

Measuring Immune Response and Cytokine Autoantibodies in Human Sepsis Samples

Robert Keith, Brooke Gilliam, Hong Luo, Harold Steiner, Yiwen Jan, Danielle Pepin, Qiang Xiao, and Wei Zheng
MilliporeSigma, St. Louis, Missouri 63103



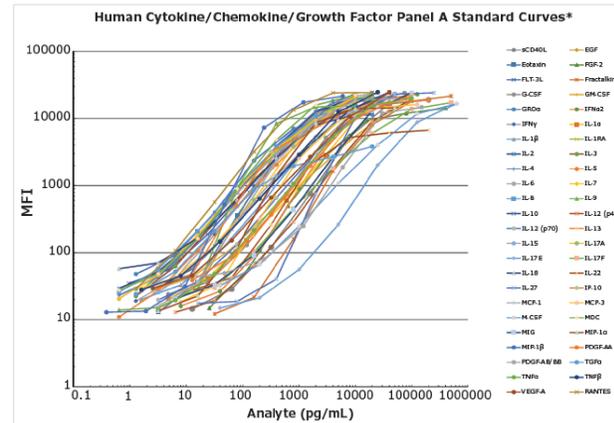
Introduction

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.

48-Plex Characteristics

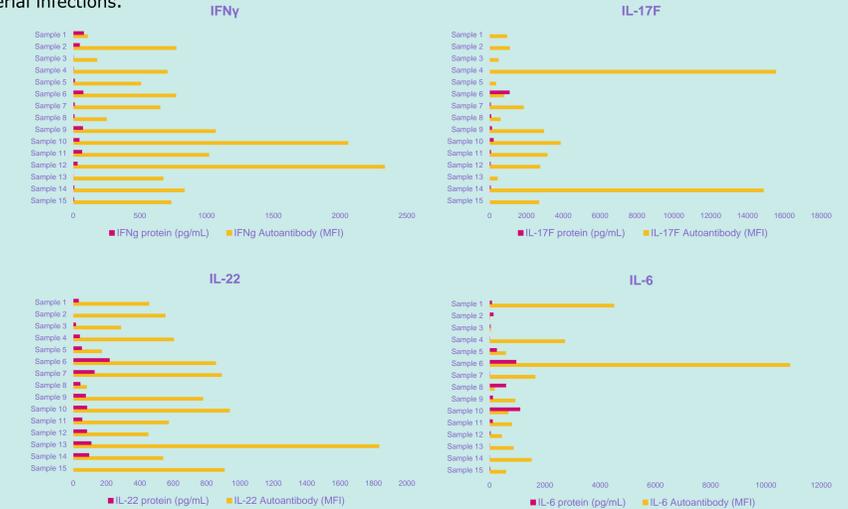


*Standard curves: Performed in MXSM-A serum matrix except *RANTES, which was in L-AB assay buffer. The kit was run using the **HCYTA-60K** overnight assay protocol.
Sensitivity: Standard curves (n=9 separate tests) were run in serum matrix, except RANTES was tested in assay buffer.
Precision: Two different 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) | | Accuracy (% Recovery) In Matrix |
|-------------|------------------------------|---------------------|-----------------|-------------|---------------------------------|
| | | | Intra-assay | Inter-assay | |
| 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 | 4.69 | 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 | 3.96 | 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 | 5.92 | 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 | 0.88 | 3.91 | 5.88 | 98 |
| IL-6 | 0.64-10.000 | 0.14 | 5.98 | 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-17EAL-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 | 12.82 | 4.54 | 6.61 | 96 |
| IP-10 | 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 |
| MIP-1β | 40-625.000 | 10.31 | 5.88 | 5.94 | 97 |
| MIP-1β | 6.4-10.000 | 0.42 | 3.47 | 4.27 | 96 |
| MIG | 6.4-100.000 | 3.97 | 6.75 | 11.07 | 95 |
| MIP-1β | 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 | 16.04 | 4.71 | 5.92 | 91 |
| PDGF-AB/BB | 9.6-150.000 | 1.59 | 6.36 | 7.29 | 93 |
| RANTES* | 1.28-20.000 | 0.98 | 3.80 | 5.39 | 102 |
| TGFα | 1.28-20.000 | 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 |

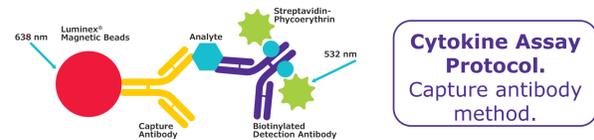
Cytokine Autoantibody Results

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.



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.

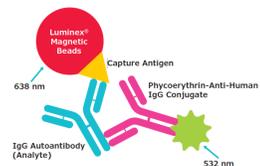


Cytokine Assay Protocol. Capture antibody method.

- 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 hour 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

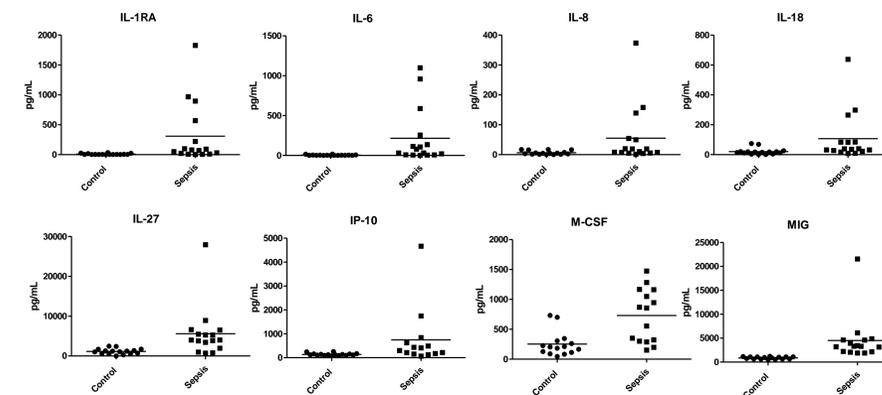


Cytokine Autoantibody Assay Protocol. Capture antigen method.

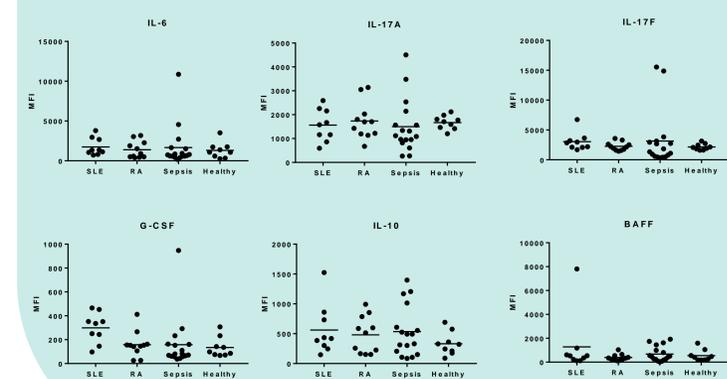


- Prewet 96-well plate with 200 µL wash buffer and decant
- + 25 µL assay buffer
- + 25 µL sample (serum or plasma); 25 µL assay buffer to background wells
- + 25 µL bead mixture
- Shake overnight at 4°C
- Wash beads x3 with wash buffer
- + 50 µL PE-IgG Conjugate
- Shake 1.5 hour at RT
- Wash beads x3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

48-Plex Sepsis vs. Normal Results



Healthy control serum/plasma samples (obtained from BioIVT 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.



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) | | | | | | | | | | | |
|---|--------|-------|-------|-------|-------|--------|-------|-------|------|-------|------------|
| | IL-1β | 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 |
| average | 2273.5 | 93.8 | 85.1 | 151.8 | 24.3 | 436.8 | 120.9 | 31.8 | 31.4 | 77.4 | 34.5 |
| min | 29.4 | 6.1 | 4.2 | 24.4 | 3.4 | 76.0 | 5.7 | 1.2 | 4.5 | 10.1 | 1.2 |
| max | 9525.8 | 353.7 | 126.8 | 499.2 | 81.8 | 1583.0 | 648.5 | 144.1 | 60.1 | 250.6 | 120.6 |
| % detectable | 100.0 | 85.0 | 100.0 | 70.0 | 100.0 | 75.0 | 90.0 | 70.0 | 80.0 | 65.0 | 85.0 |

| Normal Serum/Plasma Sample Summary Data (pg/mL) | | | | | | | | | | | |
|---|-------------|-------|---------|--------|--------|--------|-------|-------|--------|-------|-------|
| | IL-12 (p70) | 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 |
| average | 33.9 | 72.2 | 17.1 | 27.6 | 1205.7 | 2630.4 | 20.4 | 331.2 | 1204.3 | 136.3 | 256.7 |
| min | 2.1 | 3.0 | 3.0 | 0.8 | 52.5 | 1718.0 | 0.8 | 40.8 | 287.5 | 76.2 | 42.6 |
| max | 119.0 | 451.8 | 101.0 | 56.2 | 6145.6 | 4313.0 | 74.0 | 863.2 | 2472.3 | 254.5 | 756.5 |
| % detectable | 65.0 | 15.0 | 100.0 | 25.0 | 100.0 | 100.0 | 100.0 | 55.0 | 95.0 | 100.0 | 85.0 |

The life science business of Merck KGaA, Darmstadt, Germany operates as MilliporeSigma in the U.S. and Canada

Legal entity and address including country. List of product trade names are registered trademarks of Merck KGaA, Darmstadt, Germany. © 2016 Merck KGaA, Darmstadt, Germany. All rights reserved. Lit. No. MK_PS4960EN.

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.

www.sigmaaldrich.com/milliplex