

Quantitative multiplex analysis of 48 analytes using the MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A: Analyte comparisons between different kits

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Cytokines are immunomodulatory polypeptides that play key roles in both adaptive and innate immune responses. “Cytokine” is a general term used for a diverse group of soluble proteins and peptides which act as regulators under both normal and pathological conditions to modulate the functional activities of individual cells and tissues. These proteins also mediate direct interactions between cells and regulate processes taking place in the extracellular environment. Cytokines differ from hormones in that they act on a wider spectrum of target cells and include lymphokines, interferons, colony stimulating factors and chemokines. Additionally, growth factors are involved in the stimulation of target cell survival, proliferation, differentiation with effects on angiogenesis, vasculogenesis, cell migration, apoptosis, wound healing and embryogenesis.

Cytokine, chemokine and growth factor research plays a significant role in achieving a deeper understanding of the immune system and its multi-faceted response to most antigens. This deep understanding is especially relevant to those responses that make up the inflammatory process and disease states, such as infectious disease, osteoarthritis, respiratory disease, IBD, sepsis, allergic reactions, as well as neurologic, metabolic and cardiovascular disease and even cancer.

Researchers can now simultaneously measure up to 48 individual immune factors using our MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A

and Luminex® xMAP® instrumentation, saving both time and sample volume, compared to quantifying multiple protein levels using individual assays, or smaller plex assays. This is the largest kit in the MILLIPLEX® portfolio of protein immunoassays. Table 1 describes the available kits and formats. The configurable panel enables the user to choose any number of analytes within the panel to meet specific research needs. Our custom premix offering allows selection of the exact set of analytes required, with premixing of beads completed in our facility prior to shipment for ease of use. In addition, the panel is available as a fixed premixed-bead kit as either a 48-plex or a 38-plex kit. Whether configurable, custom premixed, or fixed, all kits are available for 96-well plates. We offer a format suitable for every lab, with results consistent from lot to lot.

This application note summarizes the development, analytical verification studies and subsequent test results generated by the research team during the kit development process, with a focus on comparing analyte assays contained in MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A with those in other panels within the MILLIPLEX® portfolio of products. Further experiments are also regularly conducted after the panel has been transferred to manufacturing, where it undergoes rigorous testing by our Quality Assurance/Quality Control teams.

Cat. No.	Number of Analytes	Analytes Contained	Notes
HCYTA-60K ¹	Up to 48	sCD40L, EGF, Eotaxin, FGF-2, FLT-3L, Fractalkine, G-CSF, GM-CSF, GRO α , IFN α 2, IFN γ , IL-1 α , IL-1 β , IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IL-17E/IL-25, IL-17F, IL-18, IL-22, IL-27, IP-10, MCP-1, MCP-3, M-CSF, MDC, MIG, MIP-1 α , MIP-1 β , PDGF-AA, PDGF-AB/BB, RANTES*, TGF α , TNF α , TNF β , VEGF-A	¹ Customizable panel: choose your analytes and receive individual bead vials or use our convenient service for customizing your own bead premix (additional charge applicable).
HCYTA-60K-PX38 ²	38	sCD40L, EGF, Eotaxin, FGF-2, FLT-3L, Fractalkine, G-CSF, GM-CSF, GRO α , IFN α 2, IFN γ , IL-1 α , IL-1 β , IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IL-17E/IL-25, IL-17F, IL-18, IL-22, IL-27, IP-10, MCP-1, MCP-3, M-CSF, MDC, MIG, MIP-1 α , MIP-1 β , PDGF-AA, PDGF-AB/BB, RANTES*, TGF α , TNF α , TNF β , VEGF-A	² Premix kits contain either a 37- or 47-plex bead set, plus a vial of RANTES beads.
HCYTA-60K-PXBK38 ³			³ Bulk kits match the formatting of Premix kits but arrive in Space Saver packaging.
HCYTA-60K-PX48 ²	48	sCD40L, EGF, Eotaxin, FGF-2, FLT-3L, Fractalkine, G-CSF, GM-CSF, GRO α , IFN α 2, IFN γ , IL-1 α , IL-1 β , IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IL-17E/IL-25, IL-17F, IL-18, IL-22, IL-27, IP-10, MCP-1, MCP-3, M-CSF, MDC, MIG, MIP-1 α , MIP-1 β , PDGF-AA, PDGF-AB/BB, RANTES*, TGF α , TNF α , TNF β , VEGF-A	*RANTES is always provided as a separate bead vial due to different dilution requirements for serum/plasma samples. If measuring RANTES in serum/plasma, it is recommended to use a singleplex kit including RANTES only.
HCYTA-60K-PXBK48 ³			

Table 1. Catalog numbers, formats, and analytes contained in each kit.

Materials and Methods

Development of the MILLIPLEX® Kit

The MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A kit was developed using magnetic microsphere beads from Luminex® Corporation. Each set of beads is distinguished by different ratios of two internal dyes yielding a unique fluorescent signature for each bead set. Capture antibodies or antigens

were coupled to the magnetic beads. Figure 1 shows the Luminex® methodology and instrumentation. Of note, this kit may be run on any of the Luminex® instruments, including the MAGPIX® instrument, which is limited to reading 50 specific MagPlex® magnetic microsphere bead regions¹.



Figure 1. (A) Luminex® protein detection immunoassay method. (B) Instruments for use with MILLIPLEX® kits (left to right: MAGPIX®, Luminex® 200™, FLEXMAP 3D®).

Quality Built into MILLIPLEX® Kits

Kit development and verification entails testing for selectivity and specificity to ensure negligible cross-reactivity in the tested sample types, as well as assay specificity to ensure consistent performance of an assay in singleplex vs. multiplex formats (data not shown). Buffers and diluents are optimized to enhance antibody specificity, such that only those analytes of interest are detected in samples. The serum matrix is also carefully selected and optimized for use in the standard curve when using serum or plasma samples to most closely mimic sample matrix, thus normalizing assay performance. The streptavidin-phycoerythrin (SAPE) concentration is titrated in-house for optimal signal and is provided ready-to-use with no dilution required. Additionally, all our kits are rigorously tested for shipping stability, and samples are also tested for temperature and freeze/thaw tolerance.

Sample dilution is optimized for each analyte in the kit, ensuring that biologically relevant sample values are detectable, and fall within the dynamic range of the standard curves. Samples tested for this kit include normal and disease serum and plasma samples, as well as peripheral blood mononuclear cell supernatants (PBMCs, unstimulated and stimulated with various agents, refer to “Samples Used” below). Calibrators, or Quality Controls (QCs), which are low and high dilutions of recombinant proteins for each analyte, are manufactured such that the two QCs (high and low) have optimal placement on each standard curve. QC range sheets are provided with each kit. Additionally, we always recommend users include experiment-specific samples for use as controls in each assay.

MILLIPLEX® Protocol*

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 (the overnight protocol was used for all experiments in this application note)

Wash beads with wash buffer

+ 25 µL detection antibody mixture

Shake 1 hour at RT

+ 25 µL SAPE

Shake 30 min at RT

Wash beads with wash buffer

+ 150 µL sheath fluid and read on Luminex® instrumentation

*Refer to the HCYTA-60K protocol for detailed instructions

Samples Used

For serum samples, the blood was allowed to clot for 30 minutes before centrifugation for 10 minutes at 1000 x g. The serum was removed and either assayed immediately or aliquoted and stored at -20°C. Plasma samples, with EDTA anticoagulant, were centrifuged at 1000 x g within 30 minutes of blood collection. Plasma was removed and assayed immediately or aliquoted and stored at -20°C. Frozen samples were thawed completely, vortexed and centrifuged prior to use, to remove particulates. Healthy control serum and plasma samples were obtained from BioIVT, and sepsis patient serum and plasma samples were obtained from BioIVT, Discovery and BioChemed.

For PBMC supernatants, the cells were cultured in RPMI containing 10% FBS and 1% Penicillin/Streptomycin at 10⁶ cells/mL and were incubated with either 1 µg/mL LPS or Con A (Sigma-Aldrich) for 48 hours at 37°C, after which cell-free supernatants were collected and tested. The PBMCs were either assayed immediately or aliquoted and stored at -20°C. The PBMCs were obtained from BioIVT.

Results

Workflow Improvements

An assay containing 48 individual immune factors in a single panel is a powerful tool for research. The ability to select all analytes, perhaps for screening, or to select a subset of analytes as a project continues, allows researchers the flexibility required to work efficiently. Detecting all (or a subset) of these proteins at pg/mL levels within a kit with standard curves that do not change from lot to lot makes the assay very user-friendly. Fixed standard curves from lot to lot ensures consistent sample results across a project. The MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A also has a straightforward protocol with a familiar workflow in which every step is outlined to ensure ease of use.

Another assay improvement is that both PDGF-AA and PDGF-AB/BB can be tested with neat serum and plasma samples, so there is no need for sample dilution as is the case with HCYTOMAG-60K (MILLIPLEX® Human Cytokine/Chemokine Panel 1), or for other Luminex® based kits on the market. RANTES, being highly expressed in serum and plasma samples, still requires a separate sample dilution in serum and plasma of 1:100 (please refer to the HCYTA-60K protocol for detailed instructions).

A simple assay improvement of logically arranging the bead regions and analyte names in numeric-alphabetic order, allows researchers to quickly locate specific analytes in the result output files much easier than with previous assays. This helps immensely when analyzing datasets of 42 analytes or greater.

Standard Curve Comparisons

The standard curves and standard curve ranges for the MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A are shown in Figure 2 and Table 2. Table 2 also shows the comparison panel and its corresponding standard curve range for that analyte. Adjusting the standard curves from the comparative assay allowed us to implement two major improvements to the assays, with multiple, analyte-specific improvements and changes as well. These improvements include first, the implementation of 7-point standard curves, which are commonplace for Luminex® multiplex immunoassays in the research community. Our 7-point standard curves with 1:5 serial dilutions give the assays in this kit longer dynamic ranges which still match or exceed the sensitivities of other assays in the MILLIPLEX® portfolio. The second major improvement is that specific curves were optimized for each analyte, improving the linear portion of the curve to allow more samples to be quantitated with confidence in comparison to the HCYTOMAG-60K curves (which were all set to 3.2-10,000 pg/mL).

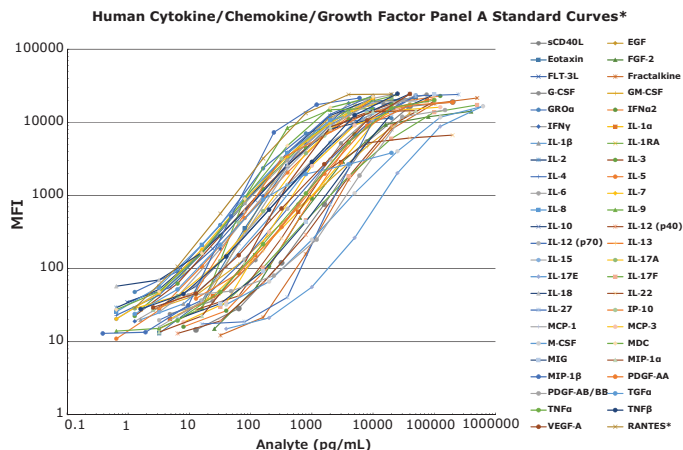


Figure 2. 48-plex standard curves were performed in MXHSM-A serum matrix except *RANTES, which was performed in L-AB assay buffer. The kit was run using the HCYTA-60K overnight assay protocol.

Analyte	HCYTA-60K Standard Curve Range (pg/mL)	Comparison Panel	Comparison Panel Standard Curve Range (pg/mL)
sCD40L	13 – 200,000	HCYTOMAG-60K	3.2 – 10,000
EGF	3 – 50,000	HCYTOMAG-60K	3.2 – 10,000
Eotaxin	3 – 50,000	HCYTOMAG-60K	3.2 – 10,000
FGF-2	26 – 400,000	HCYTOMAG-60K	3.2 – 10,000
FLT-3L	0.96 – 15,000	HCYTOMAG-60K	3.2 – 10,000
Fractalkine	32 – 500,000	HCYTOMAG-60K	3.2 – 10,000
G-CSF	4.8 – 75,000	HCYTOMAG-60K	3.2 – 10,000
GM-CSF	2.6 – 40,000	HCYTOMAG-60K	3.2 – 10,000
GRO α	1.3 – 20,000	HCYTOMAG-60K	3.2 – 10,000
IFN α 2	8 – 125,000	HCYTOMAG-60K	3.2 – 10,000
IFN γ	1.3 – 20,000	HCYTOMAG-60K	3.2 – 10,000
IL-1 α	4.8 – 75,000	HCYTOMAG-60K	3.2 – 10,000
IL-1 β	1.6 – 25,000	HCYTOMAG-60K	3.2 – 10,000
IL-1RA	1.6 – 25,000	HCYTOMAG-60K	3.2 – 10,000
IL-2	0.64 – 10,000	HCYTOMAG-60K	3.2 – 10,000
IL-3	1.3 – 20,000	HCYTOMAG-60K	3.2 – 10,000
IL-4	0.64 – 10,000	HCYTOMAG-60K	3.2 – 10,000
IL-5	0.64 – 10,000	HCYTOMAG-60K	3.2 – 10,000
IL-6	0.64 – 10,000	HCYTOMAG-60K	3.2 – 10,000
IL-7	0.64 – 10,000	HCYTOMAG-60K	3.2 – 10,000
IL-8	0.64 – 10,000	HCYTOMAG-60K	3.2 – 10,000
IL-9	0.64 – 10,000	HCYTOMAG-60K	3.2 – 10,000
IL-10	2.6 – 40,000	HCYTOMAG-60K	3.2 – 10,000
IL-12 (p40)	6.4 – 100,000	HCYTOMAG-60K	3.2 – 10,000
IL-12 (p70)	3 – 50,000	HCYTOMAG-60K	3.2 – 10,000
IL-13	6.4 – 100,000	HCYTOMAG-60K	3.2 – 10,000
IL-15	3 – 50,000	HCYTOMAG-60K	3.2 – 10,000
IL-17A	1.3 – 20,000	HTH17MAG-14K	12 – 50,000
IL-17E/IL-25	40 – 625,000	HTH17MAG-14K	120 – 500,000
IL-17F	32 – 500,000	HTH17MAG-14K	20 – 100,000
IL-18	0.64 – 10,000	HIL18MAG-66K	1.6 – 25,000

Analyte	HCYTA-60K Standard Curve Range (pg/mL)	Comparison Panel	Comparison Panel Standard Curve Range (pg/mL)
IL-22	13 – 200,000	HTH17MAG-14K	40 – 150,000
IL-27	16 – 250,000	HTH17MAG-14K	60 – 250,000
IP-10/CXCL10	2.6 – 40,000	HCYTOMAG-60K	3.2 – 10,000
MCP-1	3 – 50,000	HCYTOMAG-60K	3.2 – 10,000
MCP-3	8 – 125,000	HCYTOMAG-60K	3.2 – 10,000
M-CSF	40 – 625,000	HCYP3MAG-63K	97.7 – 100,000
MDC	0.64 – 10,000	HCYTOMAG-60K	3.2 – 10,000
MIG	6.4 – 100,000	HCYP3MAG-63K	48.8 – 50,000
MIP-1 α	3 – 50,000	HCYTOMAG-60K	3.2 – 10,000
MIP-1 β	0.38 – 6,000	HCYTOMAG-60K	3.2 – 10,000
PDGF-AA	13 – 200,000	HCYTOMAG-60K	3.2 – 10,000
PDGF-AB/BB	9.6 – 150,000	HCYTOMAG-60K	3.2 – 10,000
RANTES	1.3 – 20,000	HCYTOMAG-60K	3.2 – 10,000
TGF α	1.3 – 20,000	HCYTOMAG-60K	3.2 – 10,000
TNF α	6.4 – 100,000	HCYTOMAG-60K	3.2 – 10,000
TNF β	1.6 – 25,000	HCYTOMAG-60K	3.2 – 10,000
VEGF-A	2.6 – 40,000	HCYTOMAG-60K	3.2 – 10,000

Table 2. Standard curve ranges for each of the 48 analytes compared to that of the indicated comparison MILLIPLEX® panel assay.

Sample Detectability

In addition to improvements in the standard curves relative to the comparative analyte assays for the MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A, we also saw improvement in analyte performance in terms of sample detection. One goal in the development of this panel was to closely match sample values when evaluated against comparison MILLIPLEX® kits (HCYTOMAG-60K,

HCYP3MAG-63K, or HTH17MAG-14K, depending on the analyte). Figure 3 shows representative sample correlations between HCYTA-60K vs. the comparison panel.

Of note, three of the analyte assays have been adjusted to better match the accepted values in the literature: GRO α ^{3,5}, IL-4³ and IL-22^{2,4,6}.

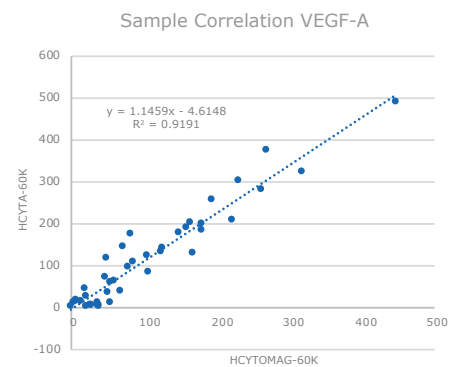
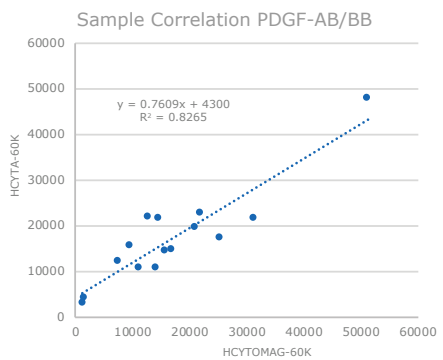
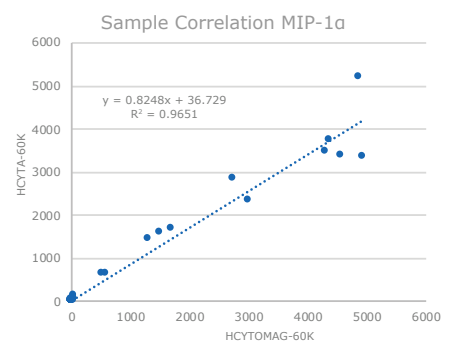
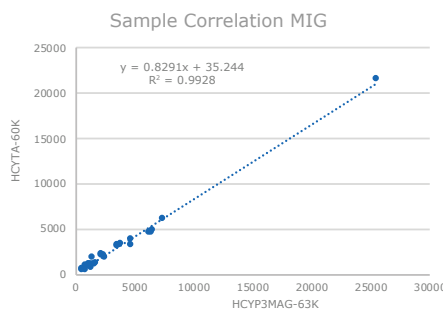
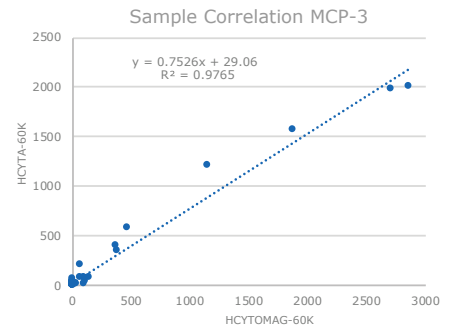
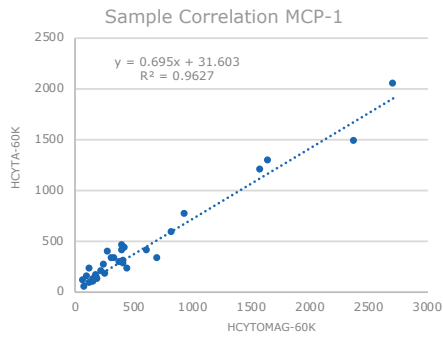
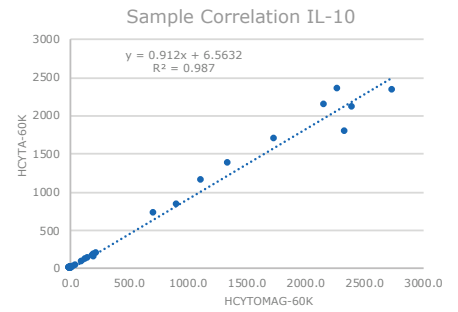
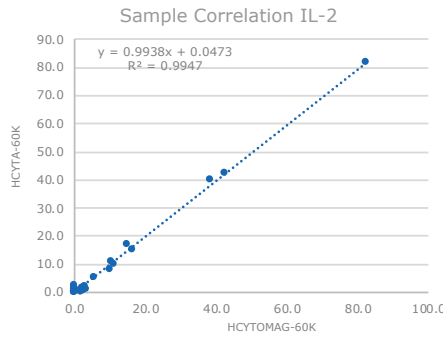
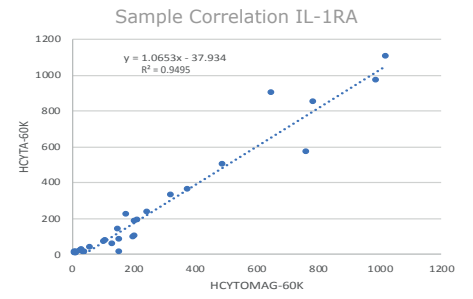
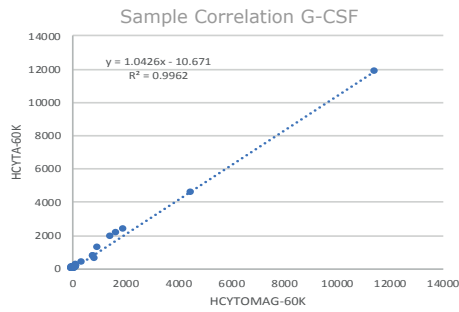


Figure 3. Representative sample correlation graphs between HCYTA-60K vs. the comparative assay from the indicated MILLIPLEX® kit. All R^2 values are ≥ 0.7 , with the exception of the assays which were adjusted to better match the accepted values in the literature.

Table 3 shows the percentage of samples detected by HCYTA-60K vs. the comparison panel. In general, sample detection in HCYTA-60K was superior to that in the comparative panel, with a few exceptions. Of the 48

analytes, 46 of them exhibited superior or comparable levels of sample detection when compared to the comparative assay from the indicated MILLIPLEX® kit.

	sCD40L	EGF	Eotaxin	FGF-2	FLT-3L	Fractalkine	G-CSF	GM-CSF
HCYTA-60K	100	92	100	81	100	86	92	67
HCYTOMAG-60K	100	92	100	81	25	11	97	92

	GRO α *	IFN α 2	IFN γ	IL-1 α	IL-1 β	IL-1RA	IL-2	IL-3
HCYTA-60K	83	72	92	83	92	100	47	44
HCYTOMAG-60K	100	86	100	36	25	75	31	17

	IL-4*	IL-5	IL-6	IL-7	IL-8	IL-9	IL-10	IL-12 (p40)
HCYTA-60K	94	97	97	100	94	94	97	97
HCYTOMAG-60K	83	22	64	42	86	28	69	39

	IL-12 (p70)	IL-13	IL-15	IL-17A	IL-17E	IL-17F	IL-18	IL-22*
HCYTA-60K	72	94	100	36	100	33	ND	69
HCYTOMAG-60K	58	28	78	78				
HCYP3MAG-63K								
HTH17MAG-14K				42	44	31		39

	IL-27	IP-10	MCP-1	MCP-3	M-CSF	MDC	MIG	MIP-1 α
HCYTA-60K	97	100	100	86	94	100	100	89
HCYTOMAG-60K		100	100	36		100		100
HCYP3MAG-63K					56		100	
HTH17MAG-14K	100							

	MIP-1 β	PDGF-AA	PDGF-BB	RANTES	TGF α	TNF α	TNF β	VEGF-A
HCYTA-60K	100	100	100	100	89	97	86	100
HCYTOMAG-60K	89	81	100	100	56	100	28	97

Table 3. Percentage of samples-detected data for the 48 analytes in HCYTA-60K vs. the comparative assay from the indicated MILLIPLEX® kit. (n=16 sepsis, n=20 normal serum/plasma samples). Undetectable samples were below minimum detectable levels for the curve analysis software for that analyte. If samples are expected to have low levels of a particular analyte, researchers should consider use of a high sensitivity kit (such as Cat. No. HSTCMAG-28SK or SMC™ kits).

*GRO α , IL-4 and IL-22 sample values now aligned with values reported in the literature for HCYTA-60K (as described).

Summary

The MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A (Cat. No. HCYTA-60K) features an exciting, new and expanded combination of 48 configurable/customizable human cytokine, chemokine and growth factor assays requiring only 25 µL of each sample. Researchers can now assay more analytes in a single well, providing up to 1,824 data points per plate, if running 38 samples in duplicate, thus saving significant time. Researchers can flexibly select the exact formats they desire, either the full 48-plex, a preconfigured 38-plex, or select only the analytes needed for a given experiment, with receipt of either individual bead vials or custom premixed beads (in which beads are mixed together prior to shipment), with curves and results consistent from lot to lot. If still more customization is desired, researchers may also engage our custom immunoassays team ([SigmaAldrich.com/customassay](https://www.sigmaaldrich.com/customassay)). This team can assist with additional analytes, conversion to 384-well format, or custom analyte development.

The MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A affords researchers multiple improvements in protocol and workflow. PDGF-AA and PDGF-AB/BB can now be tested with neat serum and plasma samples, a major improvement over the previous assays in MILLIPLEX® Human Cytokine/Chemokine Panel 1, as well as other Luminex® based assays on the market, and makes using HCYTA-60K much simpler for researchers examining these analytes. The protocol itself is written with transparency in mind. As with all of our kit protocols, the standard curve concentration for each standard

dilution (for each analyte) is listed in the protocol for convenience, and does not change from lot to lot. The protocol also contains the minDCs, percent CVs, and percent recovery of spiked standards in serum matrix obtained in our laboratories for each analyte. The last few pages of the protocol are helpful to researchers who want to know which analytes are contained in each of the kit components they receive.

Representative data shown in this application note exemplifies the value of this kit for the study of relevant disease sample biomarkers in serum and plasma biofluids, as well as in PBMCs. We demonstrate improvements in standard curve ranges when compared to other assays in the MILLIPLEX® portfolio of kits. Broad and fixed standard curve ranges have been defined and specifically optimized for each analyte, for lot-to-lot consistency.

In addition, the MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A is Cartagena regulation compliant.

We understand that sample correlation is important to researchers. As such, sample values were compared and correlated between HCYTA-60K analytes other analyte assays in our MILLIPLEX® portfolio of kits during kit development in order to provide confidence in results obtained, no matter which MILLIPLEX® analyte assay is used. This noted, it is important to understand that absolute sample values may change, but trends should remain the same. Thus, it is important for researchers to bridge assays in their own labs by comparing the two kits, and then making the switch to the larger, more flexible kit when convenient.

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