Analytical characterization of a multiplex panel measuring 45 cytokines by proximity extension assay in plasma from subjects with NSCLC

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INTRODUCTION

Cytokines are essential regulators of immune system processes and are responsible for the differentiation, proliferation and function of immune cell sub-types. Since the first approvals of immunotherapies, many more novel therapeutic strategies continue to harness components of the host immune system to drive anti-tumor activity. To characterize the pharmacodynamic and mechanistic effects of these investigational therapies, quantification of soluble cytokines has taken on increasing importance. In support of these efforts, multiplexed immunoassay panels have been developed, including proximity extension assays, expanding the available options for precise and accurate, broad-spectrum cytokine quantification from clinical biospecimens.

In this study, the analytical performance of a 45-analyte panel based on proximity extension assay technology is characterized in serum and plasma from normal donors and subjects with non-small cell lung cancer (NSCLC).[1] Initially, a sample set was screened to identify samples with analytes with different quantitative ranges and cytokines that are differentially expressed across sample groups. Selected samples were then used to characterize the assay intra- and inter-run precision.

Sample Type	Group	Number
Serum	Normal Donor	5
Plasma	Normal Donor	6
Plasma	NSCLC	27

Table 1. The study cohort included samples from normal donors. Both serum and plasma were analyzed. A larger number of study samples from subjects with NSCLC was analyzed to increase the diversity of samples investigated.

METHODS

- Serum and plasma samples from normal subjects and subjects with NSCLC (Table 1) were procured from commercial biobanks
- Samples were processed using the Olink® Target 48 Cytokine kit according to manufacturer instructions
- Briefly, 0.7 µl of serum was incubated overnight with kit affinity reagents. Following incubation, samples were prepared for extension and detection on the Olink® Signature Q100 instrument
- Each run was analyzed by a trained operator and verified to pass QC metrics

Experimental Runs

Figure 1. A layout of the experimental runs from this study is illustrated. A screening run was performed to identify samples with endogenous levels spanning analyte ranges. Selected incurred samples were then run in quadruplicate across multiple runs to characterize the assay precision across components of variability.

RESULTS

Data Completeness

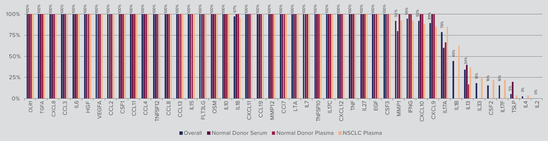

Figure 2. First-pass analysis data completeness is shown. The percent of reportable values for each sample group is shown. Values were only reported if they were between LLOQ and ULOQ and passed QC metrics. Data labels are for the overall detection rates.

- Overall data completeness for 45 analytes measured in the 33 samples was 84% (1437 out of 1710 possible data points)
- MMP1, CXCL9 and CXCL10 had 3, 4 and 3 samples respectively with values greater than ULOQ. Samples may be reanalyzed at a higher dilution to generate quantitative values for these three analytes
- 10 data points were not reportable (NaN), and the remaining data points were below LLOQ but above LOD

Differential Expression – Normal vs NSCLC

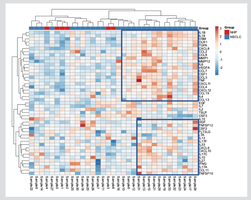

Figure 3. Unsupervised clustering of normal and NSCLC plasma samples. Analytes (rows) are clustered by expression correlation.

- Unsupervised clustering of normal donor and NSCLC samples resulted in partial clustering of normal donors into two sub-groups (Figure 3)
- A subset of NSCLC subjects clustered together (right) and shows two distinct groups of elevated cytokines. The rows are clustered according to expression correlation.
- Remaining NSCLC samples had normal levels of cytokines, similar to those measured in the normal subjects

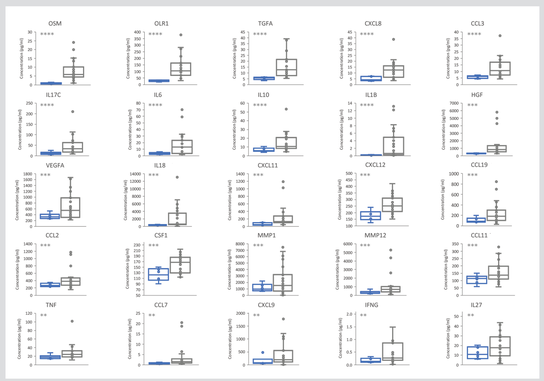

Figure 4. Box plots of all significantly regulated cytokines (p-value < 0.05) are shown.
**** p-value < 10^{-3}, *** p-value < 10^{-2}, ** p-value < 0.05.

Biological Pathways

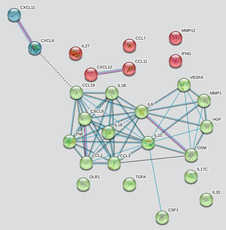

Figure 5. All significantly regulated cytokines (p-value < 0.05) were mapped into STRING database.[2]

- Differentially expressed cytokines were mapped into STRING and clustered based on co-expression and experimental/biochemical data, as well as associations annotated in curated databases
- A large grouping of cytokines are linked by co-expression to the negative prognostic IL6 and IL10[3], which are associated with poorer outcomes in subjects with NSCLC
- Other cytokines adjacent to the IL6/IL10 cluster (e.g. CXCL12) have strong prognostic characteristics and may be potential therapeutic targets[4]

Intra- and Inter-Run Precision

- 6 unique samples from the screening set were included across each of the precision runs
- Median CVs were 7.0% (Intra) and 7.7% (Inter)
- >75% of the CVs were less than 10%, and >95% were less than 15%
- The precision estimates at varying quantifiable levels are shown (Table 2) and the corresponding run 1 to run 2 concordance plot (Figure 7) is for a subset of the 45 assays

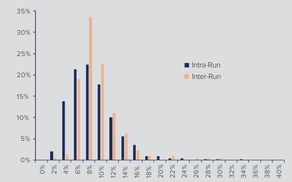

Figure 6. The distribution of CVs for all quantifiable measurements for 45 assays in 6 samples is shown. 450 intra-run CVs and 226 inter-run CVs calculated in the precision runs 2 and 3.

		IFN-γ	IL-1β	IL-6	IL-8	IL-10	TNF-α	CXCL9	CXCL10	CXCL11
Low	Mean	0.23 pg/ml	0.60 pg/ml	3.1 pg/ml	3.5 pg/ml	5.4 pg/ml	14.6 pg/ml	37.8 pg/ml	60.3 pg/ml	32.5 pg/ml
	%CV	11.8%	10.0%	4.4%	12.5%	9.7%	9.7%	5.7%	9.9%	7.8%
Mid	Mean	1.23 pg/ml	7.0 pg/ml	16.5 pg/ml	12.1 pg/ml	24.5 pg/ml	48.3 pg/ml	140 pg/ml	154.2 pg/ml	379 pg/ml
	%CV	14.5%	7.0%	5.2%	7.7%	10.4%	7.1%	5.3%	9.2%	13%
High	Mean	N/A	N/A	71.5 pg/ml	24.0 pg/ml	N/A	116.2 pg/ml	1124 pg/ml	986 pg/ml	996 pg/ml
	%CV	-	-	7.5%	7.6%	-	4.8%	13%	12%	7.8%

Table 2. Inter-run assay precision estimates are shown for a subset of analytes at varying quantitative expression levels.

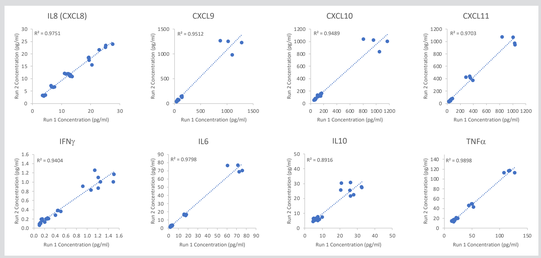

Figure 7. Inter-run concordance plots for selected assays show the correlation between runs 1 and 2.

Matrix Comparison

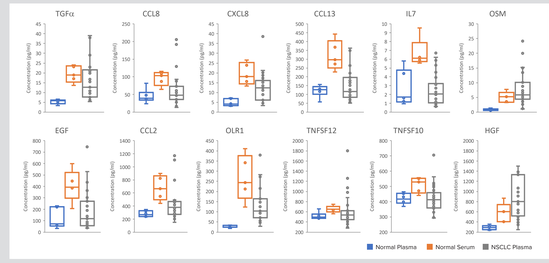

Figure 8. Box plots of all analytes with different quantified values between serum and plasma (p-value < 0.05).

- 12 / 45 cytokines in the panel had significantly different values when comparing serum and plasma from normal subjects
- When analyzing serum, additional experiments should be conducted to examine the impact of sample stability and processing times on these analytes

CONCLUSIONS

- Broad spectrum cytokine profiling is increasingly utilized to map out pharmacodynamic effects for many investigational new drugs
- The 45-cytokine panel evaluated in this study is highly sensitive, capable of detecting changes in multiple soluble cytokines in subjects with NSCLC
- The assays are precise across runs and operators, with low CVs (<15%) from < 1 pg/ml to > 1 ng/ml
- Additional pre-analytical characterization may be required when analyzing serum samples

References

1. Olink Target 48 Cytokine Validation Data v1.1; Analytical performance validation of the Target 48 Cytokine panel of 45 cytokines.
2. Szklarczyk, D.; et al.; The STRING database in 2023: protein–protein association networks and functional enrichment analyses for any sequenced genome of interest. Nucleic Acids Res. 2023 Jan. 6; 51(D1): D638-646.
3. Silva, E.M.; et al. (2017) High systemic IL-6 is associated with worse prognosis in patients with non-small cell lung cancer. PLoS ONE 12(7): e0181125.
4. Kogue, Y.; et al. Prognostic Value of CXCL12 in Non-Small Cell Lung Cancer Patients Undergoing Tumor Resection. Pharmaceuticals (Basel). 2023 Feb. 7; 16(2): 255.

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