compound (analyte) to be analyzed.

The data generated from the Incubation and Amplification Controls were first examined in each sample as a standard QC check. The results are presented in Figure 2 and show that the number of samples deviating from the median NPX was unbiased and not higher in any specific group. Thereafter, the percentage of samples that passed QC was calculated as a representation of a successful run. According to Olink criterium, the percentage of samples that passed QC should be at least 83% (5/6th) of the total number of samples in each panel. As shown in Table 1, the percentage of sample that passed QC ranges between 85-88 %, and thus, the run, on all four Olink® Explore panels, was collectively considered successful.

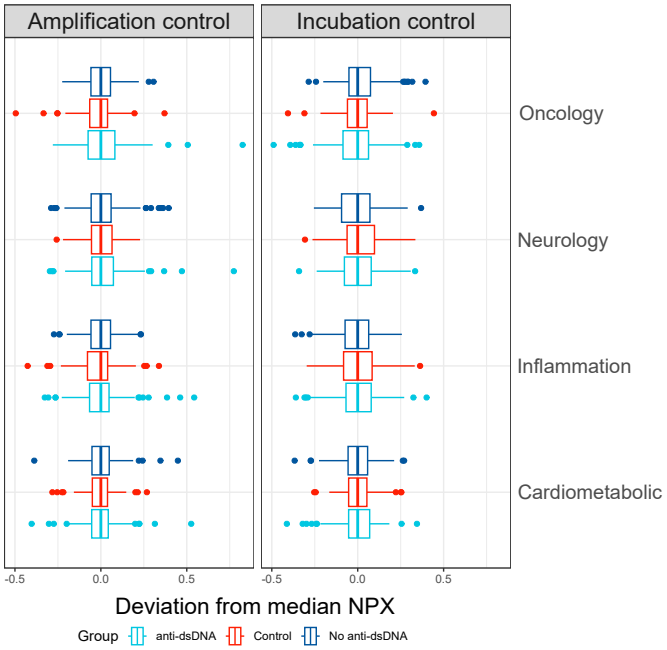

Figure 2. NPX deviation from the corresponding medians of Incubation and Amplification Controls across all samples for each Olink ® Explore 384 panel (n=88). Samples are grouped into control and patient samples (anti-dsDNA antibodies and no anti-dsDNA antibodies).

Definition

NPX is an arbitrary value that denotes Normalized Protein eXpression, and is expressed in Log2 scale. NPX is used by Olink to intuitively reflect protein abundance in the sample. The use of NPX allows users to identify changes in the level of each individual protein and compare those changes across samples within a project, enabling the establishment or identification of study-, disease-, or treatment-specific protein signatures. For more information on NPX, visit olink website (7).

Detectability

An examination of proteins detectability in each sample was performed to identify whether the presence of anti-dsDNA has affected protein assay performance. Here, detectability is presented as the fraction of protein assays above the limit of detection (LOD) in at least 50% of the samples for each Olink® Explore panel. Protein detectabilities, for all samples collectively and amongst the study groups (control and patient samples), were compared against the reference ranges calculated from previous analysis using healthy and disease plasma and serum samples. As shown in Table 1, the detectability for all samples, including samples with anti-dsDNA antibodies or autoantibodies, fall within the expected detectability range, implying that no effect on assay performance was identified.

Taken together, the QC and detectability results, clearly indicate the absence of pipetting errors, confounding effects from the samples' quality or autoantibody levels on the generated data, or any negative effects on protein assays detectability.

Absence of data patterns in SLE samples

As another validation for the high data quality generated in this run, the absence of any data-associated patterns, especially in the dataset from the anti-dsDNA antibody group, was examined. This entailed visualizing the spread of the data from all groups collectivity by plotting each sample-specific median (x-axis) against the sample-

Table 1. Percentage of samples passing quality control and percentage of protein assays above the limit of detection in at least 50% of the samples run on Olink® Explore panels.

Panel	Percentage			Detectability per Group		
Olink® Explore 384	Samples Passing QC	Overall Detectability	Expected Detectability Range	Control	No anti-dsDNA	Anti-dsDNA
Cardiometabolic	86	92	85-95	92	92	92
Inflammation	85	88	80-90	87	88	87
Neurology	86	87	80-90	86	87	86
Oncology	88	88	80-90	88	89	88

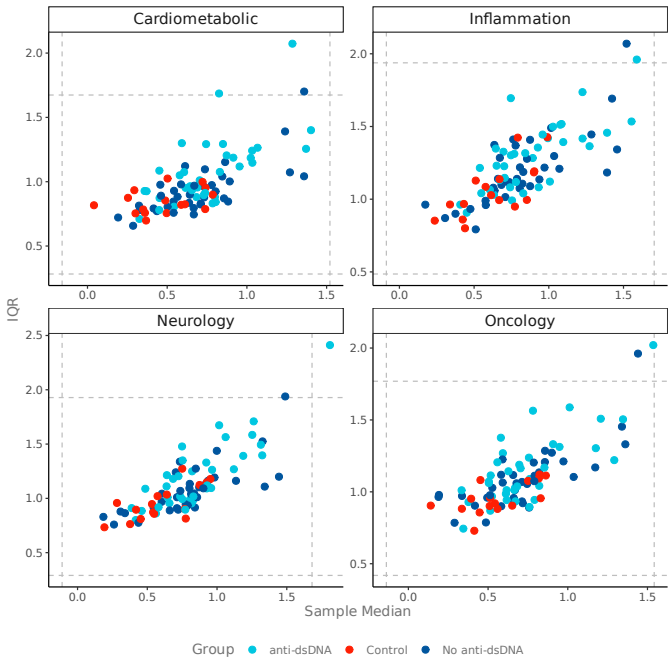

Figure 3. Interquartile range (IQR) plotted against sample median across all assays per panel for each sample. Dashed lines represent ± 0.3 standard deviations from each average. Samples outside the grid may be regarded as potential outliers.

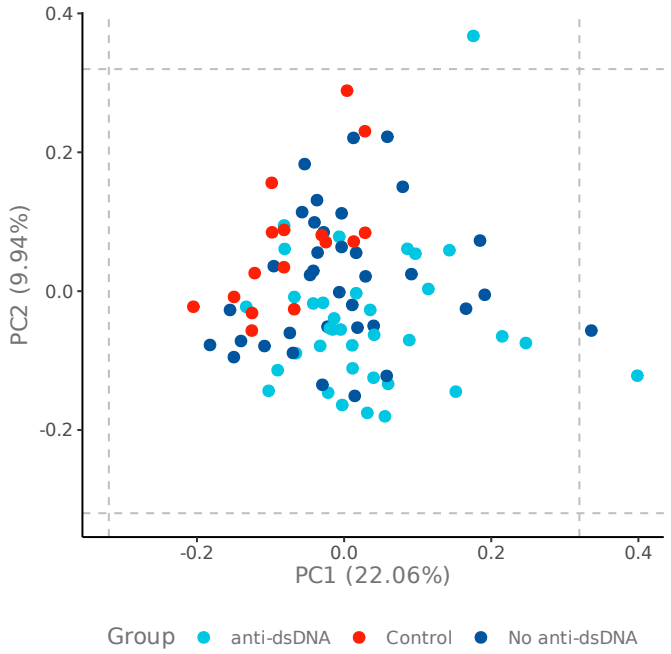

Figure 4. Projection along the first two principal components (PC) from a Principal Component Analysis (PCA) performed on all samples. Dashed lines represent ± 0.3 standard deviations from the average of each PC. Samples outside the grid may be regarded as potential outliers. Axes titles indicate the percentage of the variance the principal component explains.

specific interquartile range (y-axis) for each Olink® Explore panel (Figure 3).

No data-associated patterns could be identified within the plot, especially between the two patient groups, with and without anti-dsDNA antibodies. This result was further confirmed by a Principal Component Analysis (PCA) on the full dataset from the four Olink® Explore panels (Figure 4).

Data evaluation in relation to autoantibodies

To evaluate the global effects of anti-dsDNA antibodies on protein assays, the measured concentrations of anti-dsDNA antibodies (1-300 IU/mL) were plotted against the median NPX of all assays per sample (Figure 5). A shift in the median NPX of all assays where samples have autoantibodies or anti-dsDNA antibodies indicates potential assay interference. The results (Figure 5) show that the global median NPX per sample is stable and consistent across the different study groups (Control, SLE patients no anti-dsDNA antibodies, SLE patients with anti-dsDNA antibodies) confirming the absence of assay interference in the presence of anti-dsDNA antibodies. A further analysis combining the median NPX of all assays for all samples per panel parallels the above 'result' (Figure 6). These results collectively confirm the absence of matrix interferences in SLE samples and the compatibility of Olink® Explore technology with samples containing anti-dsDNA or autoantibodies in general.

Correlations (Olink versus Luminex)

To further confirm the absence of assay interference with sample containing autoantibodies, the results obtained with Olink® Explore were compared to Luminex measurements (bead-format antibody assays) and Spearman's correlations were calculated. First, protein measurements falling below LOD with either technology were excluded and only assays with values above LOD in more than 50% of the samples were used for the Spearman's correlation analysis (Table 2). These criteria were chosen to ensure that the results fall within the linear range of assays and that the subsequent analysis included enough samples to power the study. This resulted in 37 assays available for analysis, among which 36 produced significant correlations ($P < 0.05$), a selection of which can be seen in (Figure 7 a-j).

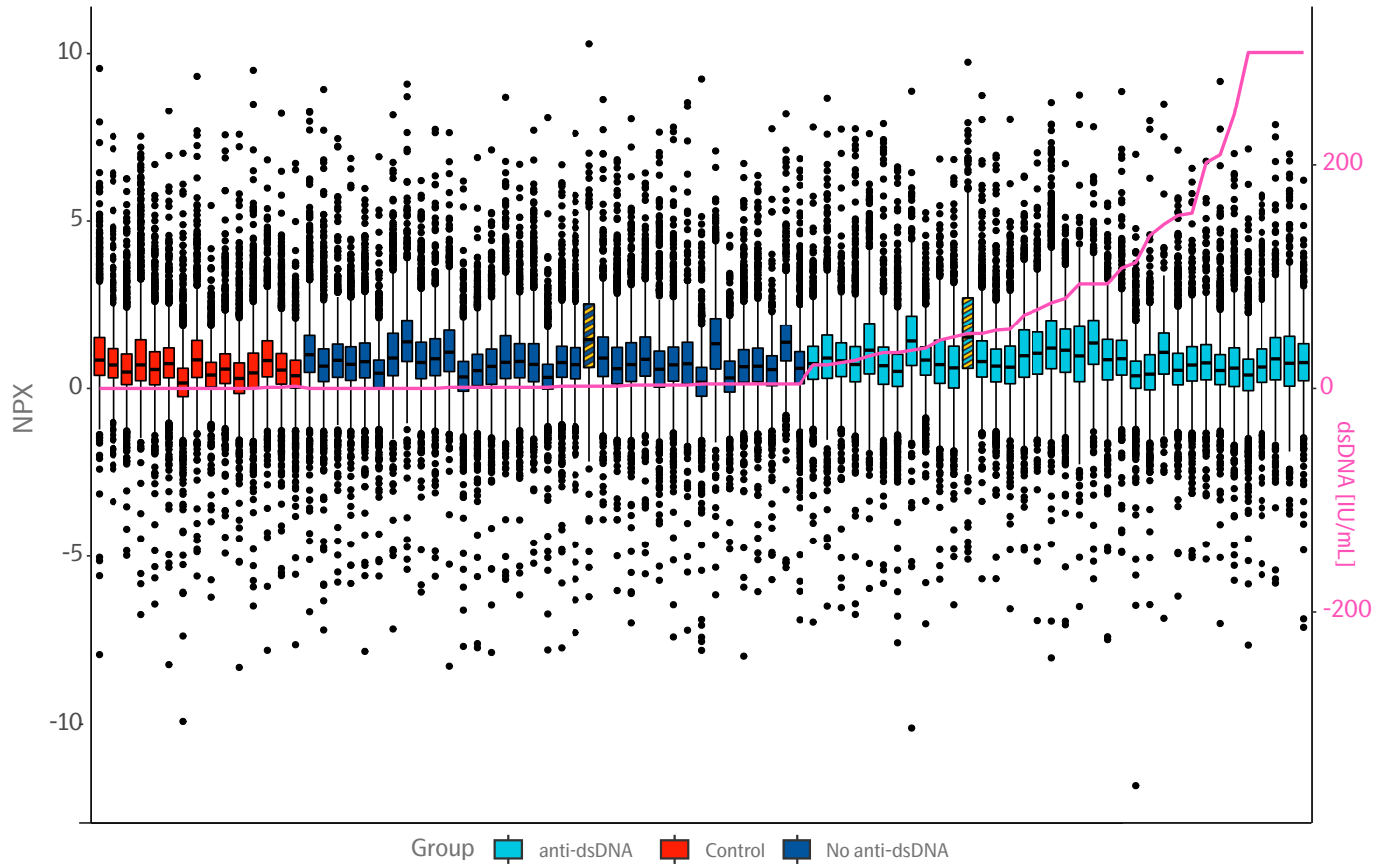

Figure 5. Boxplot showing the distribution of the median NPX of all protein assays versus the concentration of anti-dsDNA antibodies in each sample of the study groups. The box plot shows a stable distribution of the median NPX of all assays across all study groups and confirms the absence of assay interferences in general. Please note that all samples in the project including outliers are presented in this plot. Samples with outliers are marked with a patterned box.

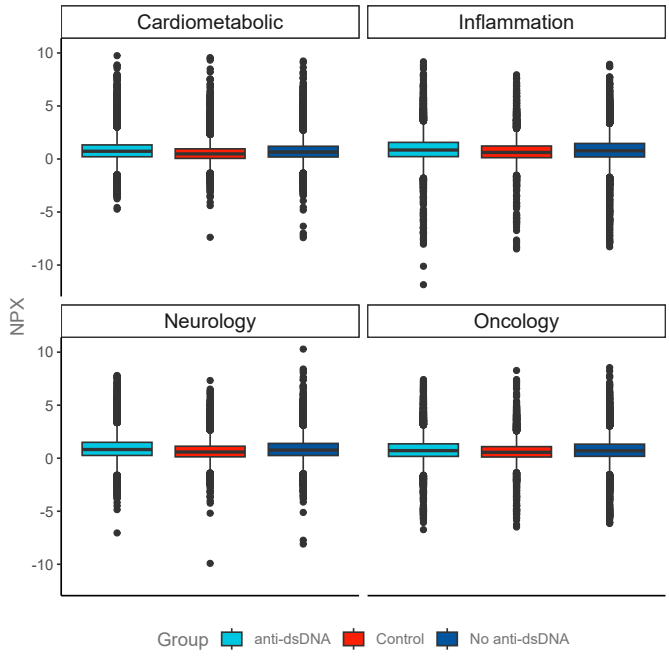

Figure 6. Median NPX for all assays and samples in the three different groups including the control group, SLE patients with no anti-dsDNA antibodies, and SLE patients with anti-dsDNA antibodies. The results are shown separately for each Olink® Explore panel.

Oncostatin M (OSM) was the only assay with weak correlation (rs = 0.09) (Figure 8), and further investigations showed that the values from Luminex assay clustered within a very narrow range indicating high dose hook effect (log2 pg/mL levels 11-12). The values obtained from Olink analysis, on the other hand, were well spread out (NPX values between -2 and 4) (Figure 8).

High Dose Hook Effect (Hooking)

A high dose hook effect is a state of antigen excess relative to the antibody probes, resulting in falsely lowered values. If overlooked, a significantly lower value can be reported and lead to misinterpretation of results.

A comparison of the differences between the correlation coefficients of the two patient groups (with and without anti-dsDNA antibodies) showed that none of the assays were statistically different between the patient groups ($P > 0.05$). This implies that correlations could be established between the two technologies regardless to the presence of anti-dsDNA in one patient group. In conclusion, the above results present additional evidence that autoantibodies, including those against dsDNA, did not impact measurements using Olink® Explore.

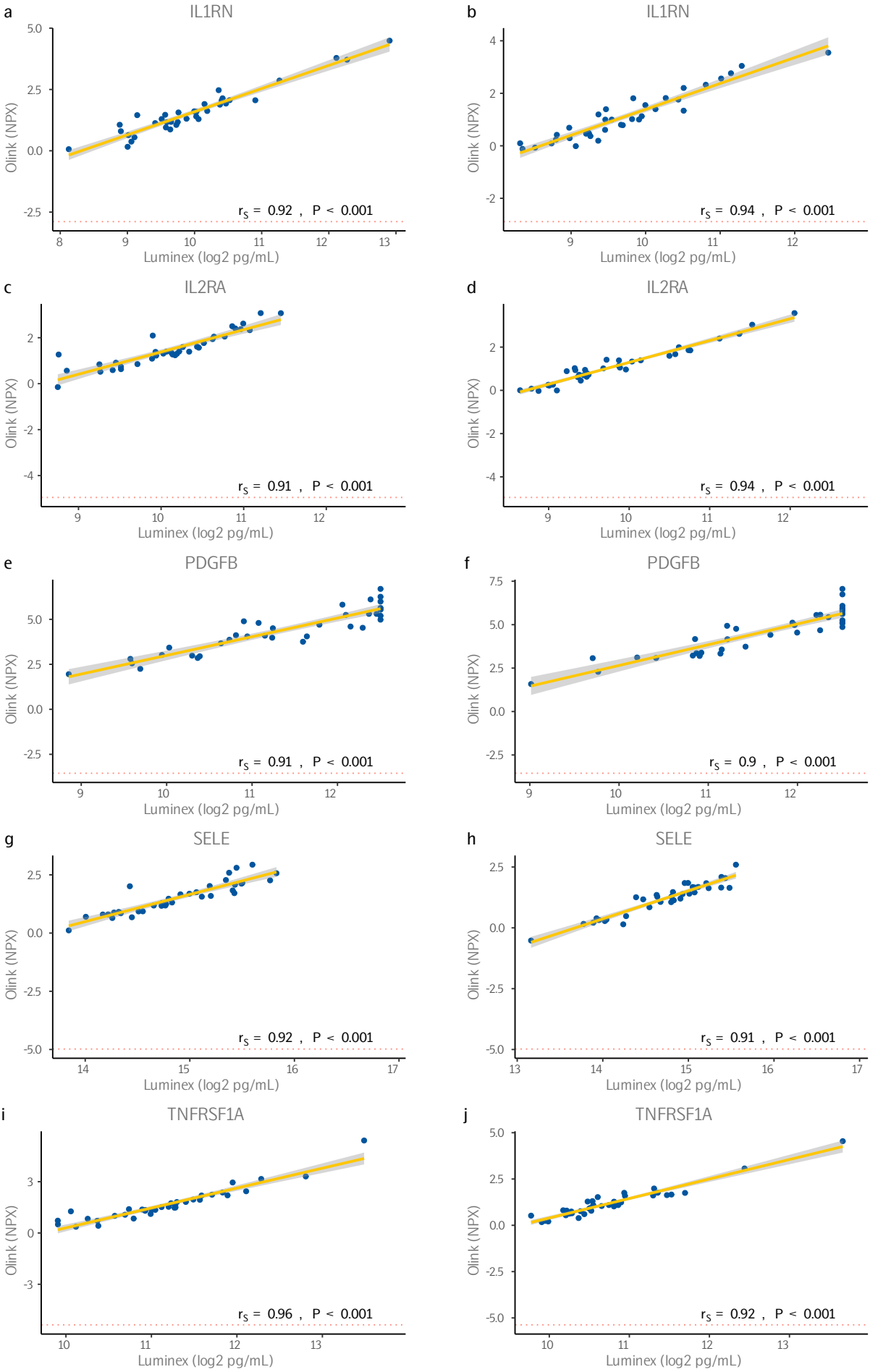

Figure 7. Correlation of assays between the Olink® Explore platform and Luminex for SLE patient group with (Left column) or without anti-dsDNA antibodies (right column). Spearman correlation coefficient was calculated for protein assays above LOD for at least 50% of the samples in both technologies. LOD for Olink protein assays is indicated by the red dotted horizontal line. The text on the bottom right of the graph shows the Spearman correlation coefficient and the Benjamini-Hochberg corrected P value from the linear regression model.

Table 2. Assays measured in both Olink Explore platform and Luminex. The percentages in the table represent cumulative detectability calculated per assay from both platforms. Thirteen assays, with cumulative detectability below 50%, were excluded from the Spearman correlation analysis.

Assay	UniProt	Percentage of Samples dsDNA antibodies		
		-ve (n=36)	+ve (n=36)	both (n=78)
IFNL1	Q8IU54	0%	0%	0%
IL2	P60568	0%	0%	0%
LIF	P15018	0%	0%	0%
IL1A	P01583	0%	3%	1%
IL4	P05112	0%	0%	1%
IL33	O95760	3%	22%	14%
IL5	P05113	31%	8%	18%
IL10	P22301	11%	31%	19%
NGF	P01138	17%	17%	19%
IL1B	P01584	22%	28%	24%
IL13	P35225	31%	22%	27%
IL17A	Q16552	33%	39%	35%
CCL7	P80098	33%	44%	36%
TNF	P01375	83%	94%	85%
IFNG	P01579	86%	97%	92%
TNFSF12	O43508	92%	100%	96%
CXCL9	Q07325	94%	89%	86%
CSF3	P09919	97%	100%	99%
CXCL13	O43927	97%	100%	99%
CCL11	P51671	100%	100%	100%
CCL2	P13500	100%	100%	100%
CCL3	P10147	100%	100%	100%
CCL4	P13236	100%	97%	99%
CD40LG	P29965	100%	100%	100%
CXCL10	P02778	100%	100%	100%
CXCL8	P10145	100%	94%	96%
FAS	P25445	100%	100%	100%
FASLG	P48023	100%	100%	100%
ICAM1	P05362	100%	100%	100%
IL12A_IL12B	P29459_P29460	100%	97%	99%
IL15	P40933	100%	100%	100%
IL16	Q14005	100%	100%	100%
IL1RN	P18510	100%	100%	100%
IL2RA	P01589	100%	100%	100%
IL6	P05231	100%	100%	100%
IL7	P13232	100%	100%	100%
KITLG	P21583	100%	100%	100%
LEP	P41159	100%	100%	100%
OSM	P13725	100%	100%	100%
PDGFB	P01127	100%	100%	100%
RETN	Q9HD89	100%	100%	100%
SDC1	P18827	100%	100%	100%
SELE	P16581	100%	100%	100%
TGFB1	P01137	100%	100%	100%
TNFRSF1A	P19438	100%	100%	100%
TNFRSF1B	P20333	100%	100%	100%
TNFSF10	P50591	100%	100%	100%
TNFSF13	O75888	100%	100%	100%
TNFSF13B	Q9Y275	100%	100%	100%
VCAM1	P19320	100%	100%	100%
VEGFA	P15692	100%	100%	100%

-ve (absent), +ve (present), both (in both groups)

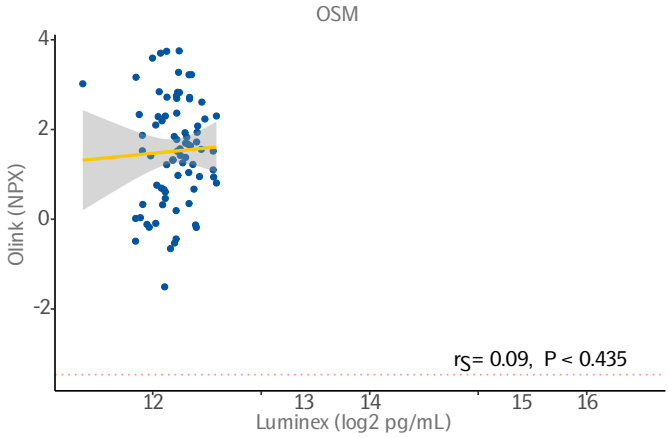

Figure 8. Correlations of Endostatin M (OSM) measurements between the Olink Explore® platform (y-axis) and Luminex (x-axis). The yellow fit line is generated from linear regression and the grey area around it represents the 95% confidence interval. LOD for Olink protein assays is indicated by the red dotted horizontal line. The text on the bottom right of the graph shows the Spearman correlation coefficient and the Benjamini-Hochberg corrected P value from the linear regression model.

Summary and Conclusions

In this study, the compatibility of samples from SLE patients with and without anti-dsDNA, was evaluated on the Olink Explore® platform to investigate any interference in the analysis. The results from internal controls, the high percentage of samples passing QC, and the excellent detectabilities seen in this study demonstrates that the presence of anti-dsDNA antibodies, did not interfere with protein assays and did not have any effects on the data and data quality. The data generated did not exhibit any deviating patterns, especially from the SLE samples, and the median NPX of all assays per sample was stable regardless of whether the samples had anti-dsDNA in high or low concentrations. A comparison of the results obtained by Olink® Explore with those obtained by Luminex showed significant correlations from all sample groups and no significant differences were seen in SLE samples with or without anti-dsDNA antibodies. The above results collectively confirm the high quality and specificity of Olink PEA technology and its resilience towards the confounding effects of anti-dsDNA antibodies. In conclusion, our results show that the Olink® Explore platform is compatible with samples from SLE patients irrespective of the presence of autoantibodies.

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