Application Note

Quantitative Multiplex Analysis of Low-level Cytokine Expression: Comparison of 384-well vs. 96-well MILLIPLEX® MAP Human High Sensitivity T Cell Panels

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Introduction

Cytokines are immunomodulatory polypeptides important in cell signaling, playing key roles in both adaptive and innate immune responses. They are classified based on their source or function, and include interleukins (act as mediators between T cells), chemokines (responsible for T cell migration), lymphokines (produced by activated T helper (T_h) cells), and myokines (produced by muscle cells). Cytokines act at the recognition, activation, and/or effector phases of an immune response to modulate the development and functional activities of T cells, B cells and myeloid cells.

Cytokines are important mediators of inflammation, immune cell development and immune cell activation during both normal and disease states. In addition, it is well documented that even low levels of chronic inflammation are involved in many clinical and subclinical disease states. According to the Centers for Disease Control and Prevention (Leading Causes of Death, cdc.gov, 2016), low-level chronic inflammation contributes to at least 7 out of the 10 leading causes of mortality in the United States, including cardiovascular disease, stroke, Alzheimer's disease, diabetes and cancer.

Consequently, research involving cytokines is critical to achieving a deeper understanding of the immune system and the complex interactions which drive inflammatory processes that directly contribute to life-threatening diseases. This deeper understanding is facilitated by the ability to reliably detect low levels of these cytokines and having the capability to study multiple cytokines simultaneously.

Based on the Luminex xMAP® technology, we offer two assays with identical analytes in two formats: 384-well format and 96-well format. The MILLIPLEX® MAP 384-well Human High Sensitivity T Cell Panel (Cat. No. HSTC384-28K) and the 96-well version, the MILLIPLEX® MAP Human High Sensitivity T Cell Panel (Cat. No. HSTCMAG-28SK) are both 21-plex multiplexed assay kits for simultaneously detecting cytokines significant to T_h1, T_h2, and T_h17 cells. Both of these panels are customizable to enable the user to choose any number of analytes within the panels to meet their specific research needs. In addition, both panels are available in a premixed-bead format as a 21-plex kit (see **Table 1** for the catalog numbers).

This application note summarizes important analytical validation studies, with a focus on comparing the performance of the 384-well assay and the 96-well assay formats.

Table 1. Ordering information for the 384-well and 96-well Human High Sensitivity T Cell Panels.

Description	384-well Kit	96-well Kit
Configurable analyte selection, up to 21 analytes	**HSTC384-28K**	**HSTCMAG-28SK**
13-plex premixed kit	—	**HSTCMAG28SPMX13**
13-plex bulk premixed kit	—	**HSTCMAG28PMX13BK**
21-plex premixed kit	**HSTCMAG384-PX21**	**HSTCMAG28SPMX21**
21-plex bulk premixed kit	**HSTCMAG384PX21BK**	**HSTCMAG28PMX21BK**

Table 2. Comparison of MILLIPLEX® MAP protocols (384-well vs. 96-well).

384-well Kit	96-well Kit
Pre-wet plate with 80 µL Wash Buffer. Shake 10 minutes. Decant.	Pre-wet plate with 200 µL Wash Buffer. Shake 10 minutes. Decant.
Reconstitute Serum Matrix to 4 mL.	Reconstitute Serum Matrix to 4 mL.
Reconstitute and further dilute Standard & QC in Serum Matrix.	Reconstitute and further dilute Standard & QC in Serum Matrix.
Add Standards and Samples to wells. • To Standard wells: Add 40 µL standard/QC or Matrix (to background), and 10 µL beads. • To Sample wells: add 20 µL Assay Buffer, 20 µL Sample, and 10 µL beads.	Add Standards and Samples to wells. • To Standard wells: Add 50 µL standard/QC or Matrix (to background), and 25 µL beads. • To Sample wells: add 25 µL Assay Buffer, 25 µL Sample, and 25 µL beads.
Centrifuge plate 1 minute at 200 x g then incubate on shaker overnight (16–18 hours) at 4 °C.	Incubate on shaker overnight (16–18 hours) at 4 °C.
Wash 3X with 80 µL Wash Buffer. Note: a plate washer is recommended.	Wash 3X with 200 µL Wash Buffer. Note: may use either a plate washer or a handheld washer.
Add 20 µL Detection Antibody cocktail. Centrifuge plate 1 minute at 200 x g.	Add 50 µL Detection Antibody cocktail.
Incubate 1 hour at RT on shaker.	Incubate 1 hour at RT on shaker.
Add 20 µL SAPE. Centrifuge plate 1 minute at 200 x g.	Add 50 µL SAPE.
Incubate 30 minutes at RT on shaker.	Incubate 30 minutes at RT on shaker.
Wash 3X with 80 µL Wash Buffer. Note: a plate washer is recommended.	Wash 3X with 200 µL Wash Buffer. Note: may use either a plate washer or a handheld washer.
Add 70 µL Sheath Fluid and shake for 5 min. Read on Luminex FLEXMAP 3D® instrument (50 µL, 50 beads per set).	Add 150 µL Sheath Fluid or Drive Fluid, shake for 5 minutes. Read on Luminex instrumentation (100 µL, 50 beads per set).

Methods

A comparison of the protocols for the 384-well and 96-well kits is shown in **Table 2**. There are minor differences in overall workflow, primarily attributable to the 384-well format (e.g., a FLEXMAP 3D® instrument is required, and a plate washer, such as the BioTek® MultiFlo™ FX Automated Washer is recommended). A 384-well plate washer assures best results. For further instructions, please refer to the kit protocols.

For serum samples, the blood was allowed to clot for 30 minutes prior to centrifugation for 10 minutes at 1000 x g. The serum was removed and either assayed immediately, or 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 stored at -20 °C. Frozen samples were thawed completely, vortexed, and centrifuged prior to use, to remove particulates. Neat samples were added directly into the assay plate. Samples were obtained from BIORECLAMATION LLC, Westbury, NY.

Results

During the development, validation and quality control of all kits, rigorous performance studies are conducted, including standard curve range, cross reactivity, recovery, precision and sensitivity. The data presented below shows that the 384-well kit performs comparably, and in some instances superior, to our popular 96-well format kit.

Standard Curves

The standard curves of the MILLIPLEX® MAP 384- and 96-well Human High Sensitivity T Cell Panels are shown in **Figure 1a** and **Figure 1b**, respectively. Both assay formats show a broad linear range with the same standard curve ranges (in pg/mL) for each analyte (**Table 3**). To get a closer look at some of the more popular individual analyte standard curves side-by-side, **Figure 2** shows a selection of the 384-well and the 96-well analytes, plotted on the same graph.

Figure 1a. Standard curves for the 384-well Kit.

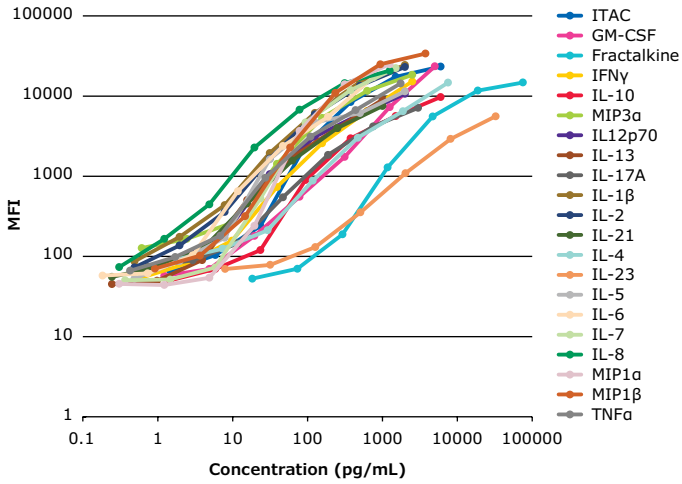


Figure 1b. Standard curves for the 96-well Kit.

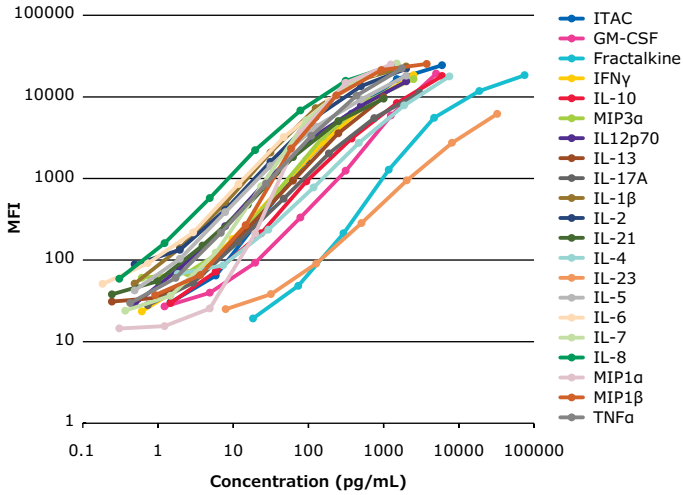


Figure 2. Select individual analyte standard curve comparisons (384-well and 96-well).

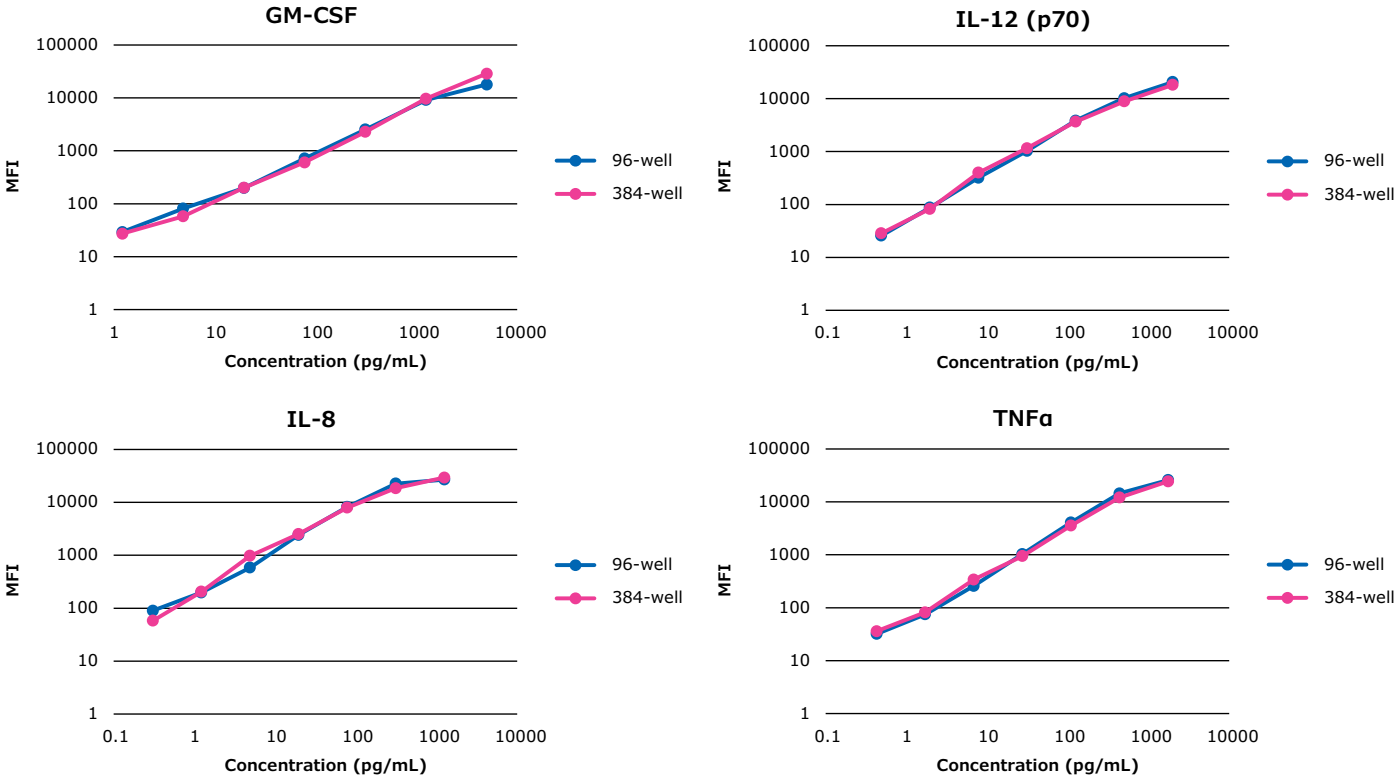


Table 3. Standard curve ranges for the 384-well (n=12) and 96-well (n=3) Kits (in pg/mL).

Standard Curve Range (384- and 96-well) (pg/mL)	
Fractalkine/CX3CL1	18.31 – 75000
GM-CSF	1.22 – 5000
IFNγ	0.61 – 2500
IL-1β	0.49 – 2000
IL-2	0.49 – 2000
IL-4	1.83 – 7500
IL-5	0.49 – 2000
IL-6	0.18 – 750
IL-7	0.37 – 1500
IL-8/CXCL8	0.31 – 1250
IL-10	1.46 – 6000
IL-12 (p70)	0.49 – 2000
IL-13	0.24 – 1000
IL-17A/CTLA8	0.73 – 3000
IL-21	0.24 – 1000
IL-23	7.93 – 32500
ITAC/CXCL11	1.46 – 6000
MIP1α/CCL3	0.31 – 1250
MIP1β/CCL3	0.92 - 3750
MIP-3α/CCL20	0.61 – 2500
TNFα	0.43 - 1750

Cross Reactivity

Potential analyte cross reactivity within the assays was investigated with a single standard cross reactivity test. Each individual standard was tested in the presence of multiplexed beads and detection antibodies. All standards had less than five percent cross reactivity with the other assays. The cross reactivity data of the 384-well format was comparable to that of the 96-well format kit (data not shown).

Assay Accuracy/Recovery

Assay accuracy was determined as the percentage of the observed concentration of known amount of standard spiked into serum matrix. The percent recoveries were between 70% and 110% for all assays. The assay accuracy data for the 384-well format was comparable to that of the 96-well format kit (data not shown).

Precision

Intra-assay precision (%CV) was determined to be less than or equal to 10% for all analytes, examining data from eight duplicates of the standard controls (data not shown). Inter-assay precision (%CV) was determined to be less than or equal to 20% for all analytes, examining data from eleven independent studies, each with duplicates of the standard controls (data not shown). The intra-assay precision data for the 384-well format was comparable to that of the 96-well format kit (data not shown).

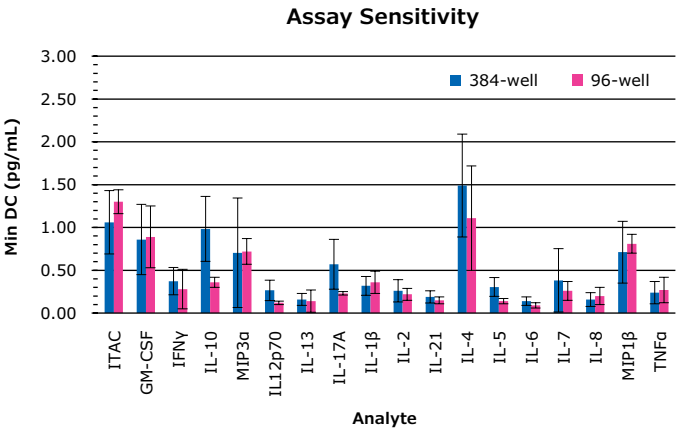
Assay Sensitivity Comparisons – 384-well vs. 96-well Kits

Based on the minimum detectable concentrations (minDCs) (determined by MILLIPLEX® Analyst Software) (**Table 4**), assay sensitivity for most assays was less than 1 pg/mL. The standard curves show a broad linear range of detection for all of the analytes in the panels (**Figure 1a** and **1b**). **Figure 3** shows minDCs for the 384-well and the 96-well assays side by side. The 384-well format offers higher throughput with the same sensitivity and sample detection, in comparison to the 96-well format.

Table 4. Minimum detectable concentrations (minDCs) in the 384-well (n=12) vs. 96-well (n=3) Kits (shown in pg/mL).

pg/mL	384-well	96-well
Fractalkine/CX3CL1	13.02	8.39
GM-CSF	0.86	0.89
IFNγ	0.37	0.28
IL-1β	0.32	0.36
IL-2	0.26	0.22
IL-4	1.49	1.11
IL-5	0.31	0.14
IL-6	0.14	0.09
IL-7	0.38	0.26
IL-8/CXCL8	0.16	0.20
IL-10	0.98	0.36
IL-12 (p70)	0.27	0.12
IL-13	0.16	0.14
IL-17A/CTLA8	0.57	0.23
IL-21	0.19	0.15
IL-23	6.91	3.36
ITAC/CXCL11	1.06	1.30
MIP1α/CCL3	1.86	0.66
MIP1β/CCL3	0.71	0.81
MIP-3α/CCL20	0.70	0.72
TNFα	0.24	0.27

Figure 3. Side by side representation of min DCs of 18 analytes determined by MILLIPLEX® Analyst Software (384-well, n=12; 96-well, n=3).



Sample Detection

Initial sample testing compared the percent sample detection between the 384-well and the 96-well formats. Eight normal (healthy control) serum samples and eight normal (healthy control) plasma samples were tested, totaling 16 samples to test the sensitivity limits of the assays. Low-level inflammation is linked to numerous clinical and subclinical diseases, but finding assays that can detect low levels of cytokines in apparently healthy individuals is a challenge. We used both kits to determine how well the panels can detect low levels of cytokines in healthy donor serum and plasma.

Both of the kit formats detected analytes at a similar frequency. **Table 5** shows the number of detectable samples (analyte by analyte), while **Table 6** shows the percentage of samples detectable in the 384-well versus the 96-well format. With respect to sample correlation, **Figure 4** shows high sample correlations between the 384-well and the 96-well kits for select analytes.

Table 5. 384-well vs. 96-well format comparison: sample detection (n=16 healthy controls).

	384-well	96-well
Fractalkine/CX3CL1	16	15
GM-CSF	14	15
IFNγ	16	16
IL-1β	14	15
IL-2	13	8
IL-4	14	13
IL-5	13	15
IL-6	16	16
IL-7	16	16
IL-8/CXCL8	16	16
IL-10	15	15
IL-12 (p70)	15	15
IL-13	14	14
IL-17A/CTLA8	16	15
IL-21	15	15
IL-23	16	15
ITAC/CXCL11	16	16
MIP1α/CCL3	15	15
MIP1β/CCL3	16	16
MIP-3α/CCL20	15	16
TNFα	16	16

Table 6. 384-well vs. 96-well format comparison: total sample detection (n=16 healthy controls), percentage of detectable samples.

Total Samples (16*21=336)	Total	% Total
384-well	317	94.3
96-well	313	93.2

Figure 4. Select sample correlation curves per analyte between the 384-well and the 96-well kits (n=16 serum and plasma samples tested).

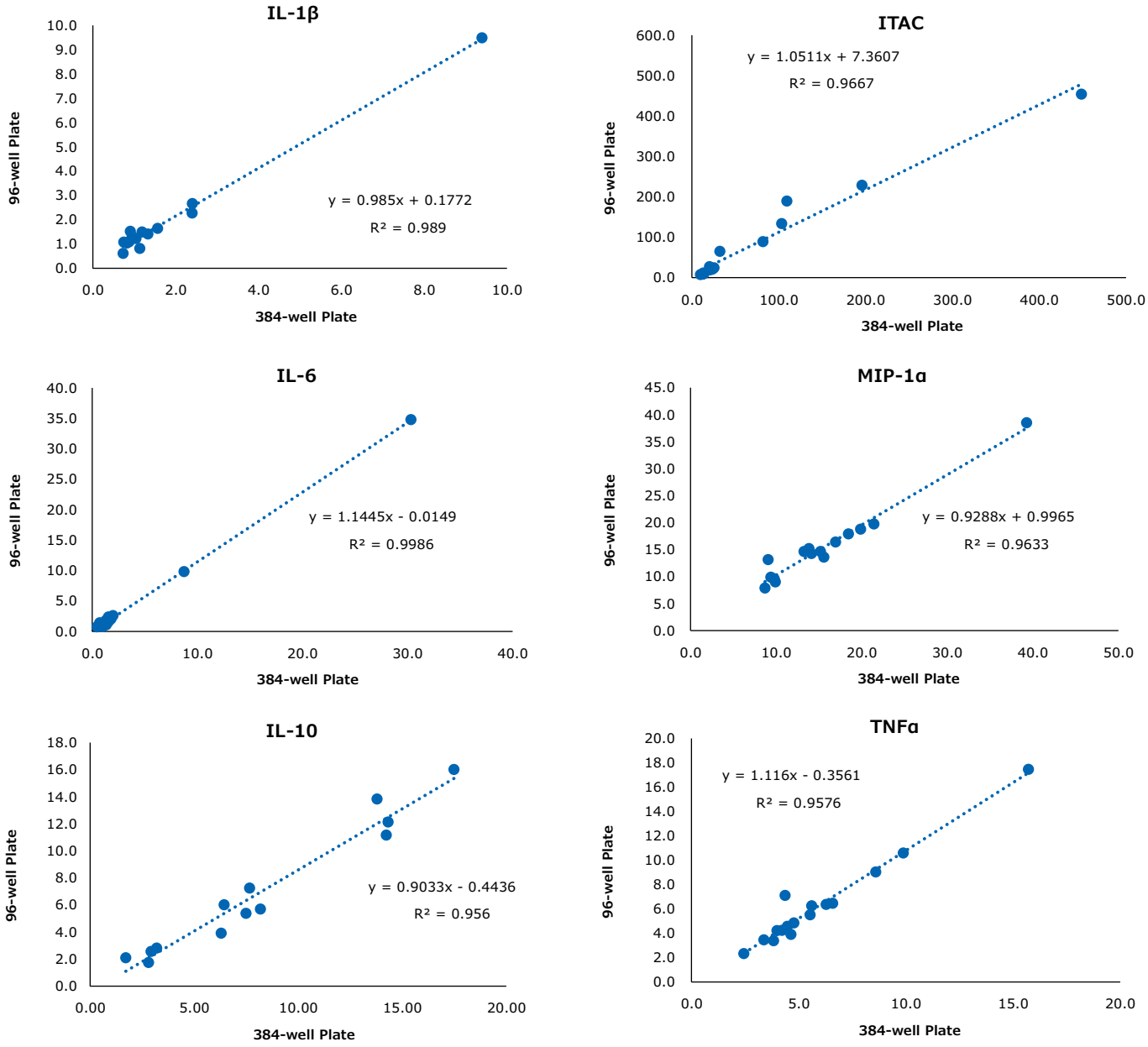
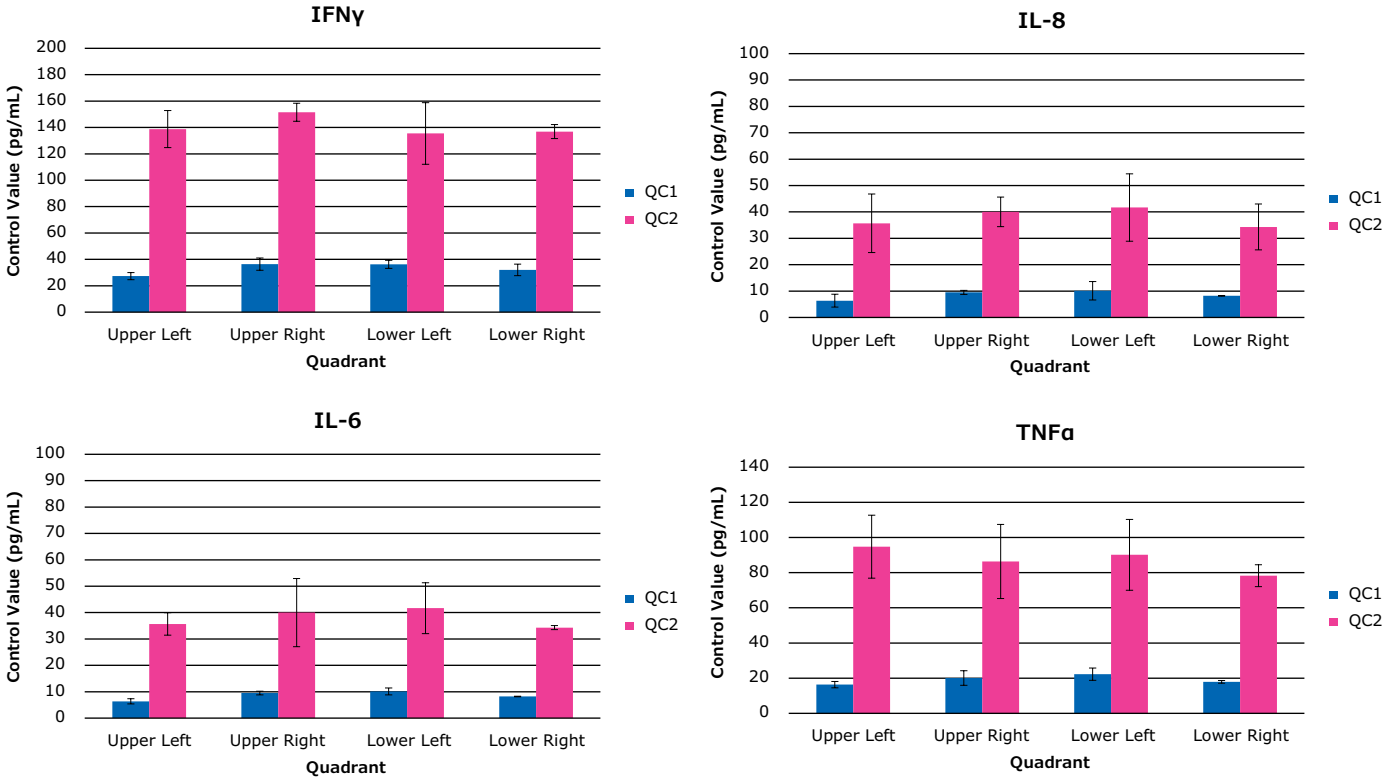


Figure 5. Control values for analytes in the 384-well Kit across four quadrants.



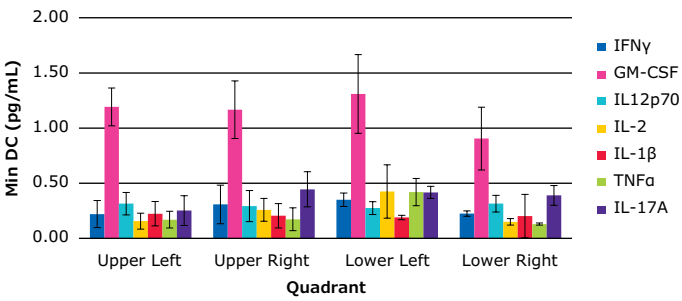
384-well Quadrant Performance

We examined the standard values obtained for specific analytes, between the four quadrants of the 384-well plate and found consistent values for the low and high concentration analyte controls (QC1 and QC2) (**Figure 5**).

Sensitivity Comparison within Four Quadrants of the 384-well Plate

The sensitivity of the 384-well kit was compared within the four quadrants of the plate for specific analytes (**Figure 6**). The 384-well kit offers higher throughput of the same analytes as in the 96-well kit, with the same sensitivity.

Figure 6. Sensitivity comparison, select analytes between the four quadrants of the 384-well Kit.



Conclusions

Low-level chronic inflammation is involved in many clinical and subclinical disease states. Consequently, investigating low levels of cytokine expression will broaden our understanding of the immune system and immune cell-mediated inflammatory processes. Our MILLIPLEX® MAP 384-well Human High Sensitivity T Cell Panel provides researchers with an analytically validated assay, superior performance, and allows very high throughput of up to 182 samples (in duplicate) in comparison to the 96-well version, which allows up to 38 samples (in duplicate), with a standard curve and quality controls in duplicate. This kit not only enables researchers to study low-level cytokine expression, but also to quantify multiple cytokine secretion levels simultaneously, in a biologically relevant context. Extensive testing shows that the 384-well format produces comparable results to our well-known 96-well kit. Researchers with access to a FLEXMAP 3D® system can use the MILLIPLEX® MAP 384-well kit to increase throughput to approximately 17,000 data points in a single day.

Since its launch in 2014, the MILLIPLEX® MAP Human High Sensitivity T Cell Panel in the 96-well format has become one of the most popular kits on the market. Now, with the release of the 384-well Human High Sensitivity T Cell Panel, researchers can use the same highly sensitive technology to test more samples, faster. It is our hope that these kits will contribute to advancing our understanding of cardiovascular disease, stroke, Alzheimer's, diabetes and cancer – ultimately leading to new therapies for these life-threatening diseases.

To place an order or receive technical assistance

In the U.S. and Canada, call toll-free
1-800-645-5476

For other countries across Europe
and the world, please visit:
emdmillipore.com/offices

For Technical Service, please visit:
emdmillipore.com/techservice

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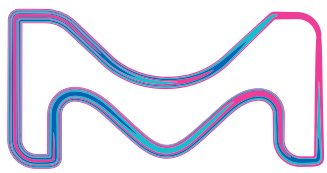

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