Measuring Immune Response and Cytokine Autoantibodies in Human Sepsis Samples

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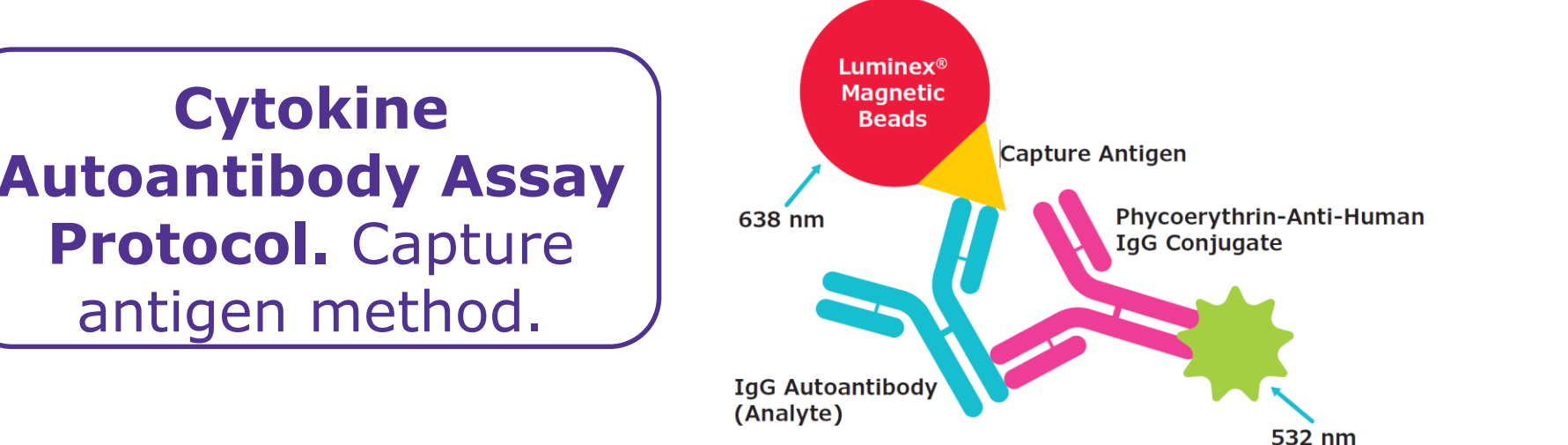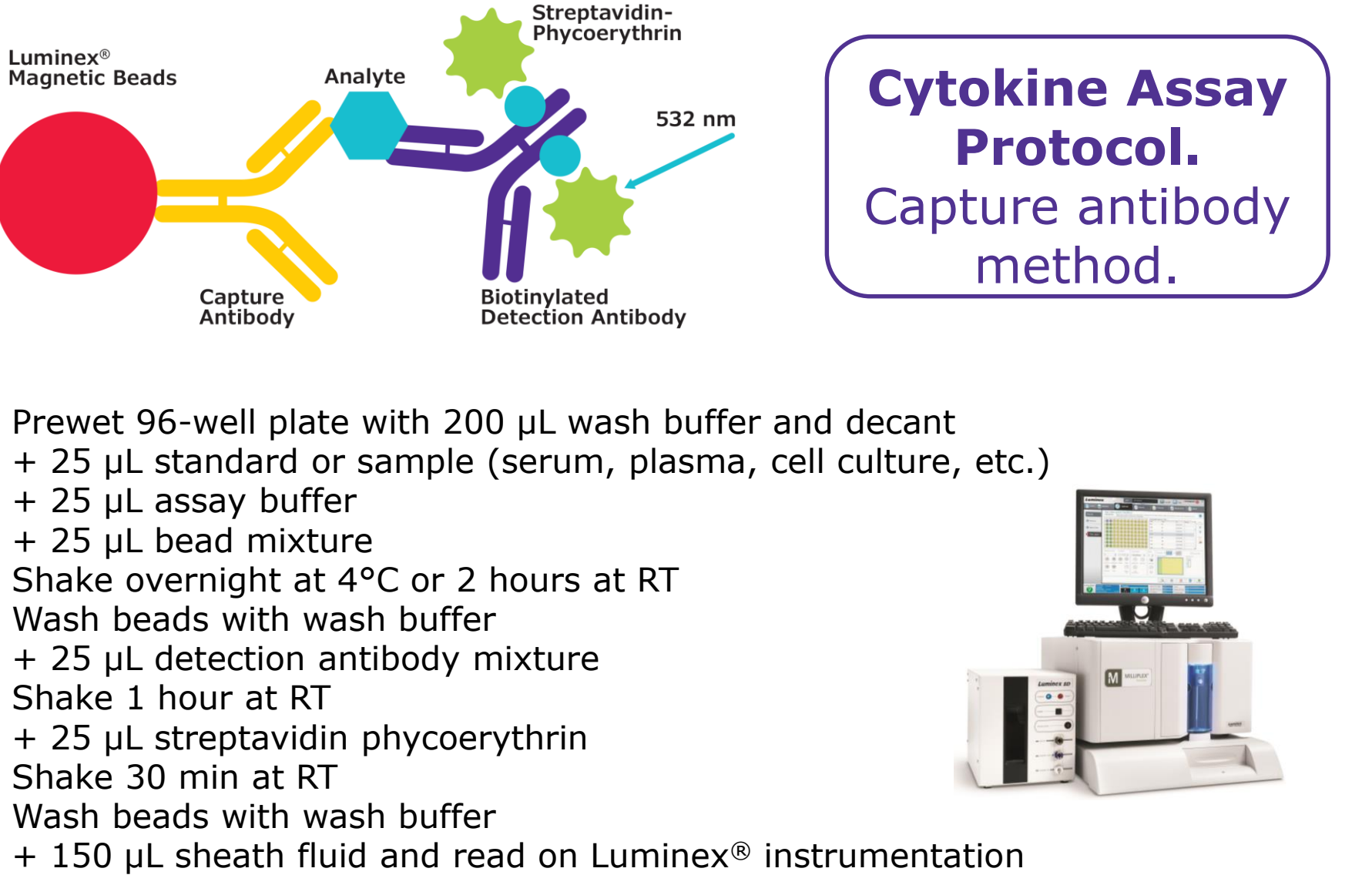**MILLIPORE SIGMA**

Introduction

Cytokine, chemokine and growth factor research plays a significant role in achieving a deeper understanding of the immune system as well as many disease states such as inflammatory disease, allergic reactions, IBD, sepsis, and cancer. The ability to test for multiple factors simultaneously in a single sample is a valuable tool to researchers. MILLIPLEX® Human Cytokine/Chemokine/ Growth Factor Panel A (Cat. No. HCYTA-60K) combines tests for 48 individual immune factors that previously have not been together in a single panel.

Anti-cytokine antibodies occur frequently and are present in healthy individuals and patients with acquired immunodeficiency and autoimmune diseases. Cytokines offering protection against microbes can be targeted by cytokine autoantibodies, leading to life-threatening infections. Measuring cytokine autoantibodies may be useful for disease monitoring and efficacy of treatment. We are developing a MILLIPLEX® Human Cytokine Autoantibody Panel (Cat. No. HCYTAAB-17K) consisting of 15 autoantibody immunoassays for use in serum and plasma samples. We measured expression of autoantibodies in serum and plasma from patients with SLE, RA, and sepsis along with healthy controls.
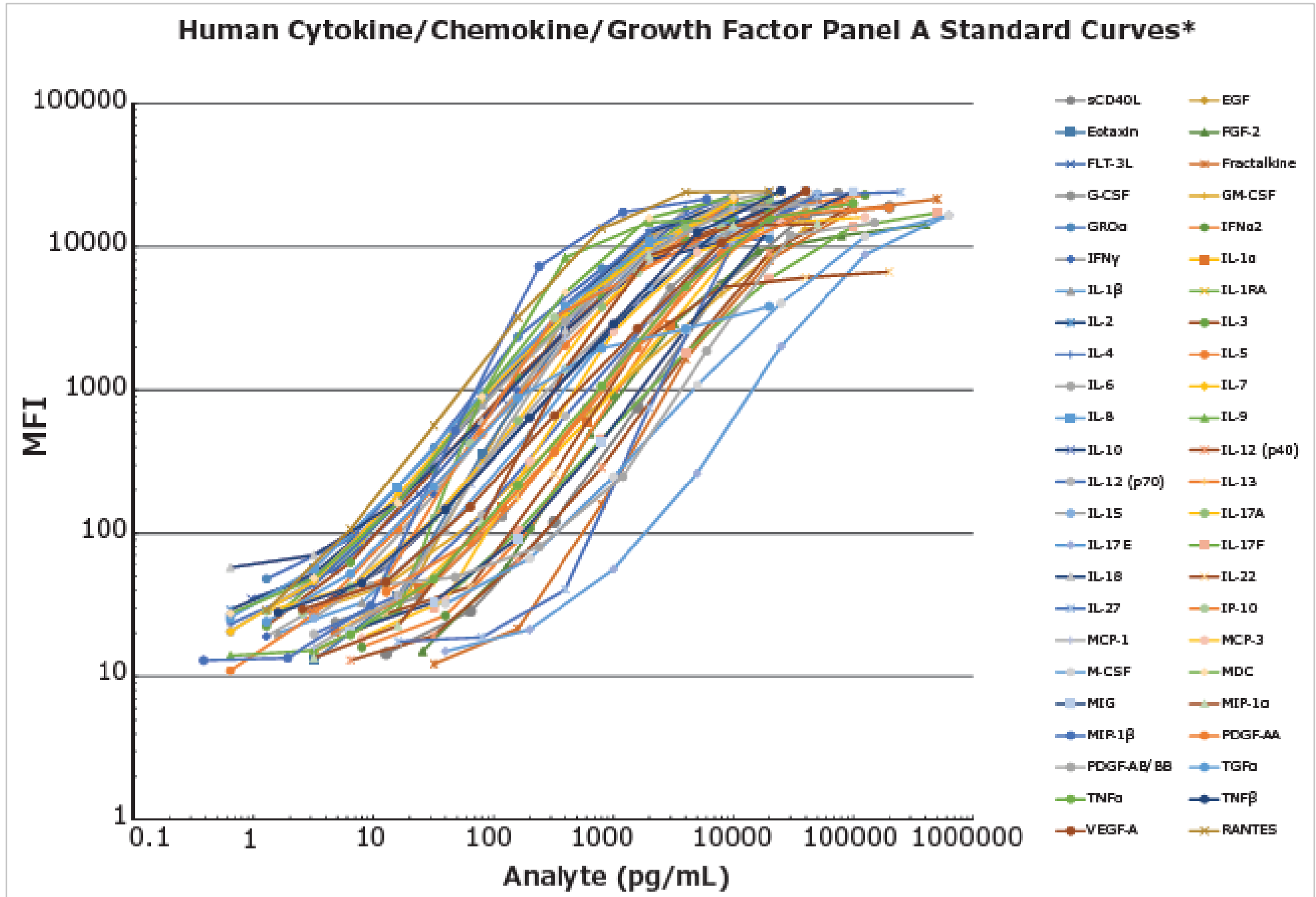
The use of these two panels allows researchers to gain information they may not have otherwise been able to access elucidating the relationship between cytokine levels and the corresponding autoantibody levels.

Methods

Microspheres. We used magnetic microsphere beads from Luminex® Corp. Each set of beads is distinguished by different ratios of two internal dyes yielding a unique fluorescent signature to each bead set. Capture antibodies or antigens were coupled to the magnetic beads.


Cytokine Assay Protocol. Capture antibody method.

Prewet 96-well plate with 200 μL wash buffer and decant
+ 25 μL standard or sample (serum, plasma, cell culture, etc.)
+ 25 μL assay buffer
+ 25 μL bead mixture
Shake overnight at 4°C or 2 hours at RT
Wash beads with wash buffer
+ 25 μL detection antibody mixture
Shake 1 hour at RT
+ 25 μL streptavidin phycoerythrin
Shake 30 min at RT
Wash beads with wash buffer
+ 150 μL sheath fluid and read on Luminex® instrumentation

Cytokine Autoantibody Assay Protocol. Capture antigen method.

Prewet 96-well plate with 200 μL wash buffer and decant
+ 25 μL assay buffer
+ 25 μL sample (serum or plasma); 25 μL assay buffer to background wells
+ 25 μL bead mixture
Shake overnight at 4°C
Wash beads x3 with wash buffer
+ 50 μL PE-IgG Conjugate
Shake 1.5 hour at RT
Wash beads x3 with wash buffer
+ 150 μL sheath fluid and read on Luminex® instrumentation

48-Plex Characteristics

Human Cytokine/Chemokine/Growth Factor Panel A Standard Curves*

*Standard curves: Performed in MXHSM-A serum matrix except *RANTES, which was in L-AB assay buffer. The kit was run using the HCYTA-60K overnight assay protocol.
Sensitivity: Standard curves (n=9 separate tests) were run in serum matrix, except RANTES was tested in assay buffer.
Precision: Two different standard controls were tested in serum matrix for intra-assay (n=8) and inter-assay (n=9) studies.
Accuracy: Three different standard controls were spiked into serum matrix (n=6).

Analyte	Standard Curve Range (pg/mL)	Sensitivity (pg/mL)	Precision (%CV) Intra-assay	Precision (%CV) Inter-assay	Accuracy (% Recovery) In Matrix
sCD40L	12.8-200,000	6.40	4.20	18.39	95
EGF	3.2-50,000	3.19	1.97	4.57	101
Eotaxin	3.2-50,000	3.07	3.93	5.27	96
FGF-2	25.6-400,000	22.40	4.73	4.33	106
Flt3 Ligand	0.96-15,000	0.85	3.11	4.66	95
Fractalkine	32-500,000	29.61	3.17	5.36	98
G-CSF	4.8-75,000	3.53	9.29	7.71	97
GM-CSF	2.56-40,000	1.60	5.82	4.78	100
GROα	1.28-20,000	1.06	3.06	3.93	99
IFNα2	8-125,000	6.43	4.35	4.39	98
IFNγ	1.28-20,000	0.86	10.10	12.74	94
IL-1α	4.8-75,000	2.22	5.00	5.11	98
IL-1β	1.6-25,000	0.52	3.65	5.02	92
IL-1RA	1.6-25,000	1.26	4.00	6.29	97
IL-2	0.64-10,000	0.27	4.02	4.82	95
IL-3	1.28-20,000	0.29	3.26	5.62	100
IL-4	0.64-10,000	0.20	4.55	5.94	95
IL-5	0.64-10,000	0.17	3.99	5.42	99
IL-6	0.64-10,000	0.14	5.06	6.13	96
IL-7	0.64-10,000	0.14	4.85	4.61	97
IL-8	0.64-10,000	0.52	3.07	5.05	96
IL-9	0.64-10,000	2.82	3.47	5.13	101
IL-10	2.56-40,000	0.88	3.91	5.88	98
IL-12 (p40)	6.4-100,000	3.19	3.35	4.68	95
IL-12 (p70)	3.2-50,000	0.88	3.53	5.99	96
IL-13	6.4-100,000	2.42	11.67	7.40	95
IL-15	3.2-50,000	0.71	4.91	5.69	96
IL-17A	1.28-20,000	0.70	4.72	5.14	93
IL-17E/IL-25	40-625,000	21.25	5.03	5.45	93
IL-17F	32-500,000	28.56	4.63	6.08	95
IL-18	0.64-10,000	0.53	4.38	4.68	97
IL-22	12.8-200,000	12.62	4.54	6.61	96
IL-27	16-250,000	47.50	7.24	4.82	96
IP-10	2.56-40,000	2.13	4.14	9.11	99
MCP-1	3.2-50,000	3.07	3.91	4.25	101
MCP-3	8-125,000	8.58	3.51	4.76	104
M-CSF	40-625,000	31.94	4.38	5.04	97
MDC	0.64-10,000	0.42	3.47	4.27	96
MIG	6.4-100,000	3.97	6.75	11.07	95
MIP-1α	3.2-50,000	3.72	2.92	3.12	104
MIP-1β	0.38-6,000	0.34	3.56	4.73	99
PDGF-AA	12.8-200,000	10.31	5.88	5.98	102
PDGF-AB/BB	9.6-150,000	16.05	4.71	5.92	91
RANTES*	1.28-20,000	1.59	6.36	7.29	93
TGFα	1.28-20,000	0.98	3.80	5.39	102
TNFα	6.4-100,000	5.30	4.36	5.77	101
TNFβ	1.6-25,000	0.77	3.50	7.12	99
VEGF-A	2.56-40,000	0.96	4.94	5.73	99

48-Plex Sepsis vs. Normal Results

IL-1RA, IL-6, IL-8, IL-18, IL-27, IP-10, M-CSF, MIG scatter plots (Control vs. Sepsis)
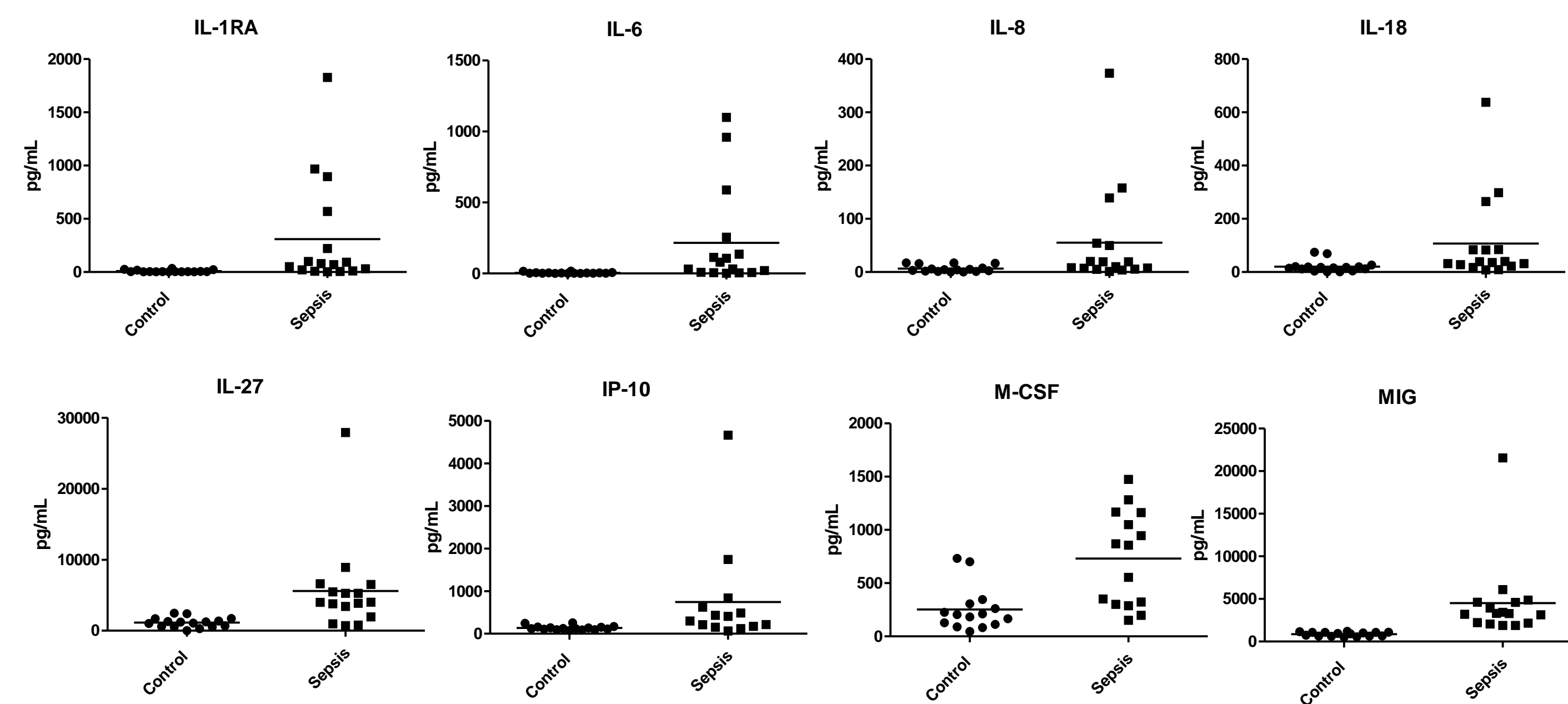
Healthy control serum/plasma samples (obtained from BioIVT) and sepsis patient serum/plasma samples (obtained from BioIVT, Discovery, and BioChemed) were tested neat (25 μL/well) in the HCYTA-60K panel.
Healthy control serum/plasma samples, N=20. Sepsis serum/plasma samples, N=16.

Cytokine Autoantibody Results

Fifteen sepsis serum/plasma samples were compared between the MILLIPLEX® Human Cytokine/Chemokine/ Growth Factor Panel A (Cat. No. HCYTA-60K) and the MILLIPLEX® Human Cytokine Autoantibody Panel (Cat. No. HCYTAAB-17K). Panel A cytokine concentrations are reported in pg/mL, and cytokine autoantibody levels are reported in MFI. Particular cytokine autoantibodies may be related to increased susceptibility to infection. Both IL-17A, IL-17F, and IFNγ autoantibodies play a neutralizing role, hampering the protective immune response. The data shown below supports this notion, with decreased cytokine levels in patients with increased anti-cytokine autoantibody levels. Anti-IL-6 and anti-IL-22 antibodies have also been associated with severe and recurrent bacterial infections.

IFNγ, IL-17F, IL-22, IL-6 bar charts comparing protein (pg/mL) and autoantibody (MFI) across Samples 1–15

IL-6, IL-17A, IL-17F, G-CSF, IL-10, BAFF scatter plots (SLE, RA, Sepsis, Healthy)

As noted above, Anti-IL-6, IL-17A, and anti-IL-17F autoantibody levels are elevated in sepsis samples when compared to autoimmune disease and healthy subjects. Anti-cytokine autoantibodies have also been associated with autoimmune diseases and can indicate disease improvement or increased severity. Anti-BAFF autoantibodies have been linked to decreased disease activity in SLE and anti-G-CSF autoantibodies have been associated with SLE and RA, specifically in those patients with neutropenia. Anti-IL-10 autoantibodies have also been associated with SLE.

Serum/Plasma Sample Testing

Serum/Plasma samples were tested neat (25 μL/well) except for RANTES for which samples were diluted 1:100 in assay buffer.

Normal Serum/Plasma Sample Summary Data (pg/mL)

	sCD40L	EGF	Eotaxin	FGF-2	FLT-3L	Fractalkine	G-CSF	GM-CSF	GROα	IFNα2	IFNγ	IL-1α
N	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
average	2273.5	6.1	42.3	24.4	436.8	120.9	31.8	31.4	77.4	34.5	39.8	
min	29.4	6.1	42.3	24.4	3.4	76.0	5.7	1.2	4.5	10.1	1.2	1.9
max	9505.8	353.7	126.8	499.2	81.6	1583.0	648.5	144.1	60.1	250.6	120.8	189.5
% detectable	100.0	85.0	100.0	70.0	100.0	75.0	90.0	70.0	80.0	65.0	85.0	75.0

Sepsis Serum/Plasma Sample Summary Data (pg/mL)

N	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0
average	1896.9	63.2	70.3	68.6	28.9	214.8	1250.4	13.5	62.2	24.4	29.4	22.6
min	24.0	17.0	72.0	12.5	91.0	17.0	23.0	22.5	49.0	14.0	18.0	17.0
max	7097.4	196.5	137.2	138.0	50.9	446.5	11850.0	55.8	222.5	88.5	79.1	78.6

Normal Serum/Plasma Sample Summary Data (pg/mL)

	IL-12 (p70)	IL-13	IL-15	IL-17A*	IL-17E	IL-17F	IL-18	IL-22	IL-27	IP-10	MCP-1	MCP-3
N	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
average	33.9	72.2	17.1	27.6	1205.9	2630.4	20.4	331.2	1204.3	136.3	256.7	103.9
min	2.1	3.0	3.0	0.8	52.5	1718.0	0.8	40.8	287.5	76.2	42.6	11.1
max	119.0	451.8	101.0	56.2	6145.6	4313.0	74.0	863.2	2472.3	254.5	756.5	405.1
% detectable	65.0	19.0	100.0	25.0	100.0	20.0	100.0	55.0	95.0	100.0	100.0	85.0

Sepsis Serum/Plasma Sample Summary Data (pg/mL)

N	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0
average	6.1	23.9	17.3	5.0	487.0	212.7	107.1	75.0	5587.5	1663.7	573.2	31.3
min	1.6	8.3	5.4	0.8	45.5	8.0	15.6	714.0	65.5	107.4	11.1	
max	31.2	70.2	40.3	19.4	2921.9	1079.0	637.6	218.2	27900.0	14532.0	2041.3	84.3

Normal Serum/Plasma Sample Summary Data (pg/mL)

	IL-1β	IL-1RA	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-9	IL-10	IL-12(p40)
N	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
average	25.3	8.8	12.1	1.6	4.2	7.3	4.6	3.9	7.3	21.1	26.3	127.5
min	0.7	1.7	0.4	0.6	0.2	0.3	0.5	0.4	1.0	2.5	0.9	6.5
max	102.6	34.6	30.3	3.3	20.8	33.3	17.1	12.9	17.2	55.5	167.2	744.6
% detectable	85.0	100.0	30.0	45.0	90.0	100.0	95.0	100.0	90.0	100.0	95.0	100.0

Sepsis Serum/Plasma Sample Summary Data (pg/mL)

N	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0
average	9.5	309.3	1.8	2.0	1.9	4.6	215.4	9.1	55.1	14.8	162.8	96.3
min	1.0	3.1	0.4	0.3	0.8	1.5	2.0	1.2	4.9	1.2	35.8	
max	41.4	1828.2	10.7	5.9	7.5	28.6	1099.0	28.3	373.5	39.6	1796.5	247.1

Normal Serum/Plasma Sample Summary Data (pg/mL)

	M-CSF	MDC	MIG	MIP-1α	MIP-1β	PDGF-AA	PDGF-AB/BB	RANTES	TGFα	TNFα	TNFβ	VEGF-A
N	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
average	252.8	842.9	869.7	193.2	22.9	2273.4	15919.9	69838.5	118.5	82.4	72.0	130.6
min	46.3	440.0	501.4	4.3	6.3	296.8	3086.1	10762.0	0.9	7.1	0.8	3.2
max	732.5	1592.0	1205.6	1434.5	39.7	7147.8	32160.0	129934.0	755.2	425.0	352.8	379.9
% detectable	95.0	100.0	100.0	85.0	100.0	100.0	100.0	100.0	80.0	100.0	75.0	100.0

Sepsis Serum/Plasma Sample Summary Data (pg/mL)

N	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0
average	731.3	729.0	4512.0	24.9	47.8	3285.0	20776.9	116456.5	24.0	61.1	8.1	173.1
min	150.6	328.5	1881.5	5.4	13.5	1480.0	10630.4	26792.0	2.2	10.6	1.2	37.3
max	1474.4	2802.0	21550.0	87.9	155.2	5573.2	47940.0	293916.0	137.6	293.5	32.8	489.9

Summary

The MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A (Cat. No. HCYTA-60K) features an exciting new large combination of 48 configurable human cytokine, chemokine and growth factor assays requiring only 25 μL of each sample. Representative data shown here exemplifies the value of this kit for the study of relevant disease sample biomarkers in serum and plasma biofluids.

This study demonstrates the value of using multiplex technology to evaluate multiple autoantibodies, in this case with the MILLIPLEX® Human Cytokine Autoantibody Panel (Cat. No. HCYTAAB-17K) allowing for an extensive autoantibody profile to evaluate patients with autoimmune or infectious disease. Anti-cytokine autoantibodies may have an activating or neutralizing affect and can aid in monitoring disease activity.

The use of these two types of capture methods exemplifies the flexibility of the Luminex® platform, and the power of analyzing multiple analytes.

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