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Application Note

Look Beyond Traditional Immune Factors With A Novel MILLIPLEX® 48-Plex Cytokine Immunoassay

Introduction

Cytokines, chemokines, and growth factors are key mediators of immune system functions capable of signaling through autocrine, paracrine, and endocrine mechanisms. Their pleiotropic immunomodulatory properties allow these biomolecules to react to diverse stimuli and regulate the immune response, either by promoting or inhibiting inflammation. Growing research continues to highlight the role of the immune system in every facet of human health and disease. The MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel B offers a unique combination of 48 biomarkers that can be simultaneously analyzed in a small sample volume. **Table 1** details the available kit formats and included analytes.

This application note outlines key analytical studies and results to demonstrate the reliable and accurate performance of MILLIPLEX® Human Cytokine/ Chemokine/Growth Factor Panel B. This includes analyte specificity, parallelism, and sample detectability compared to multiplex kits from other brands and to our MILLIPLEX® kits containing the same analytes.

MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel B			
Cat. Nos.	**HCYTB-60K**	**HCYTB-60K-PX48 HCYTB-60K-PXBK48 (Bulk packaging)**	**HCYTB-60K-PX38 HCYTB-60K-PXBK38 (Bulk packaging)**
Description	Select your analytes, custom premixing available	48-plex fixed premixed beads	38-plex fixed premixed beads
Analytes Included	6Ckine, sCD137, sFAS, sFASL, APRIL, BAFF, BCA-1, CCL28, CTACK, CXCL16, ENA-78, Eotaxin-2, Eotaxin-3, GCP-2, Granzyme A, Granzyme B, HMGB1, I-309, IFNβ, IFNω, IL-11, IL-16, IL-20, IL-21, IL-23, IL-24, IL-28A, IL-29, IL-31, IL-33, IL-34, IL-35, I-TAC, LIF, Lymphotactin, MCP-2, MCP-4, MIP-1δ, MIP-3α, MIP-3β, MPIF-1, Perforin, SCF, SDF-1, TARC, TPO, TRAIL, TSLP	6Ckine, sCD137, sFAS, sFASL, APRIL, BAFF, BCA-1, CTACK, ENA-78, Eotaxin-2, Eotaxin-3, Granzyme A, Granzyme B, HMGB1, I-309, IFNβ, IFNω, IL-16, IL-20, IL-21, IL-23, IL-28A, IL-29, IL-31, IL-33, I-TAC, LIF, MCP-2, MCP-4, MIP-1δ, MIP-3α, Perforin, SCF, SDF-1, TARC, TPO, TRAIL, TSLP

Table 1. MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel B kit catalog numbers, formats, and analytes included in each kit.

Methods

MILLIPLEX® Immunoassays and Data Analysis

The MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel B **(Cat. No. HCYTB-60K)** assays were performed in 96-well plates according to the product manual. All assays were run on the Luminex® 200™ instrument and data was acquired via xPONENT® v. 4.3 software. Data analysis was performed for all immunoassays using the Belysa® Immunoassay Curve Fitting Software **(Cat. No. 40-122)**. Figures were prepared in GraphPad Prism and Microsoft Excel.

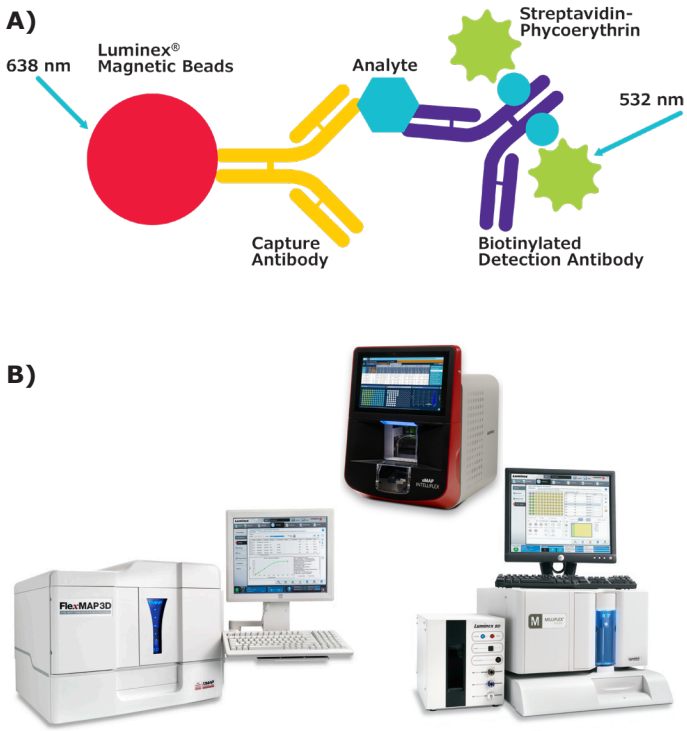

Figure 1. (A) MILLIPLEX® protein detection method. **(B)** Luminex® instrumentation for use with MILLIPLEX® multiplex kits, including xMAP® INTELLIFLEX® RUO and DRSE, FLEXMAP 3D®, Luminex® 200™, and MAGPIX® (not pictured) platforms.

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Sample Preparation

Blood Samples

Matched human plasma and serum samples from healthy donors, as well as serum or plasma samples from donors with sepsis, psoriasis, and rheumatoid arthritis (RA), were obtained from BioIVT, Westbury, NY. Colorectal cancer (CRC) serum samples were obtained from Discovery Life Sciences, Huntsville, AL.

For serum samples, the blood was allowed to clot for 30 minutes before centrifugation for 10 minutes at 1,000 x g. The serum was removed and either assayed immediately or aliquoted and stored at -80°C. Plasma samples with EDTA anticoagulant were centrifuged at 1,000 x g within 30 minutes of blood collection. Plasma was removed and assayed immediately or aliquoted and stored at -80°C. Frozen samples were thawed completely, vortexed, and centrifuged before use to remove particulates. Samples were run neat or diluted, according to the respective kit protocols.

Cell Culture Supernatants

Supernatants from the culture of peripheral blood mononuclear cells (PBMCs) were obtained from BioIVT, Westbury, NY. The PBMCs were obtained from healthy donors and cultured in a cell culture medium (RPMI, 10% FBS, 1% penicillin/streptomycin) at $1x10^6$ cells/mL. Cells were stimulated for 48 hours with 1 µg/mL lipopolysaccharide (LPS), 5 µg/mL concanavalin A (ConA), or left unstimulated as a control. Cultures were centrifuged to remove cell debris, and supernatants were aliquoted and stored at -80°C immediately.

Results

Analyte Specificity

In multiplex immunoassays, it is important that analytes are specific to confirm accurate sample detectability. Standards, capture antibodies, and detection antibodies were evaluated to confirm minimal cross-reactivity between analytes. **Figure 2** shows analyte specificity for a selection of analytes included in this kit, including sFas, BAFF, Granzyme A, Granzyme B, IL-33, and Perforin. All 48 analytes in this panel demonstrated comparable performance. Analyte specificity is a standard test for all MILLIPLEX® multiplex assays and the results are reported in each kit protocol.

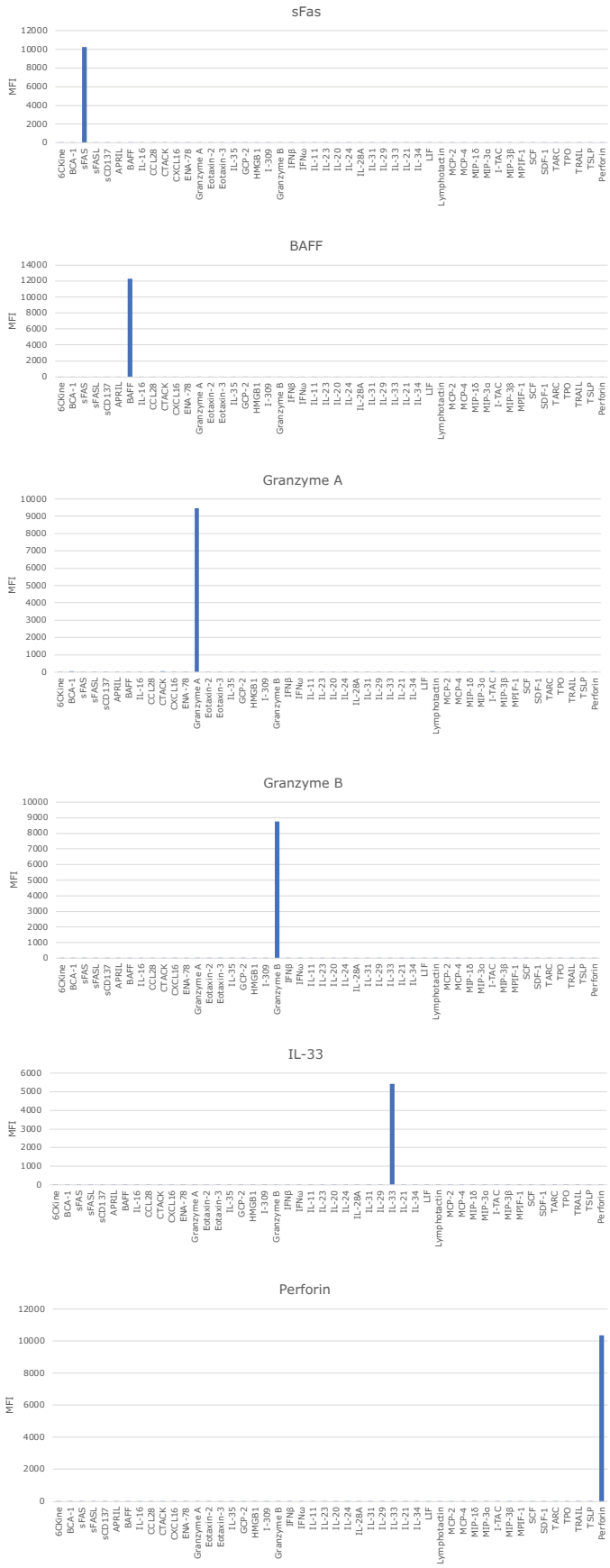


Figure 2. Representative analyte specificity for select analytes in MILLIPLEX® Human Cytokine Panel B. There was no or negligible cross-reactivity between the antibodies for any of the analytes in this kit.

Parallelism

Parallelism is the parallel relationship between the standard curve and samples that have been diluted serially to determine dilution effects on sample measurement.[1] To evaluate parallelism, samples should contain high levels of endogenous analyte so that the analyte can still be quantified in the serially diluted sample. For MILLIPLEX® Human Cytokine/Chemokine/ Growth Factor B, parallelism was analyzed in neat samples that were diluted 1:2, 1:4, and 1:8, with representative data shown for six analytes in **Figure 3.** Back calculated concentrations in the samples did not exceed ± 30% and did not exceed ± 20% for APRIL, MPIF-1, and TRAIL.

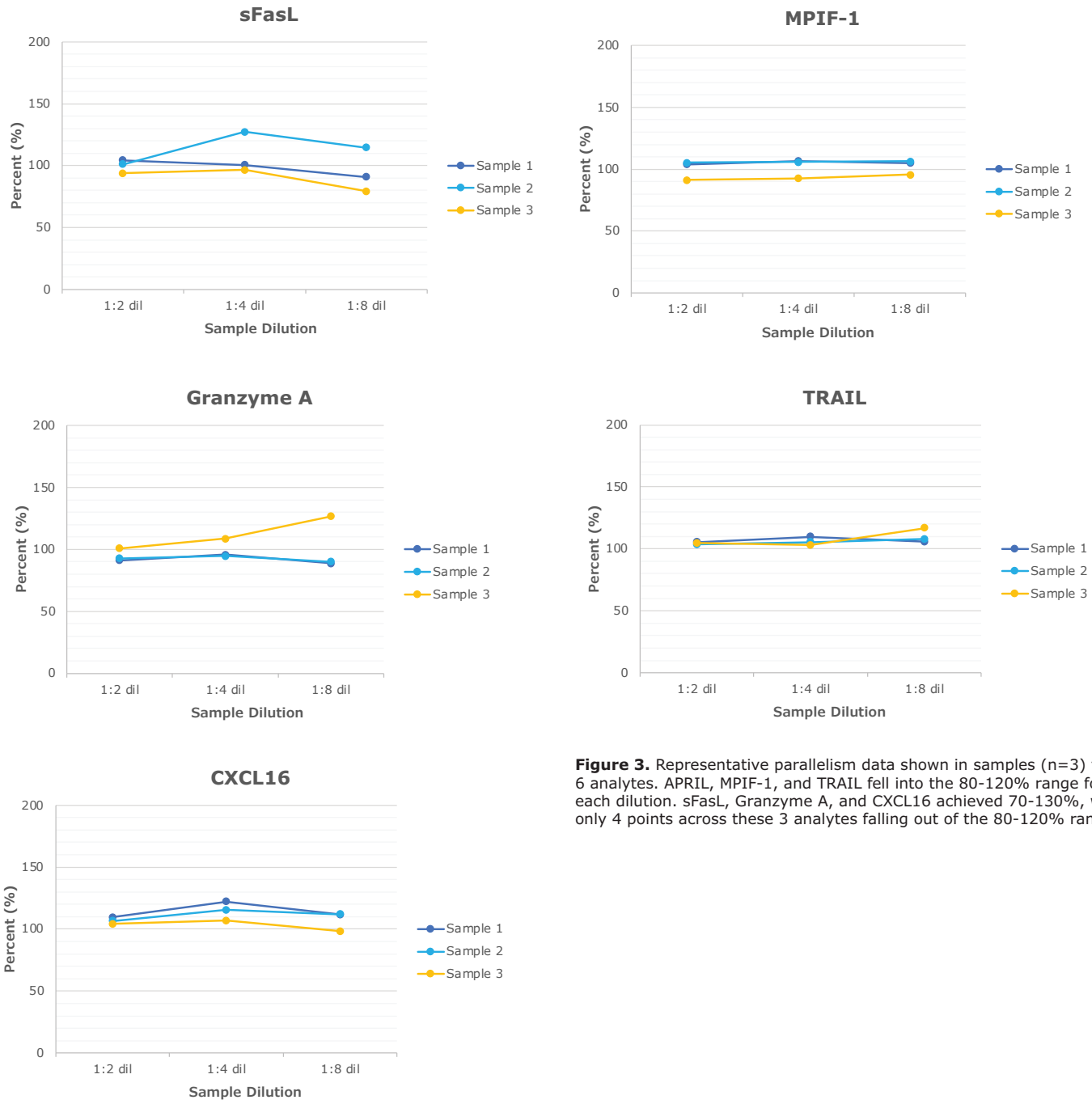


Figure 3. Representative parallelism data shown in samples (n=3) for 6 analytes. APRIL, MPIF-1, and TRAIL fell into the 80-120% range for each dilution. sFasL, Granzyme A, and CXCL16 achieved 70-130%, with only 4 points across these 3 analytes falling out of the 80-120% range.

Sample Detectability and Correlation

Brand Comparison

The MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel B 48-plex kit was tested against other Luminex® Brand kits, including Brand 1 which offers a discovery assay and a performance assay that reportedly includes a higher level of assay verification. In this comparison, the Brand 1 discovery assays are referred to as Brand 1D, and the performance assays are referred to as Brand 1P. MILLIPLEX® Human Cytokine Panel B was also compared to other Luminex® partner kits, here referred to as Brand 2 and Brand 3.

Granzyme B in MILLIPLEX® Human Cytokine Panel B demonstrated 100% sample detectability in both healthy and disease serum and plasma samples **(Figure 4A)**. Conversely, Brand 1P only had 2 detectable disease samples and none of the healthy samples read above background. The discovery assay from the same Brand showed better sample detectability that was comparable in sample range for healthy and disease state samples to MILLIPLEX® Human Panel B. According to a literature search, typical Granzyme B levels should range between 2-50 pg/mL,[2,3] with the MILLIPLEX® panel healthy serum and plasma sample average at 8.9 pg/mL.

For TNF-related apoptosis-inducing ligand (TRAIL), all healthy and disease samples were detectable using MILLIPLEX® Human Cytokine Panel B. Brand 1P had 9/16 (56%) detectable disease samples. As an immune response regulator in sepsis, reduced TRAIL levels in sepsis have been associated with poor outcomes.[4] MILLIPLEX® Human Cytokine Panel B followed this trend of reduced TRAIL concentration in disease state samples, including sepsis, which was also demonstrated less prominently in other Brand multiplex panels **(Figure 4B)**. Healthy TRAIL levels have been shown to average around 50-100 pg/mL, which is in line with the MILLIPLEX® Human Cytokine Panel B results.

MIP-3β was detectable in 100% of healthy serum and plasma samples using the MILLIPLEX® Human Cytokine Panel B kit. When compared to other Brand kits, average results were consistent with kits from Brand 2 and Brand 3 and were in line with literature ranges.[5] The kits from Brand 1D and 1P demonstrated false positive results in two samples, reading nearly 3-fold higher than the other healthy samples across the 5 multiplex kits **(Figure 4C)**. The kits from Brands 2 and 3 both had detectable levels of MIP-3β in 89% of serum and plasma samples, including disease state samples (data not shown).

CXCL6/GCP-2 demonstrated 100% sample detectability using the MILLIPLEX® Human Cytokine Panel B kit with average values in line with Brand kits 2 and 3. The MILLIPLEX® Panel B was also in line with literature ranges of 40-350 pg/mL.[6] As with other analytes, the kit from Brand 1D again showed false positive results with the average healthy sample concentration nearly four times higher than other assays **(Figure 4D)**. Only 55% of healthy samples were detectable using the multiplex assay from Brand 2, which had an overall 58% sample detectability rate when also considering disease state samples (data not shown).

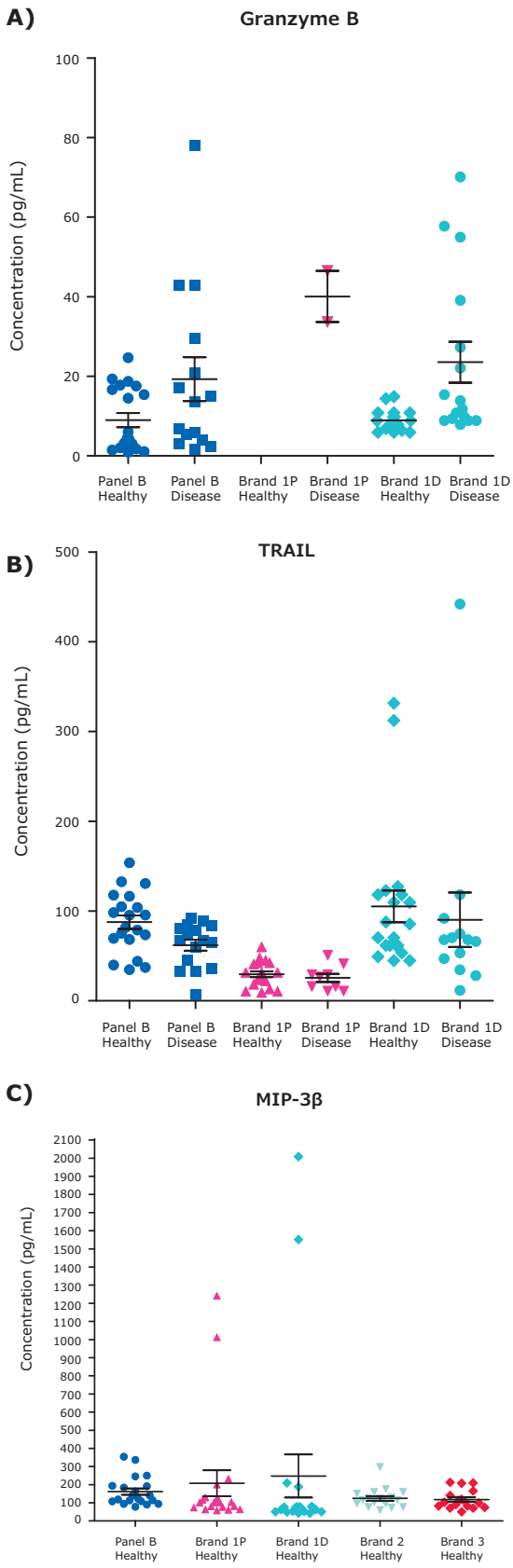


Figure 4. MILLIPLEX® Human Cytokine Panel B sample concentration comparison with other Luminex® Brand multiplex kits. Representative data are shown for **(A)** Granzyme B, **(B)** TRAIL, and **(C)** MIP-3β. Test samples included healthy (n=20) and disease (sepsis and RA; n=16) serum and plasma samples.

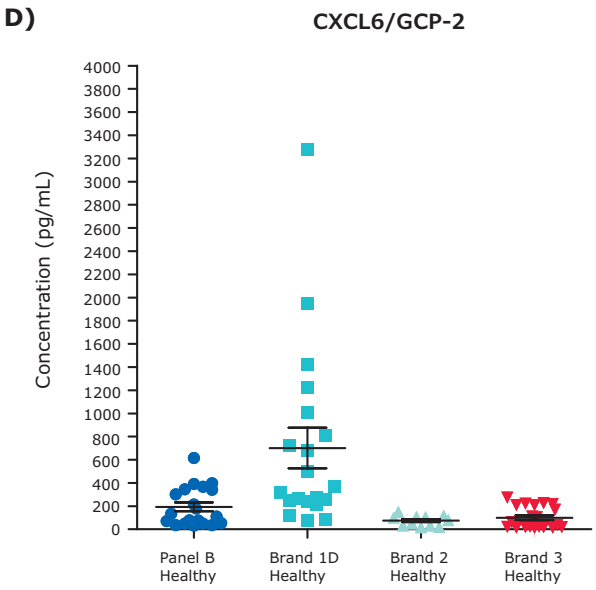
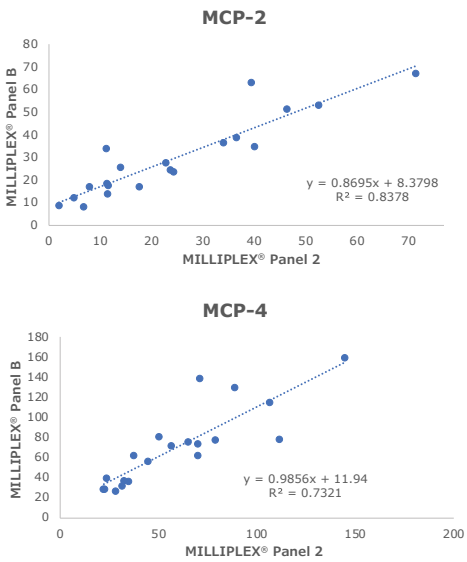


Figure 4 (continued) MILLIPLEX® Human Cytokine Panel B sample concentration comparison with other Luminex® Brand multiplex kits. Representative data are shown for **(D)** CXCL6/GCP-2. Test samples included healthy (n=20) and disease (sepsis and RA; n=16) serum and plasma samples.

Comparison to Other MILLIPLEX® Kits

MILLIPLEX® Human Cytokine Panel B was evaluated against other MILLIPLEX® Human Cytokine panels to ensure sample values matched previous kit performance. **Figure 5** shows sample correlation data from MILLIPLEX® Human Cytokine Panel B compared to common analytes from MILLIPLEX® Human Cytokine Panel 2 **(Cat. No. HCYP2MAG-62K)**, MILLIPLEX® Human Cytokine Panel 3 **(Cat. No. HCYP3MAG-63K)**, and MILLIPLEX® Human CD8+ T Cell Panel **(Cat. No. HCD8MAG-15K)**. In some instances, sample concentrations in MILLIPLEX® Human Panel B may not match previous kits but are now more in line with literature ranges. This includes analytes like BAFF,[7] HMGB1,[8] SCF,[9] IL-28A,[10] and IL-23.[11]



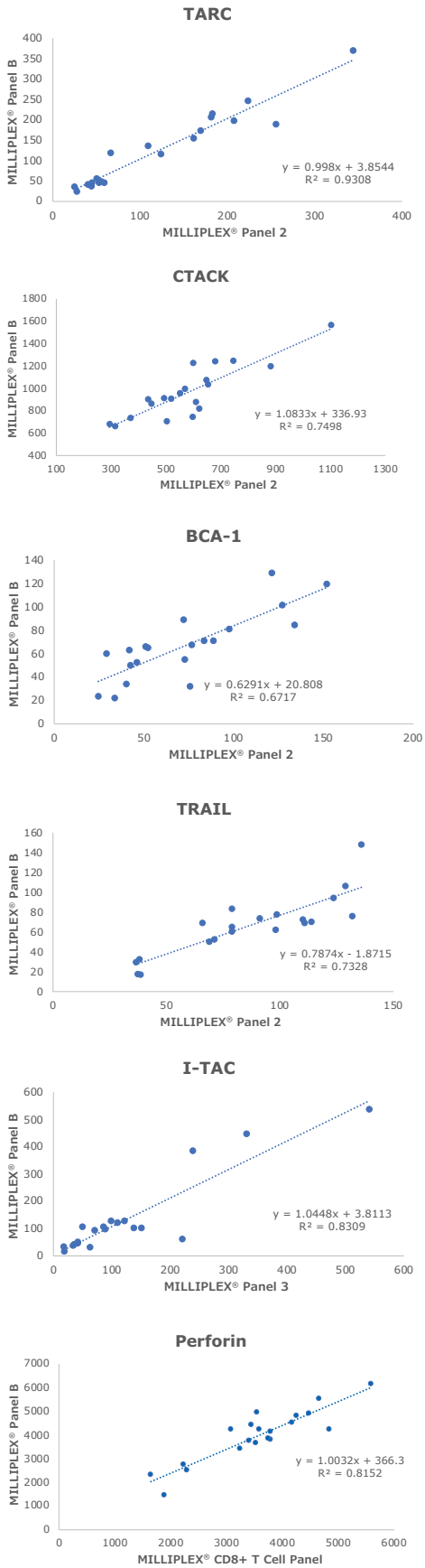
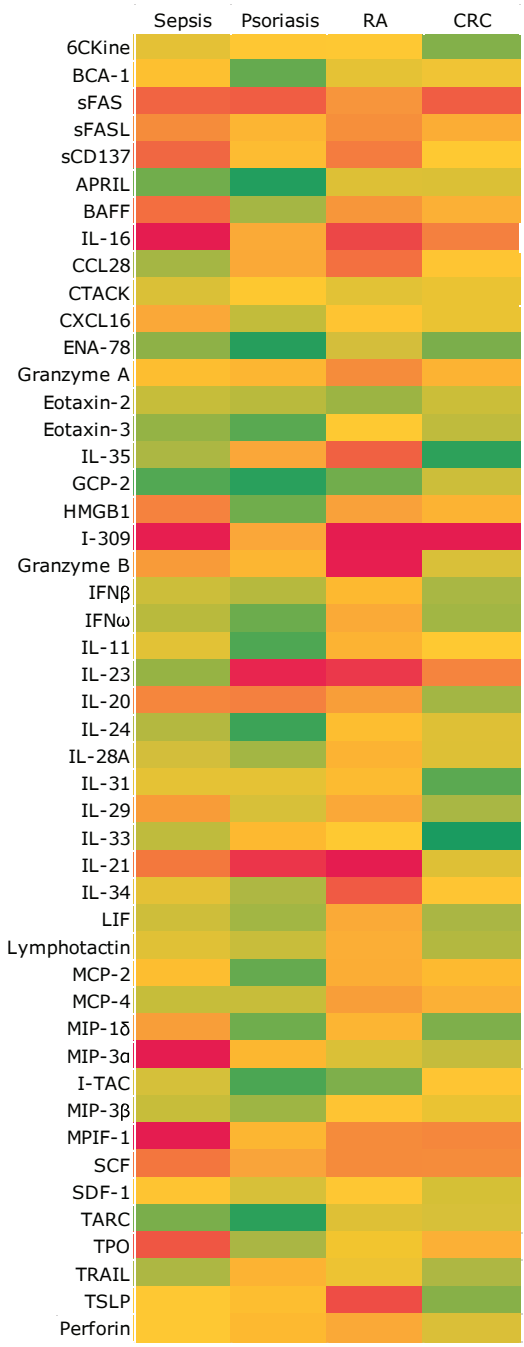


Figure 5. Representative sample correlation graphs between MILLIPLEX® Human Cytokine Panel B and the comparable MILLIPLEX® kit indicated in each graph.

Patterns of Cytokine Recognition in Human Sera Samples

MILLIPLEX® Human Cytokine Panel B was used to measure sera samples from healthy controls and various disease states, including sepsis, psoriasis, RA, and CRC. The heat map shows relative levels of induction for each analyte in **Figure 6**. IL-21 showed increased induction in RA and psoriasis, along with IL-34 in RA. IL-34 is a pro-inflammatory cytokine that has been shown to exhibit elevated expression in RA samples.[12] BAFF and TPO exhibited elevated levels in sepsis samples. Members of the TNFα superfamily, including BAFF, have been shown to play a role in the pathogenesis of sepsis.[13] Additionally, increased TPO levels have also been reported in sepsis samples.[14]

A)



B)

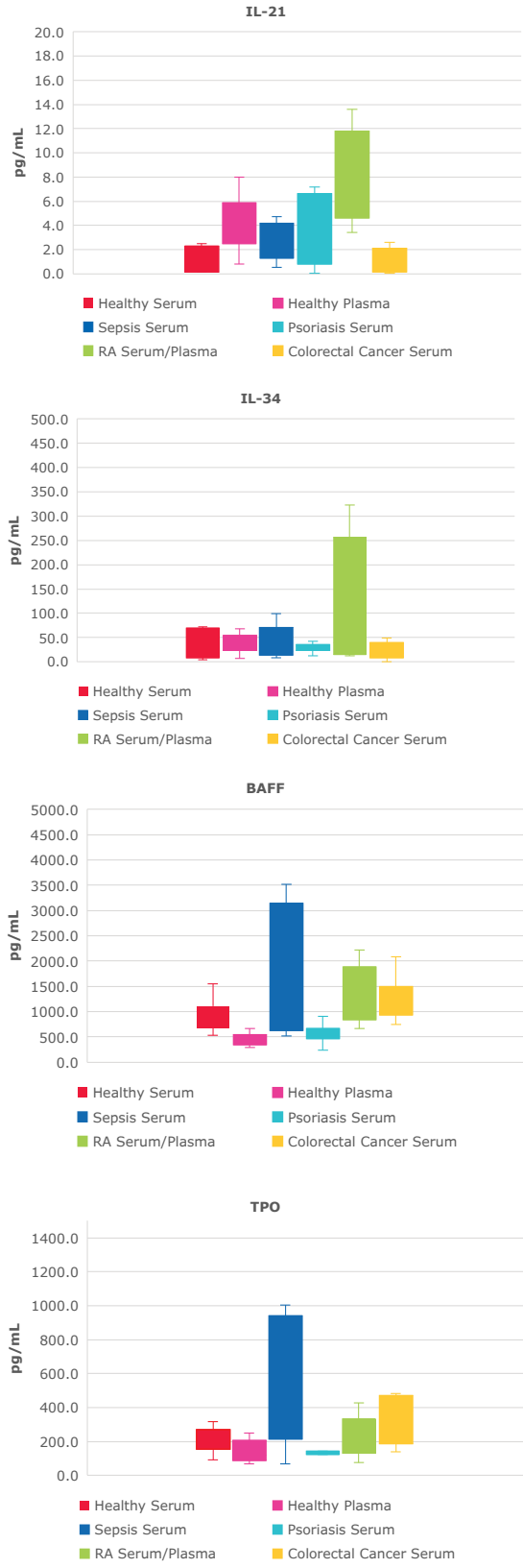


Figure 6. (A) Heat map showing relative levels of induction for each cytokine in sepsis, psoriasis, RA, and CRC serum. The data is presented as a ratio of disease over healthy concentrations in a Red-Yellow-Green pattern showing higher values in Red and lower values in Green. **(B)** IL-21, IL-34, BAFF, and TPO average concentrations were stratified by healthy and disease samples.

Patterns of Cytokine Regulation in Human PBMC Supernatants

Five matched sets of human PBMCs were treated with LPS or Con A for 48 hours or left unstimulated, then the supernatant was collected and evaluated using MILLIPLEX® Human Cytokine Panel B. The heat map shows the relative response for a subset of proteins, with Red representing induction, Green representing repression, and Yellow indicating proteins with comparatively muted responses to treatment **(Figure 7)**. BCA-1, IL-23, MCP-2, MIP-3α, and MIP-3β demonstrated induction in both LPS and ConA compared to unstimulated PBMC supernatants.

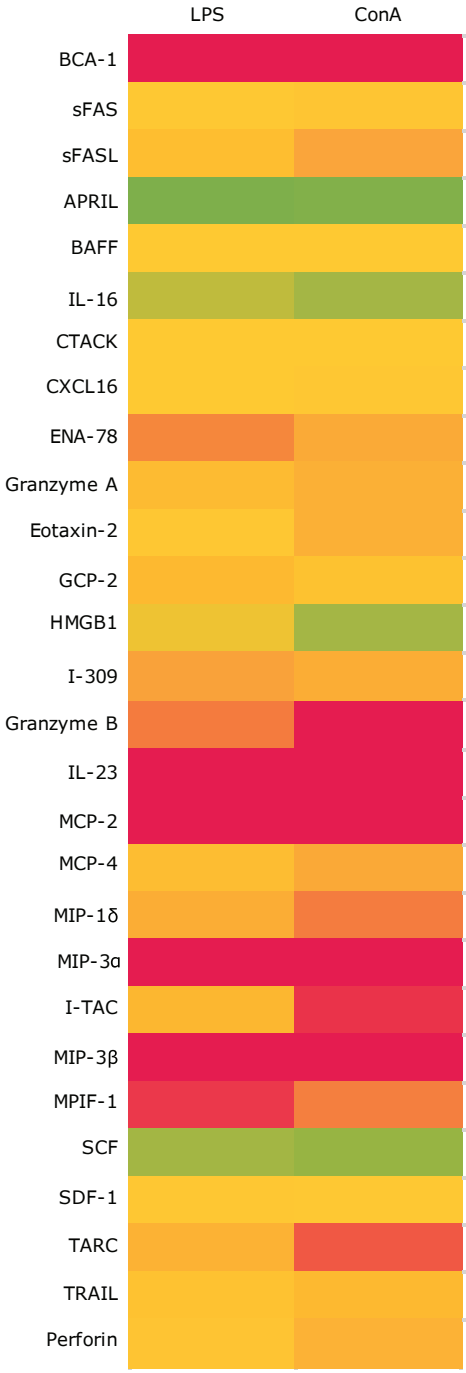


Figure 7. Heat map showing relative response for analytes induced by LSP, ConA, or both compared to unstimulated PBMCs.

Summary

The MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel B **(Cat. No. HCYTB-60K)** is a novel, multiplexed immunoassay using Luminex® xMAP® technology capable of detecting up to 48 different analytes relevant for human health and immune regulation studies. MILLIPLEX® immunoassay kits provide a powerful tool for examining many sample types relevant to human disease and critical research areas. Here we demonstrate the detection of important analytes in various states of induction in a selection of sample types pertinent to work in immunology, drug discovery, and beyond.

Acknowledgments

Thank you to Brooke Gilliam and Anthony Saporita, Ph.D. for writing the application note, to Sasha Williams and Harold Steiner for generating data, and to Irene Zollars, PharmD, and Danielle Pepin for their thoughtful reviews.

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