

Multiplex Analysis of Inflammatory Markers Using Bio-Plex Pro[™] Human Th17 Cytokine Assays

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Introduction

Th17 cells are an important subclass of T-helper lymphocytes that produce IL-17 and other cytokines. Since 2005, the Th17 immune response pathway has been a rapidly expanding field of research, with 83% of literature citations appearing in just the past three years.

Studies in humans and mouse models indicate that Th17 cytokines promote host defense against extracellular microbes such as bacteria and fungi. Recent reports suggest a further role for Th17 cytokines in the protection against protozoa and viruses (O'Quinn et al. 2008 and Hartigan-O'Connor et al. 2011).

The Th17 pathway has also been implicated in the pathogenesis of chronic inflammatory conditions including autoimmune diseases (rheumatoid arthritis, lupus, psoriasis, multiple sclerosis, Crohn's disease, and type 1 diabetes), allergy, the acceptance or rejection of transplanted organs, and the development and progression of cancer (Wilke et al. 2011).

Using Luminex xMAP technology, we have developed a panel of magnetic bead-based assays consisting of sixteen (15+1) markers that are directly relevant to the Th17 immune response pathway. The assays are configured to provide the option of testing as a premixed 15-plex panel or as custom blends of selected assays based on specific end-user needs. Magnetic beads are employed to enable automation of wash steps and to ensure compatibility with all Luminex-based life science research instruments.

The validation studies summarized in this tech note cover all 16 markers in singleplex and multiplex formats. The assays were evaluated in serum, plasma, and cell culture matrices. Testing of patient samples was carried out in collaboration with Pharmasan Labs, Inc.

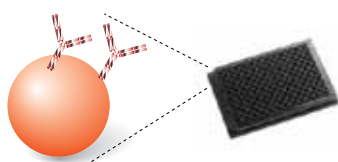
Table 1. Bio-Plex Pro human Th17 cytokine panel.

Assays	Premixed 15-Plex	Singleplex	Bead Regions
IL-1 β	•		39
IL-4	•		52
IL-6	•		19
IL-10	•		56
IL-17A	•		76
IL-17F	•		44
IL-21	•		47
IL-22	•		18
IL-23	•		43
IL-25	•		55
IL-31	•		62
IL-33	•		46
IFN- γ	•		21
sCD40L	•		29
TNF- α	•		36
IL-17A/F		•	66

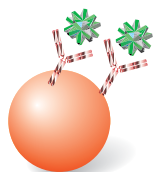
Method

The Th17 assays were designed on magnetic beads according to a capture sandwich immunoassay format. The capture antibody-coupled beads were first incubated with antigen standards, samples, or controls (60 min) followed by incubation with biotinylated detection antibodies (30 min). After washing away the unbound biotinylated antibodies, the beads were incubated with a reporter streptavidin-phycoerythrin conjugate (SA-PE) for 10 min. Following the removal of excess SA-PE, the beads were passed through the Bio-Plex suspension array reader, which measures the fluorescence of the beads and of the bound SA-PE (Figure 1). All washes were performed using a Bio-Plex Pro wash station. Data acquisition was performed using Bio-Plex Manager[™] 6.0 at a low PMT setting. The t-test function in Bio-Plex Data Pro[™] software was used to perform statistical analysis on sample data.

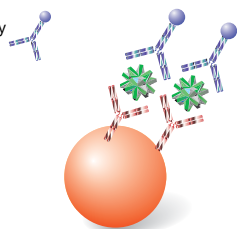
Step One:
Dispense capture beads
Wash plate 2 times



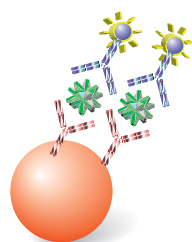
Step Two:
Add samples
Incubate
Wash plate 3 times



Step Three:
Add biotinylated detection antibody
Incubate
Wash plate 3 times



Step Four:
Add streptavidin-PE reporter dye
Incubate
Wash plate 3 times



Step Five:
Resuspend beads
Perform fluorescent sorting
Analyze data

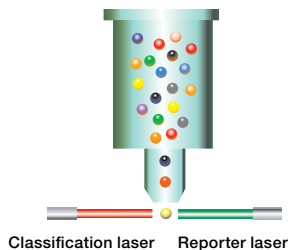


Fig. 1. Schematic representation of a sandwich-based Bio-Plex assay workflow.

Performance Characteristics

The validation demonstrated excellent performance in terms of sensitivity, precision, accuracy, and robustness in sample measurement (Table 2).

Table 2. Assay performance characteristics.

Parameters	Technical Performance
Assay sensitivity	≤ 3 pg/ml (analyte dependent)
Assay precision	Intra-assay CV ≤7% Inter-assay CV ≤11%
Assay accuracy	Standard point recovery within 80–120%
Assay specificity	Cross-reactivity ≤10%
Assay parallelism	% Difference of curve slopes ≤19%
Linearity of dilutions	R-squared (R ²) ≥ 0.97

Assay working range, also known as reportable range, is defined as an interval between the upper limit of quantification (ULOQ) and the lower limit of quantification (LLOQ) in which both intra-assay precision and standard curve accuracy are demonstrated. The Th17 assays are designed to meet an intra-assay precision of ≤10% and a standard curve recovery of 80–120%. With the exception of IL-4 and IL-22, all targets recorded 4 logs in dynamic range (Table 3). This hallmark feature differentiates the Bio-Plex assays from the 2 to 3 logs dynamic range typically recorded on a conventional ELISA standard curve.

To evaluate the precision profile of the assays, intra-assay precision (within-run %CV) was calculated from three replicates at each standard dilution point obtained from a representative assay. Inter-assay precision (between-run %CV) was derived from three independent assays of a series of spiked controls prepared in a serum matrix (Table 4).

Assay accuracy (recovery) was calculated as the percentage of the observed concentration value of a target antigen relative to the expected value. This parameter was investigated in both serum and RPMI cell culture medium by evaluating the recovery of the standard curve plus a series of spike controls prepared in serum matrix. The recovery data showed that in the majority of the assays at least seven of the eight standard points are robust within the specified 80–120% recovery range. This was supported by similar findings on the seven levels of spike controls (Table 5).

Table 3. Assay working range.

Analyte	IL-1β	IL-4	IL-6	IL-10	IL-17A	IL-17F	IL-21	IL-22
ULOQ	3,994.0	1,361.2	27,078.0	32,674.0	19,682.0	18,667.7	147,023.0	11,916.6
LLOQ	0.2	1.3	1.7	2.0	1.2	3.0	9.0	3.9
Log dynamic range	4	3	4	4	4	4	4	3
Analyte	IL-23	IL-25	IL-31	IL-33	IFN-γ	sCD40L	TNF-α	IL-17A/F
ULOQ	120,389.0	16,375.0	63,397.0	68,483.0	20,779.0	39,457.0	9,270.0	6,631.0
LLOQ	7.4	1.0	3.9	4.2	2.5	2.4	0.6	1.6
Log dynamic range	4	4	4	4	4	4	4	4

Table 4. Intra- and inter-assay precision.

Assays	Intra-Assay Precision, %CV			Inter-Assay Precision, %CV	
	Serum Matrix		RPMI Matrix	Serum Matrix-Multiplex	
	Multiplex	Singleplex	Multiplex	Standard points	Spike controls
IL-1 β	3.5	3.7	2.8	2.4	3.8
IL-4	2.4	3.9	3.3	2.5	8.6
IL-6	2.9	4.4	5.4	5.4	7.9
IL-10	2.8	3.8	4.2	2.4	6.2
IL-17A	2.2	5.6	4.1	2.6	6.2
IL-17F	4.3	6.0	4.1	5.6	8.2
IL-21	3.3	5.3	4.0	1.9	6.7
IL-22	2.2	2.9	2.6	2.2	5.7
IL-23	3.8	3.9	6.1	4.1	9.3
IL-25	2.9	3.3	3.4	1.8	5.0
IL-31	3.9	3.6	6.6	3.0	5.5
IL-33	2.7	6.2	2.7	1.4	5.3
IFN- γ	3.3	6.9	4.2	3.9	10.1
sCD40L	2.8	4.1	2.1	2.2	8.7
TNF- α	4.1	4.6	2.4	1.6	5.1
IL-17A/F	4.3	4.3	4.7	4.2	5.8

Note: The intra-assay %CV values represent a mean of eight standard points. The inter-assay %CV values for spike controls represent a mean of seven control data points.

Assay specificity was examined by subjecting specific test reagents to single-detection cross-reactivity studies. This was conducted by testing the individual detection antibody in the presence of multiplexed antigens and capture beads. Table 6 shows that the degree of cross-reactivity reported among the 15 targets was negligible and that these assays can be easily multiplexed.

Table 6. Single-detection antibody cross-reactivity study.

Detection Antibody	% Cross-Reactivity														
	IL-1 β	IL-4	IL-6	IL-10	IL-17A	IL-17F	IL-21	IL-22	IL-23	IL-25	IL-31	IL-33	IFN- γ	sCD40L	TNF- α
IL-1 β	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IL-4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IL-6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.8	0.0	0.0	0.0	0.0
IL-10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IL-17A	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IL-17F	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IL-21	0.0	0.0	0.0	0.0	0.9	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IL-22	0.0	0.0	0.5	0.0	0.0	0.1	0.0	0.0	0.0	0.8	0.2	0.0	0.0	0.0	0.7
IL-23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
IL-25	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
IL-31	0.0	0.0	0.0	0.0	2.5	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
IL-33	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.0
IFN- γ	0.0	0.0	0.1	0.1	0.0	0.2	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.2
sCD40L	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TNF- α	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 5. Standard curve recovery and recovery of spike controls.

Assays	Standard Curve Recovery, %			Spike Control, %		
	Plate 1	Plate 2	Plate 3	Plate 1	Plate 2	Plate 3
IL-1 β	100	100	100	97	95	93
IL-4	101	101	100	101	92	85
IL-6	100	101	100	100	93	94
IL-10	100	100	100	98	93	88
IL-17A	100	100	100	97	93	87
IL-17F	96	98	99	98	88	88
IL-21	99	100	100	92	97	92
IL-22	96	109	109	97	95	91
IL-23	101	100	100	100	92	84
IL-25	100	100	100	96	94	91
IL-31	100	100	100	89	94	87
IL-33	100	100	100	98	95	92
IFN- γ	99	101	102	89	90	78
sCD40L	100	101	100	99	91	85
TNF- α	100	100	100	98	95	91
IL-17A/F	—	98	98	—	96	—

Note: Recovery values represent the mean of eight standard points for the standard curve and the mean of seven points for a series of spike controls.

Dilution linearity was investigated in these assays to ensure that analytes present in concentrations above the ULOQ can be diluted and measured accurately within the assay working range. Linearity of dilution was examined by diluting spiked samples with human serum or plasma in a 1:3 serial dilution. Thus, serum or plasma content remains constant throughout the dilution series. The observed and expected sample concentrations within assay working range were plotted for each analyte. The correlation coefficient (R^2) value generated by linear regression analysis reflects the linearity of dilution for that assay. In both serum and plasma matrices, the R^2 values were 0.971 or higher for all the assays. Similar results were obtained with the singleplex assays (Table 7).

Table 7. Linearity of dilution in serum and plasma matrices.

Analyte	Linearity, R^2			
	Serum		Plasma	
	Multiplex	Singleplex	Multiplex	Singleplex
IL-1 β	1.000	0.999	1.000	0.997
IL-4	0.999	1.000	1.000	0.998
IL-6	0.993	0.999	0.992	0.999
IL-10	1.000	1.000	0.999	0.999
IL-17A	1.000	0.999	0.999	0.998
IL-17F	0.999	0.997	0.996	0.993
IL-21	0.999	1.000	0.994	0.990
IL-22	0.986	0.974	0.987	0.994
IL-23	0.999	1.000	1.000	1.000
IL-25	1.000	0.998	1.000	1.000
IL-31	1.000	1.000	1.000	1.000
IL-33	0.999	0.998	1.000	1.000
IFN- γ	0.971	0.994	0.973	0.992
sCD40L	1.000	0.998	1.000	0.996
TNF- α	1.000	0.999	0.999	1.000
IL-17A/F	—	1.000	—	1.000

Parallelism analysis was performed in both serum and plasma matrices to ensure that the reference standards and the samples are biologically comparable to each other. This was investigated by comparing the slope values of a spike concentration response curve prepared with either serum or plasma with that of a standard curve prepared in standard diluent. In both sample matrices, all 16 assays demonstrated a parallel dose response between the standard curve and the spiked concentration response curve. Overall, the percentage difference in slope value was <20% for all the assays (Table 8).

Table 8. Parallelism analysis in serum and plasma matrices.

Analyte	Standard diluent	Slope		Slope	
		Spiked serum	% Difference in slope	Spiked plasma	% Difference in slope
IL-1 β	0.91	0.95	4.7	0.88	3.8
IL-4	1.28	1.57	18.8	1.29	1.0
IL-6	0.95	1.10	13.6	0.88	7.7
IL-10	1.09	1.16	6.4	1.12	2.6
IL-17A	0.96	0.91	4.8	0.86	10.7
IL-17F	1.37	1.53	10.5	1.29	5.7
IL-21	1.03	0.96	7.6	0.98	6.1
IL-22	1.03	1.12	8.5	1.00	2.6
IL-23	1.08	1.13	4.7	1.03	4.4
IL-25	0.97	1.02	4.6	0.91	6.1
IL-31	1.00	1.01	0.8	0.92	9.0
IL-33	0.95	1.05	9.1	1.00	5.3
IFN- γ	1.17	1.21	3.4	1.08	8.5
sCD40L	1.01	1.07	5.9	0.93	9.1
TNF- α	1.01	1.01	0.2	0.97	4.4
IL-17A/F	1.40	1.60	12.9	1.45	3.5

Assay sensitivity, defined as limit of detection (LOD), was evaluated by adding two standard deviations to the mean median fluorescence intensity (MFI) of ten zero standard replicates. In the 16 assays investigated, 13 targets (IL-1 β , IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-22, IL-25, IL-31, IL-33, IFN- γ , sCD40L, and TNF- α) recorded ≤ 1 pg/ml, two targets (IL-23 and IL-17A/F) were at ≤ 2 pg/ml, and one (IL-21) was at ≤ 3 pg/ml (Table 9). Overall, the results in the RPMI matrix were comparable to values derived from the serum matrix.

Table 9. Limit of detection (pg/ml) in serum and RPMI matrices.

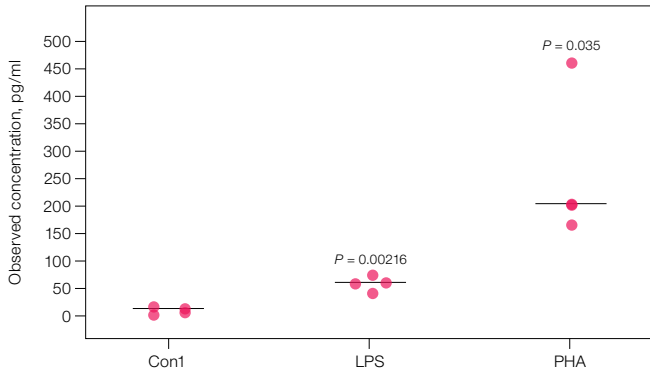
Analyte	Serum matrix	RPMI
IL-1 β	0.02	0.08
IL-4	0.52	0.75
IL-6	0.67	0.20
IL-10	0.30	0.02
IL-17A	0.49	0.70
IL-17F	0.80	0.50
IL-21	2.13	2.35
IL-22	0.30	0.51
IL-23	1.55	1.14
IL-25	0.07	0.21
IL-31	0.49	1.88
IL-33	0.58	0.27
IFN- γ	0.43	0.46
sCD40L	0.41	0.08
TNF- α	0.07	0.11
IL-17A/F	1.28	0.89

Validation with Patient Samples

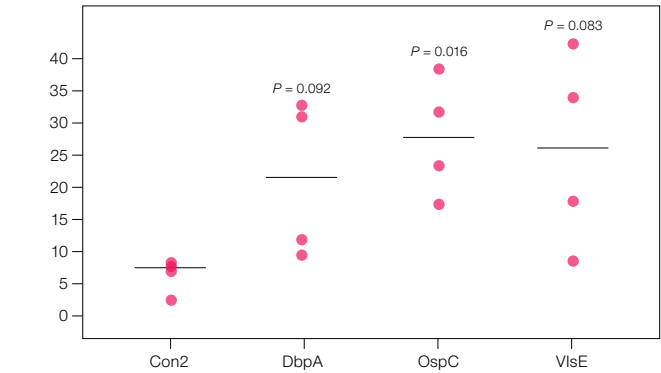
The human Th17 cytokine panel was validated in two separate studies of clinically relevant samples. The first study, performed in collaboration with Pharmasan Labs, Inc., tested the ability to detect native analytes released from peripheral blood mononuclear cells (PBMC). Figure 2 shows the observed concentrations of IL-17A, IL-17F, and IL-23 in culture supernatants after stimulation with various mitogens and

antigens. Similar expression patterns were also observed for IL-6, IL-10, IL-31, IL-33, sCD40L, and TNF- α (data not shown). The second study involved collecting measurements in serum and plasma from different disease states. Figure 3 depicts the levels of IL-6, IL-10, IL-33, and sCD40L in sera from patients diagnosed as normal or as having one of several autoimmune conditions.

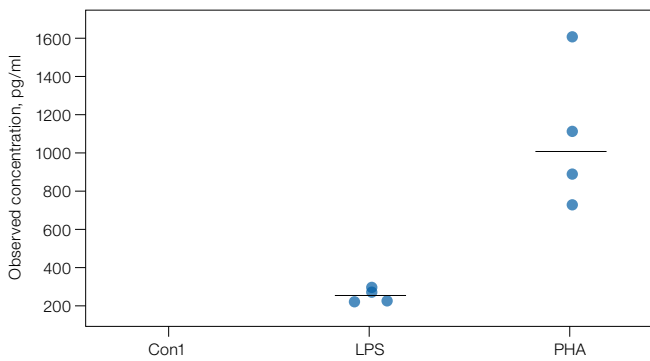
IL-17A



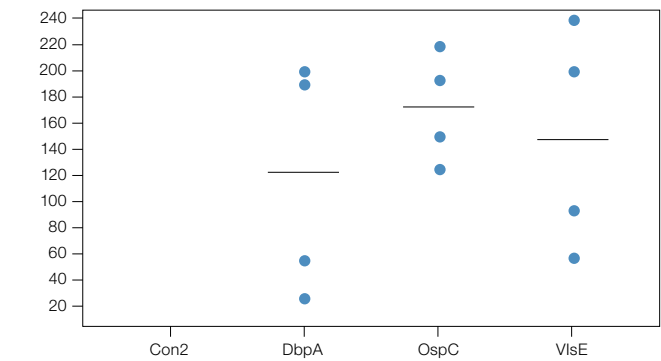
IL-17A



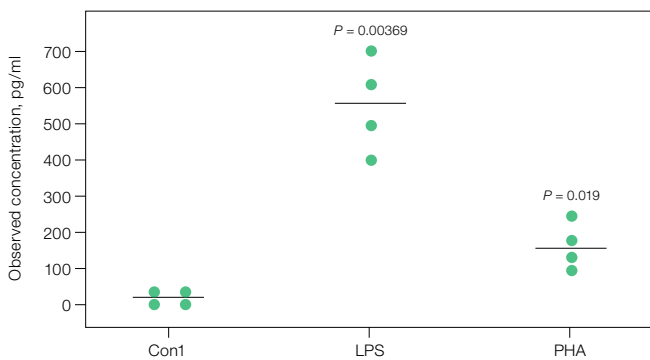
IL-17F



IL-17F



IL-23



IL-23

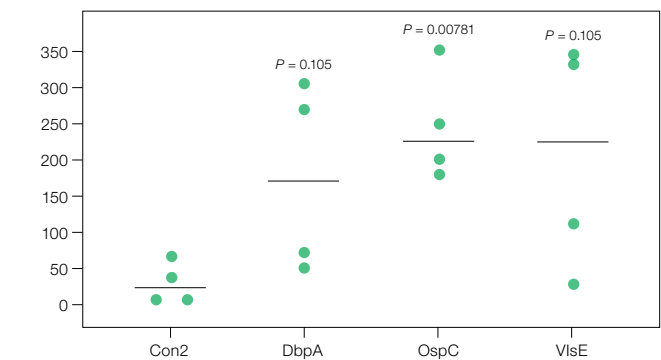


Fig. 2. Cytokine release from stimulated PBMC. Cells (1×10^6 /ml) were cultured in the presence or absence of mitogens and antigens at the following final concentrations: phytohemagglutinin (PHA 5 μ g/ml), lipopolysaccharide (LPS 5 μ g/ml), decorin-binding protein A (DbpA 6 μ g/ml), outer surface protein C (OspC 12.5 μ g/ml), and variable major protein-like sequence (VlsE-1 12.5 μ g/ml) purified from *Borrelia burgdorferi*. Con1 and Con2 refer to the matching, non-stimulated controls used in each experiment. Bio-Plex Data Pro software was used to calculate statistical differences by t-test. Plots without a p-value indicated that the control levels were undetectable. Sample testing and raw data provided by Pharmasan Labs, Inc.

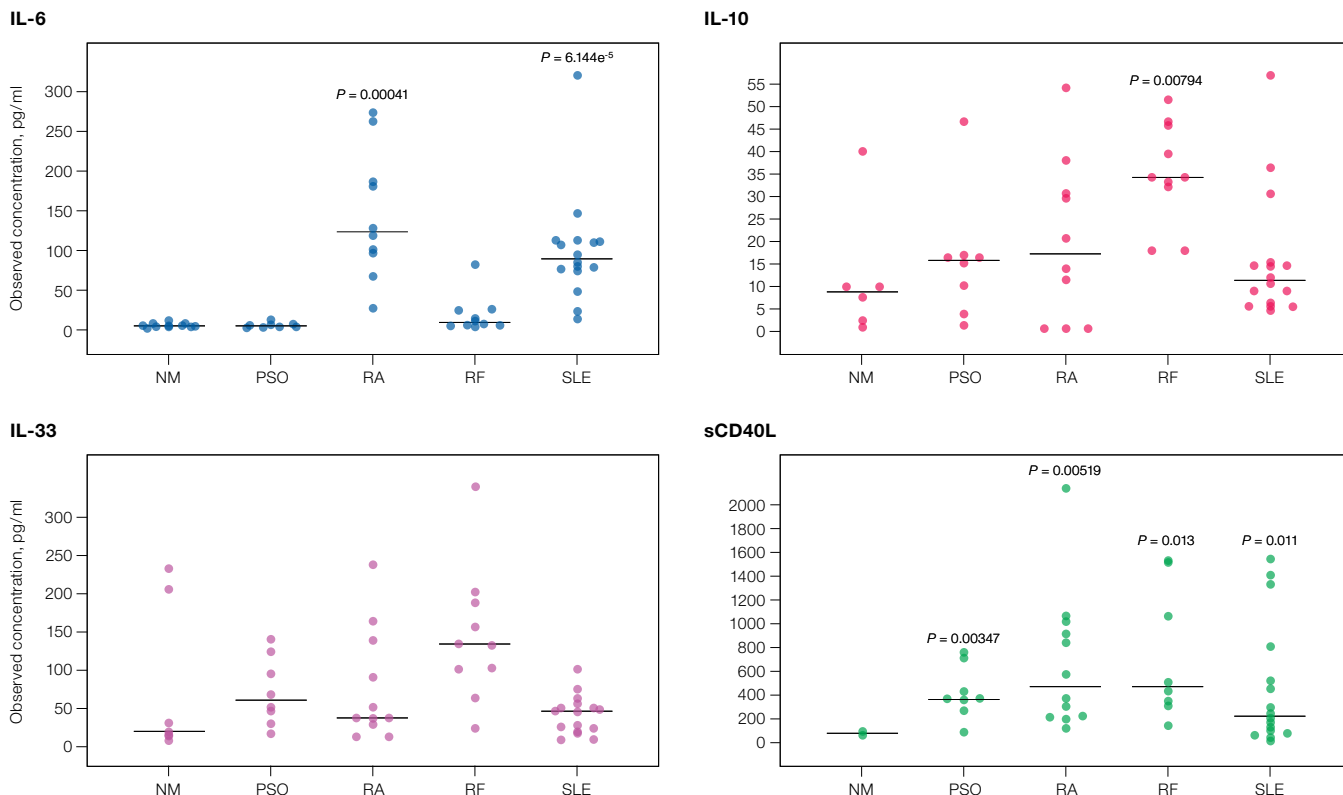


Fig. 3. Serum cytokine levels from individuals with psoriasis (PSO, N = 8), rheumatoid arthritis (RA, N = 14), rheumatoid factors (RF, N = 10), and systemic lupus erythematosus (SLE, N = 18). Non-matching normal (NM, N = 11) serum samples were used as the baseline reference. Bio-Plex Data Pro software was used to calculate statistical differences by t-test. Only p-values with statistical significance are shown.

Conclusions

The newly configured Bio-Plex Pro human Th17 cytokine assays were designed on magnetic beads to provide rapid, robust, and economical collection of multiplex data. All 16 assays validated in this study showed excellent precision, accuracy, specificity, and sensitivity. By measuring multiple analytes simultaneously, the Th17 panel helps to reduce assay time, cost, and sample volume compared to more traditional methods such as ELISA. This high throughput multiplex approach is rapidly becoming a standard in basic research and clinical laboratories.

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